Lipids in Photosynthesis

Advances in Photosynthesis and Respiration

VOLUME 30

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The scope of our series, beginning with volume 11, reflects the concept that photosynthesis and respiration are intertwined with respect to both the protein complexes involved and to the entire bioenergetic machinery of all life. *Advances in Photosynthesis and Respiration* is a book series that provides a comprehensive and state-of-the-art account of research in photosynthesis and respiration. Photosynthesis is the process by which higher plants, algae, and certain species of bacteria transform and store solar energy in the form of energy-rich organic molecules. These compounds are in turn used as the energy source for all growth and reproduction in these and almost all other organisms. As such, virtually all life on the planet ultimately depends on photosynthetic energy conversion. Respiration, which occurs in mitochondrial and bacterial membranes, utilizes energy present in organic molecules to fuel a wide range of metabolic reactions critical for cell growth and development. In addition, many photosynthetic organisms engage in energetically wasteful photorespiration that begins in the chloroplast with an oxygenation reaction catalyzed by the same enzyme responsible for capturing carbon dioxide in photosynthesis. This series of books spans topics from physics to agronomy and medicine, from femtosecond processes to season long production, from the photophysics of reaction centers, through the electrochemistry of intermediate electron transfer, to the physiology of whole organisms, and from X-ray crystallography of proteins to the morphology or organelles and intact organisms. The goal of the series is to offer beginning researchers, advanced undergraduate students, graduate students, and even research specialists, a comprehensive, up-to-date picture of the remarkable advances across the full scope of research on photosynthesis, respiration and related processes.

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Lipids in Photosynthesis

Essential and Regulatory Functions

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Lipids in the dimeric complex of cyanobacterial photosystem II, as viewed from the cytoplasmic side towards the slightly tilted membrane plane. Lipids are shown by spheres, with carbon atoms in yellow and oxygen atoms in red. The protein components of the two monomers of photosystem II are shown in different shades of grey. Additional cofactors are shown in orange (carotenoids), green (chlorophylls), magenta (plastoquinone) and blue (heme).

Image generated by Dr. Jan Kern from pdb entries 3BZ1 and 3BZ2 (Guskov et al., 2009; see Chapter 10), using the PyMol program.

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This book is dedicated to one of the leading plant biologists of the twentieth century

Andrew Alm Benson

Who made major breakthroughs

In understanding the carbon fixation cycle, known as the Calvin-Benson cycle

and

For his major discoveries in the structure and the function of chloroplast lipids (phosphatidylglycerol and sulfolipids, among others)

From the Series Editor

Advances in Photosynthesis and Respiration

Volume 30: Lipids in Photosynthesis

Essential and Regulatory Functions

I am delighted to announce the publication, in the Advances in Photosynthesis and Respiration (AIPH) Series, of *Lipids in Photosynthesis: Essential and Regulatory Functions*. Two distinguished authorities from Japan have edited this volume: Hajime Wada (University of Tokyo) and Norio Murata (National Institute for Basic Biology, Okazaki). This book is produced as a sequel to volume 6 of the Series *Lipids in Photosynthesis: Structure, Function and Genetics*, edited by Paul-André Siegenthaler and Norio Murata, and published in 1998. I list below information on the 29 volumes thus far published in the AIPH series.

Published Volumes (2009–1994)

- *Volume 29* (2009): ***Photosynthesis* in silico**: ***Understanding Complexity from Molecules,*** edited by Agu Laisk, Ladislav Nedbal, and Govindjee, from Estonia, The Czech Republic and USA. Twenty chapters, 508 pp, Hard cover, ISBN: 978-1-4020-9236-7
- *Volume 28* (2009): ***The Purple Phototrophic Bacteria,*** edited by C. Neil Hunter, Fevzi Daldal, Marion C. Thurnauer and J. Thomas Beatty, from UK, USA and Canada. Forty-eight chapters, 1014 pp, Hardcover, ISBN: 978-1-4020-8814-8
- *Volume 27 (2008)*: ***Sulfur Metabolism in Phototrophic Organisms,*** edited by Christiane Dahl, Rüdiger Hell, David Knaff and Thomas Leustek, from Germany and USA. Twenty-four chapters, 551 pp, Hardcover, ISBN: 978-4020-6862-1
- *Volume 26* (2008): ***Biophysical Techniques in Photosynthesis, Volume II***, edited by Thijs Aartsma and Jörg Matysik, both from The Netherlands. Twenty-four chapters, 548 pp, Hardcover, ISBN:978-1-4020-8249-8
- *Volume 25* (2006): ***Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications***, edited by Bernhard Grimm, Robert J. Porra, Wolfhart Rüdiger, and Hugo Scheer, from Germany and Australia. Thirty-seven chapters, 603 pp, Hardcover, ISBN: 978-1-40204515-8
- *Volume 24* (2006): ***Photosystem I: The Light-Driven Plastocyanin: Ferredoxin Oxidoreductase***, edited by John H. Golbeck, from USA. Forty chapters, 716 pp, Hardcover, ISBN: 978-1-40204255-3
- *Volume 23* (2006): ***The Structure and Function of Plastids***, edited by Robert R. Wise and J. Kenneth Wise, from USA. Twenty-seven chapters, 575 pp, Softcover, ISBN: 978-1-4020-6570-6; Hardcover, ISBN: 978-1-4020-4060-3
- *Volume 22* (2005): ***Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase***, edited by Thomas J. Wydrzynski and Kimiyuki Satoh, from Australia and Japan. Thirty-four chapters, 786 pp, Hardcover, ISBN: 978-1-4020-4249-2
- *Volume 21* (2005): ***Photoprotection, Photoinhibition, Gene Regulation, and Environment***, edited by Barbara Demmig-Adams, William W. III Adams and Autar K. Mattoo, from USA. Twenty-one chapters, 380 pp, Hardcover, ISBN: 978-14020-3564-7
- *Volume 20* (2006): ***Discoveries in Photosynthesis***, edited by Govindjee, J. Thomas Beatty, Howard Gest and John F. Allen, from USA, Canada and UK. One hundred and eleven chapters, 1304 pp, Hardcover, ISBN: 978-1-4020-3323-0
- *Volume 19* (2004): ***Chlorophyll a Fluorescence: A Signature of Photosynthesis***, edited

by George C. Papageorgiou and Govindjee, from Greece and USA. Thirty-one chapters, 820 pp, Hardcover, ISBN: 978-1-4020-3217-2

- *Volume 18* (2005): ***Plant Respiration: From Cell to Ecosystem***, edited by Hans Lambers and Miquel Ribas-Carbo, from Australia and Spain. Thirteen chapters, 250 pp, Hardcover, ISBN: 978-14020-3588-3
- *Volume 17* (2004): ***Plant Mitochondria: From Genome to Function***, edited by David Day, A. Harvey Millar and James Whelan, from Australia. Fourteen chapters, 325 pp, Hardcover, ISBN: 978-1-4020-2399-6
- *Volume 16* (2004): ***Respiration in Archaea and Bacteria: Diversity of Prokaryotic Respiratory Systems***, edited by Davide Zannoni, from Italy. Thirteen chapters, 310 pp, Hardcover, ISBN: 978- 14020-2002-5
- *Volume 15 (2004)*: ***Respiration in Archaea and Bacteria: Diversity of Prokaryotic Electron Transport Carriers***, edited by Davide Zannoni, from Italy. Thirteen chapters, 350 pp, Hardcover, ISBN: 978-1-4020-2001-8
- *Volume 14* (2004): ***Photosynthesis in Algae***, edited by Anthony W. Larkum, Susan Douglas and John A. Raven, from Australia, Canada and UK. Nineteen chapters, 500 pp, Hardcover, ISBN: 978-0-7923-6333-0
- *Volume 13* (2003): ***Light-Harvesting Antennas in Photosynthesis***, edited by Beverley R. Green and William W. Parson, from Canada and USA. Seventeen chapters, 544 pp, Hardcover, ISBN: 978- 07923-6335-4
- *Volume 12* (2003): ***Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism***, edited by Christine H. Foyer and Graham Noctor, from UK and France. Sixteen chapters, 304 pp, Hardcover, ISBN: 978-07923-6336-1
- *Volume 11* (2001): ***Regulation of Photosynthesis***, edited by Eva-Mari Aro and Bertil Andersson, from Finland and Sweden. Thirty-two chapters, 640 pp, Hardcover, ISBN: 978-0-7923-6332-3
- *Volume 10* (2001): ***Photosynthesis: Photobiochemistry and Photobiophysics***, authored by Bacon Ke, from USA. Thirty-six chapters, 792 pp, Softcover, ISBN: 978-0-7923-6791-8; Hardcover: ISBN: 978-0-7923-6334-7
- *Volume 9* (2000): ***Photosynthesis: Physiology and Metabolism***, edited by Richard C. Leegood, Thomas D. Sharkey and Susanne von Caemmerer, from UK, USA and Australia. Twenty-four chapters, 644 pp, Hardcover, ISBN: 978-07923-6143-5
- *Volume 8* (1999): ***The Photochemistry of Carotenoids***, edited by Harry A. Frank, Andrew J. Young, George Britton and Richard J. Cogdell, from UK and USA. Twenty chapters, 420 pp, Hardcover, ISBN: 978-0-7923-5942-5
- *Volume 7* (1998): ***The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas***, edited by Jean David Rochaix, Michel Goldschmidt-Clermont and Sabeeha Merchant, from Switzerland and USA. Thirty-six chapters, 760 pp, Hardcover, ISBN: 978-0-7923-5174-0
- *Volume 6* (1998): ***Lipids in Photosynthesis: Structure, Function and Genetics***, edited by Paul-André Siegenthaler and Norio Murata, from Switzerland and Japan. Fifteen chapters, 332 pp, Hardcover, ISBN: 978-0-7923-5173-3
- *Volume 5* (1997): ***Photosynthesis and the Environment***, edited by Neil R. Baker, from UK. Twenty chapters, 508 pp, Hardcover, ISBN: 978- 07923-4316-5
- *Volume 4* (1996): ***Oxygenic Photosynthesis: The Light Reactions,*** edited by Donald R. Ort, and Charles F. Yocum, from USA. Thirty-four chapters, 696 pp, Softcover: ISBN: 978-0-7923-3684-6; Hardcover, ISBN: 978-0-7923-3683-9
- *Volume 3* (1996): ***Biophysical Techniques in Photosynthesis***, edited by Jan Amesz and Arnold J. Hoff, from The Netherlands. Twenty-four chapters, 426 pp, Hardcover, ISBN: 978-0-7923-3642-6
- *Volume 2* (1995): ***Anoxygenic Photosynthetic Bacteria***, edited by Robert E. Blankenship, Michael T. Madigan and Carl E. Bauer, from USA. Sixty-two chapters, 1331 pp, Hardcover, ISBN: 978-0-7923-3682-8
- *Volume 1* (1994): ***The Molecular Biology of Cyanobacteria***, edited by Donald R. Bryant, from USA. Twenty-eight chapters, 916 pp, Hardcover, ISBN: 978-0-7923-3222-0

Further information on these books and ordering instructions can be found at http://www.springer.com/series/5599 Contents of volumes 1–29 can be found at <http://www.life.uiuc.edu/govindjee/photosynSeries/ttocs.html>. Special discounts are available to members of the International Society of Photosynthesis

Research, ISPR http://www.photosynthesisresearch.org/>:

See http://www.springer.com/ispr

About Volume 30: Lipids in Photosynthesis: Essential and Regulatory Functions

As mentioned earlier, two distinguished authorities from Japan have edited this volume: Hajime Wada (University of Tokyo) and Norio Murata (National Institute for Basic Biology, Okazaki).

This book provides an essential summary of an exciting decade of research on relationships between lipids and photosynthesis. This book, designed both for scientists whose work focuses on photosynthesis and lipids and for graduate students who are developing an interest in the field, brings together extensively cross-referenced and peer-reviewed chapters by prominent researchers. The topics covered include the structure, molecular organization and biosynthesis of fatty acids, glycerolipids and non-glycerolipids in plants, mosses, lichens, algae, and cyanobacteria, as well as in chloroplasts and mitochondria. Several chapters deal with the manipulation of the extent of unsaturation of fatty acids and the effects of such manipulation on photosynthesis and responses to various forms of stress. The final chapters focus on lipid trafficking, signaling and advanced analytical techniques. Ten years ago, Paul André Siegenthaler and Norio Murata edited *Lipids in Photosynthesis: Structure, Function and Genetics*, which has been a unique and authoritative book in the field. The new volume, *Lipids in Photosynthesis: Essential and Regulatory Functions*, belongs, beside its predecessor, on every plant and microbiological researcher's bookcase.

Hajime Wada and Norio Murata have done an outstanding job in attracting 52 authors from nine countries (France; Germany; Israel; Japan; Mexico; Russia; Singapore; UK; and USA) to write 20 authoritative chapters on the topic of this book.

I thank each and every one of the 52 authors (see List of contributors on pp. xxiii-xxv) for their highly authoritative contributions to the field of lipids in photosynthesis. I am delighted to recognize that six of the 22 authors of volume 6 have returned to write for volume 30 (Christoph Benning, Maryse A. Block, John Browse, Eric Maréchal, Norio Murata, and Hajime Wada).

Hajime Wada and Norio Murata have introduced, in their *Preface*, the various chapters, that have been included, in this outstanding and much-needed book.

Content of Volume 6, Advances in Photosynthesis and Respiration

As volume 30 (the current volume) is a sequel to volume 6, it is beneficial for the readers of the new volume to consult and cite chapters in the earlier volume. To help the readers use directly the citations to various chapters, I present below the chapter numbers followed by the names of the authors, the titles of chapters, editors' names, title of the series, volume number, publisher, city, and page numbers in that book. Please note that this volume was published by Kluwer, which was later acquired by Springer, the publisher of the current volume.

Chapter 1: N. Murata and P.-A. Siegenthaler (1998) Lipids in photosynthesis: an overview. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 1–8

Chapter 2: J. Joyard, E. Maréchal, C. Miège, M.A. Block, A.J. Dorne and R. Douce (1998) Structure, distribution and biosynthesis of glycerolipids from higher plant chloroplasts. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 9–14

Chapter 3: J.L. Harwood (1998) Membrane lipids in algae. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 15–29

Chapter 4: H. Wada and N. Murata (1998) Membrane lipids in cyanobacteria. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 31–40

Chapter 5: C. Benning (1998) Membrane lipids in anoxygenic photosynthetic bacteria. In:

Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 41–46

Chapter 6: W.P. Williams (1998) The physical properties of thylakoid membrane lipids and their relation to photosynthesis. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 47–69

Chapter 7: P-A. Siegenthaler (1998) Molecular organization of acyl lipids in photosynthetic membranes of higher plants. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 71–77

Chapter 8: P-A. Siegenthaler and A. Trémolières (1998) Role of acyl lipids in the function of photosynthetic membranes in higher plants. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 79–98

Chapter 9: A. Trémolières and P-A. Siegenthaler (1998) Reconstitution of photosynthetic structures and activities with lipids. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 99–117

Chapter 10: B. de Krujiff, R. Pilon, R. van't Hof and R. Demel (1998) Lipid-protein interactions in chloroplast protein import. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 119–137

Chapter 11: E. Selstam (1998) Development of thylakoid membranes with respect to lipids. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 139–154

Chapter 12: D. Facciotti and V. Knauf (1998) Triglycerides as products of photosynthesis. Genetic engineering, fatty acid composition and structure of triglycerides. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 155–175

Chapter 13: Z. Gombos and N. Murata (1998) Genetic engineering of the unsaturation of membrane glycerolipid: effects on the ability of the photosynthetic machinery to tolerate temperature stress. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 177–192

Chapter 14: P. Vijayan, J.M. Routaboul and J. Browse (1998) A genetic approach to investigating membrane lipid structure and photosynthetic function. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 193–204

Chapter 15: J.L. Harwood (1998) Involvement of chloroplast lipids in the reaction of plants submitted to stress. In: Siegenthaler PA and Murata N (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht, pp. 205–222

Future Advances in Photosynthesis and Respiration and Other Related Books

The readers of the current series are encouraged to watch for the publication of the forthcoming books (not necessarily arranged in the order of future appearance):

- *C-4 Photosynthesis and Related CO_2 Concentrating Mechansims* (Editors: Agepati S. Raghavendra and Rowan Sage);
- *Photosynthesis: Perspectives on Plastid Biology, Energy Conversion and Carbon Metabolism* (Editors: Julian Eaton-Rye and Baishnab Tripathy);
- *Abiotic Stress Adaptation in Plants: Physiological, Molecular and Genomic Foundation* (Editors: Ashwani Pareek, Sudhir K. Sopory, Hans J. Bohnert and Govindjee);
- *The Chloroplast: Biochemistry, Molecular Biology and Bioengineering* (Editors: Constantin Rebeiz, Hans Bohnert, Christoph Benning, Henry Daniell, J. Kenneth Hoober, Hartmut Lichtenthaler, Archie R. Portis and Baishnab C. Tripathy);

- *Functional Genomics and Evolution of Photosynthetic Systems* (Editors: Robert Burnap and Willem Vermaas)

In addition to these contracted books, the following topics, among others, are under consideration:

- Cyanobacteria
- Biohydrogen Production
- ATP Synthase and Proton Translocation
- Interactions between Photosynthesis and other Metabolic Processes
- Carotenoids II
- Green Bacteria and Heliobacteria
- Ecophysiology
- Photosynthesis, Biomass and Bioenergy
- Global Aspects of Photosynthesis
- Artificial Photosynthesis

Readers are encouraged to send their suggestions for future volumes (topics, names of future editors, and of future authors) to me by E-mail (gov@ illinois.edu) or fax (1-217-244-7246).

In view of the interdisciplinary character of research in photosynthesis and respiration, it is my earnest hope that this series of books will be used in educating students and researchers not only in Plant Sciences, Molecular and Cell Biology, Integrative Biology, Biotechnology, Agricultural Sciences, Microbiology, Biochemistry, Chemical Biology, Biological Physics, and Biophysics, but also in Bioengineering, Chemistry, and Physics.

I take this opportunity to thank and congratulate Hajime Wada and Norio Murata for their outstanding editorial work; they have done an outstanding job not only in editing, but also in organizing this book for Springer, and for their highly professional dealing with the typesetting process and their help in preparing this editorial. I have already thanked (see above) all the 52 authors of this book: without their authoritative chapters, there would be no such volume.

I give special thanks to Peter Pushpanathan for directing the typesetting of this book: his expertise has been crucial in bringing this book to completion. We owe Jacco Flipsen, Noeline Gibson and André Tournois (of Springer) thanks for their friendly working relation with us that led to the production of this book. Thanks are also due to Jeff Haas (Director of Information Technology, Life Sciences, University of Illinois at Urbana-Champaign, UIUC), Feng Sheng Hu (Head, Department of Plant Biology, UIUC) and my dear wife, Rajni Govindjee for constant support.

April 10, 2009

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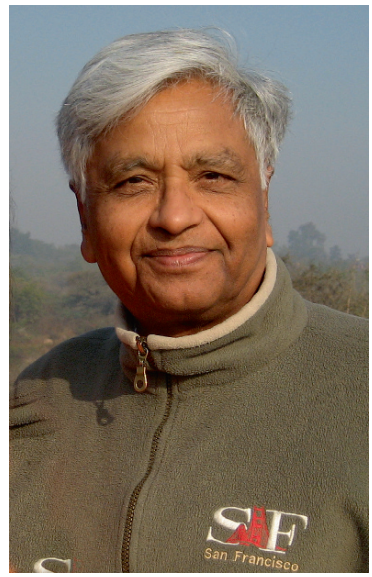

Govindjee, Series Editor

Govindjee was born on October 24, 1932, in Allahabad, India. Since 1999, he has been Professor Emeritus of Biochemistry, Biophysics and Plant Biology at the University of Illinois at Urbana-Champaign (UIUC), Urbana, IL, USA. He obtained his B.Sc. (Chemistry and Biology) and M.Sc. (Botany; Plant Physiology) in 1952 and 1954, from the University of Allahabad. He studied 'Photosynthesis' at the UIUC, under Robert Emerson, and Eugene Rabinowitch, obtaining his Ph.D. in 1960, in Biophysics. He is best known for his research on the excitation energy transfer, light emission, the primary photochemistry and the electron transfer in "Photosystem II" (PS II, water-plastoquinone oxido-reductase). His research, with many collaborators, has included the discovery of a short-wavelength form of chlorophyll (Chl) a functioning in the Chl b-containing system, now called PS II; of the two-light effect in Chl a fluorescence; and of the two-light effect (Emerson Enhancement) in NADP reduction in chloroplasts. His major achievements include an understanding of the basic relationships between Chl a fluorescence and photosynthetic reactions; an unique role of bicarbonate on the electron acceptor side of PS II, particularly in the protonation events involving the Q_B binding region; the theory of thermoluminescence in plants; the first picosecond measurements on the primary photochemistry of PS II; and the use of Fluorescence Lifetime Imaging Microscopy (FLIM) of Chl a fluorescence in understanding photoprotection, by plants, against excess light. His current focus is on the "History of Photosynthesis Research", in 'Photosynthesis Education', and in the 'Possible Existence of Extraterrestrial Life' He has served on the faculty of the UIUC for ~ 40 years. Govindjee's honors include: Fellow of the American Association of Advancement of Science (AAAS); Distinguished Lecturer of the School of Life Sciences, UIUC; Fellow and Lifetime member of the National Academy of Sciences (India); President of the American Society for Photobiology (1980-1981); Fulbright Scholar and Fulbright Senior Lecturer; Honorary President of the 2004 International Photosynthesis Congress (Montréal, Canada); the first recipient of the Lifetime Achievement Award of the Rebeiz Foundation for Basic Biology, 2006; Recipient of the Communication Award of the International Society of Photosynthesis Research, 2007; and the Liberal Arts & Sciences Lifetime Achievement Award of the UIUC, 2008. Further, Govindjee was honored in 2007, through 2 special volumes of Photosynthesis Research, celebrating his 75th birthday and for his 50-year dedicated research in 'Photosynthesis' (Guest Editor: Julian Eaton-Rye); (2) in 2008, through a special International Symposium on 'Photosynthesis in a Global Perspective', held in November, 2008, at the University of Indore, India. Govindjee is coauthor of 'Photosynthesis' (John Wiley, 1969); and editor of many books, published by several publishers including Academic Press and Kluwer Academic Publishers (now Springer).

Contents

Preface

Photosynthesis, the driving force for life on earth, has been studied by biochemists, biophysicists, plant physiologists, and agronomists. Similarly, lipids have attracted the attention of researchers in plant physiology, photosynthesis and agronomy. Moreover, even though glycolipids are among the most abundant natural compounds in the biosphere, glycolipids in chloroplast membranes were discovered only in the 1950s. Subsequently, however, studies of glycolipids have proceeded at an exponentially increasing rate and copious information is now available on the chemical structures and physico-chemical properties of glycerolipids, as well as on their occurrence and distribution among many genera of cyanobacteria, algae, mosses, and higher plants. Considerable efforts have been made to elucidate the biosynthetic pathways to glycerolipids and fatty acids and, more recently, interest in glycerolipids has shifted towards their roles in the structures and the functions of the thylakoid and chloroplast envelope membranes. In addition, the development of the methods and concepts of molecular biology has yielded answers to many questions that are related to the genes and enzymes that participate in the synthesis of membrane and storage lipids and in the regulation of such synthesis.

Several excellent books are available that deal with recent research on photosynthesis, and there are also a number of books that summarize recent progress in plant-lipid research. In 1998, Paul-André Siegenthaler and one of us (NM) edited a book entitled "Lipids in Photosynthesis: Structure, Function and Genetics" in the "Advances in Photosynthesis and Respiration" (series editor, Govindjee). This book emphasized the importance of lipids in photosynthetic processes and the importance of photosynthesis in the synthesis of lipids. In the years following the publication of this earlier volume, great advances have been made in this area of research. In particular, genetic and genomic approaches revealed the importance of individual lipid species in photosynthesis and its regulation. We hope that researchers and students of photosynthesis will read it to gain a fuller appreciation of the new findings and concepts that have accumulated over the past decade. Readers will also find updated information about various lipids, such as the major phospholipids and sphingolipids that appear to be involved in the regulation of photosynthesis. This book is intended for a wide audience but it is designed specifically for advanced undergraduate and graduate students and for researchers in photosynthesis who are interested in the functions and roles of membrane and storage lipids.

Chapter 1 provides a brief overview of findings made during the last decade by exploitation of newly available information and techniques, such as genome sequences, mutagenesis and transgenes, and physical and electron-microscopic methods. Readers are directed to appropriate chapters in which each specific topic is covered in depth. We have also made an effort to provide cross-references between chapters to help readers access all relevant materials on each specific topic.

The other chapters encompass several major themes: The structure, molecular organization, and biosynthesis of fatty acids in plants (Chapter 2); the structure, composition and biosynthesis of membrane glycerolipids in higher plant chloroplasts (Chapter 3), in plant mitochondria (Chapter 4), in lower plants, algae, lichens and mosses (Chapter 6), in the green alga *Chlamydomonas reinhardtii* (Chapter 7), and in cyanobacteria (Chapter 8); the structure, composition, and biosynthesis of membrane nonglycerolipids, namely, sphingolipids in photosynthetic organisms (Chapter 5) and heterocyst-specific glycolipids in cyanobacteria (Chapter 9); the molecular structural organization of glycerolipids in the photosynthetic apparatus (Chapter 10); the roles of glycerolipids in the photosynthetic membranes of higher plants and cyanobacteria (Chapters 11 and 12); the dynamics and architecture of thylakoid membranes with emphasis on their lipids (Chapters 13 and 14); the genetic manipulation of the unsaturation of membrane lipids and its effects on gene expression and photosynthesis (Chapters 15 and 17); lipid trafficking

in chloroplasts and cells of higher plants (Chapter 16); the genetic manipulation of the biosynthesis of triacylglycerol in plant seeds (Chapter 19); the functions of products of oxidation of membrane lipids as signaling molecules in higher plants (Chapter 18); and, finally, the development of advanced methods, such as mass spectrometry, for the analysis of lipids from photosynthetic organisms (Chapter 20).

The publication of this book has involved the efforts of many people to whom we would like to express our gratitude. First and foremost, we thank the authors of individual chapters for their contributions and for their cooperation during revision of their manuscripts. We are also grateful to the reviewers who read the various chapters and whose advice and suggestions did so much to improve the quality of the book. They are, in alphabetical order, Mats Andersson (Göteborg University), Christoph Benning (Michigan State University), Douglas Bruce (Brock University), Andrew Cossins (University of Liverpool), Peter Dörmann (University of Bonn), Ivo Feussner (Georg-August-University Göttingen), James W. Golden (Texas A&M University), Govindjee (University of Illinois at Urbana-Champaign), Ernst Heinz (University Hamburg), Michael Jones (University of Bristol), Daniel V. Lynch (Williams College), Johnathan Napier (Rothamsted Research), Ikuo Nishida (Saitama University), John Ohlrogge (Michigan State University), Norihiro Sato (Tokyo University of Pharmacy and Life Sciences), Kushnir Sergei (Ghent University), Antoni R. Slabas (University of Durham), Willem Vermass (Arizona State University), and Ruth Welti (Kansas State University). Finally, we thank Govindjee, the series editor of "Advances in Photosynthesis and Respiration", for inviting us to produce this book and for placing his trust in our efforts. We express our gratitude to Noeline Gibson, Jacco Flipsen, André Tournois, all of Springer, Dordrecht, for their help in the planning and the production of this book. We also thank Peter Pushpanathan, of SPi Technologies Pvt. Ltd, Pondicherry, India, for his valuable help during the typesetting and the final stages of this book.

April 10, 2009

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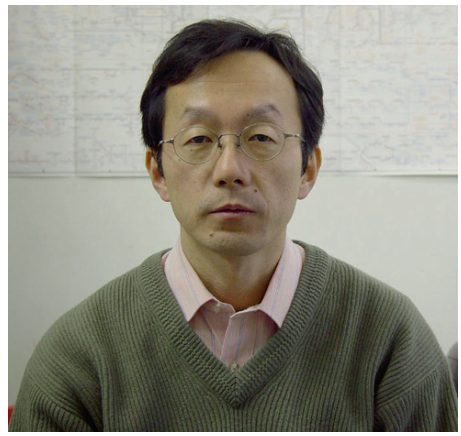

Hajime Wada

Hajime Wada was born in 1959 in Miyazaki, Japan. He is now a professor in the Department of Life Sciences of the University of Tokyo, Japan. He received his bachelor's degree in 1981 from the Tokyo University of Agriculture and Technology, and his master's degree in 1983 from the University of Tokyo, where his work with Professor Norio Murata inspired a lifelong interest in plant biology. To learn more about plant biology, he moved to the National Institute for Basic Biology (Japan) and began graduate studies under Murata's supervision. He started his doctoral studies, working on a cyanobacterium *Synechocystis* sp. PCC 6803; he focused on the desaturation of fatty acids that are bound to membrane lipids, which is related to cold adaptation in cyanobacteria. He succeeded in isolating mutants that were defective in the desaturation of fatty acids and then cloned the genes for the desaturases responsible for the introduction of double bonds into fatty acids. Cloning of these genes allowed subsequent modification of the unsaturation of membrane lipids by transformation of cells with such genes, as well as investigations of the roles of unsaturated fatty acids in photosynthesis and cold tolerance in genetically modified strains of cyanobacteria. He demonstrated that desaturation of fatty acids is required for the acclimation of cyanobacteria to cold and that cold tolerance can be manipulated by transformation with genes for desaturases. In 1990, he received his doctorate from the University of Tokyo and then he became a member of the faculty at the National Institute for Basic Biology (1991-1993), at Kyushu University (1993-2002), and at the University of Tokyo (2002-present). He continues to study plant lipids and photosynthesis. His achievements include the identification of novel genes involved in lipid biosynthesis; the discovery of fatty acid synthesis in mitochondria and its role in plant cells; and the discovery of important functions of lipids in plant development and in the assembly of the photosynthetic apparatus. His current research is focused on the regulation of biosynthetic pathways to lipids and the functions of lipids in photosynthetic organisms, which uses a variety of molecular genetic, biochemical, and physiological approaches.

Norio Murata

Norio Murata was born in 1940 in Tokyo, Japan. He is a Professor Emeritus of the National Institute for Basic Biology, Okazaki, Japan. He obtained his bachelor's degree in 1964 at the University of Tokyo. Under the supervision of Professors Atusi Takamiya and Mitsuo Nishimura, he started his doctoral studies by examining chlorophyll fluorescence in photosynthetic organisms in the chloroplasts and in thylakoid membranes isolated from them. His work focused on the emission and action spectra of fluorescence and on the kinetic analysis of fluorescence at both room temperature and at the temperature of liquid nitrogen. He estimated the sizes of electron pools on the acceptor side of Photosystem II (PS II) by analyzing the kinetics of fluorescence at room temperature. He assigned three bands of fluorescence at liquid nitrogen temperature to photosystems I and II, and he discovered the state transitions, the energy-dependent quenching of fluorescence and the transport of electrons to P680 on the donor side of PSII at low temperature. Murata received his doctoral degree from the University of Tokyo in 1969 and then spent twenty months (1969-1970) at the Carnegie Institution of Washington, Department of Plant Biology, working with Dr. Stacy C. French. Upon his return to Japan, he was made an assistant professor in the Department of Biophysics and Biochemistry at the University of Tokyo (1970-1978), and he was promoted to associate professor in the Department of Biology of the same University (1978-1985). During 1970s and early 1980s, he discovered and characterized the 33-kDa protein of PS II (now known as PsbO) and investigated the important roles of membrane lipids in cyanobacteria and, in particular, of phosphatidylglycerol in plants in the tolerance to cold. In 1985, he was appointed Professor at the National Institute for Basic Biology. From 1985 to 2005, he and his collaborators reported several important results, for example, (1) the introduction of transgenes for modification of membrane lipids and the resultant enhancement of the ability of cyanobacteria and plants to tolerate cold stress (the first successful molecular genetic improvement of stress tolerance); (2) the introduction of genes for glycinebetaine-synthesizing enzymes and the enhancement of the tolerance of model, agricultural and horticultural plants to many kinds of environmental stress; (3) the discovery of several histidine kinases that play important roles in the stress signalling transduction pathways in cyanobacteria; and (4) the elucidation of a mechanism that explains the tolerance and sensitivity of the photosynthetic machinery to environmental stresses. For his pioneering research, he received a Doctor of Science (honoris causa) of the University of Neuchâtel (1996) and of the University of Quebec (2004), and he was elected an honorary member of the Hungarian Academy of Science (1998).

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Color Plates

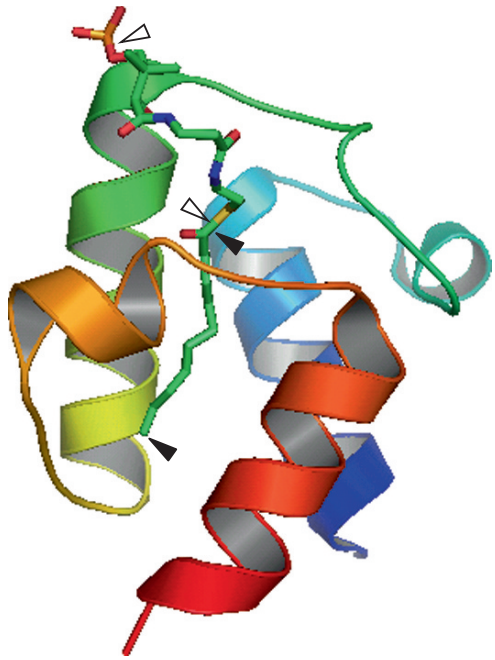


Fig. 1. Acylated *E. coli* ACP. A cartoon of ACP main chain, colored in rainbow depiction from N- to C-terminus, is shown. A C10 acyl chain (*solid arrows*) attached via a pantotheine arm (*open arrows*) to Ser36 is inserted into a central cavity of the four helix bundle. See Chapter 2, p. 22.

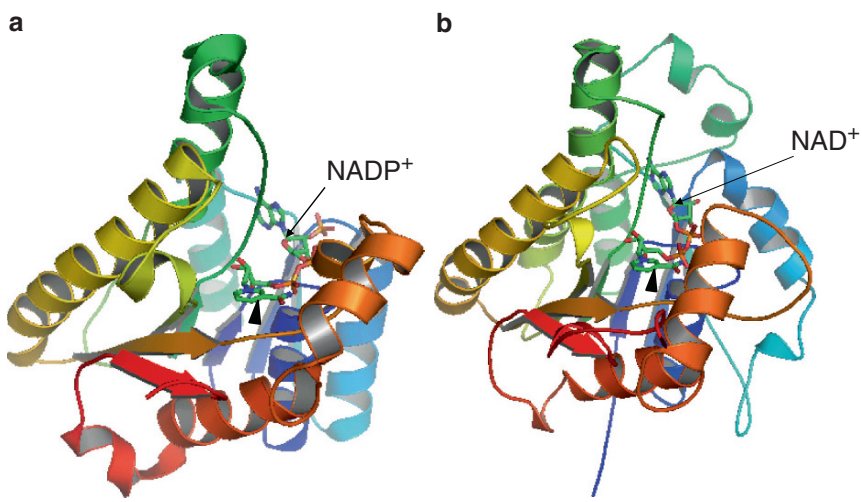


Fig. 2. Structure of *B. napus* acyl-ACP reductases. (**a**) A monomer of *B. napus* 3-ketoacyl-ACP reductase is shown, with the enzyme depicted and colored as for ACP in Fig. 4, Chapter 2. The view is into the active site from the nicotinamide ring (solid arrow) end of a bound $NADP^+$ cofactor. (**b**) A monomer of *B. napus* enoyl-ACP reductase, depicted, colored and viewed as for 3-ketoacyl-ACP reductase, but this time with a bound NAD^+ cofactor. See Chapter 2, p. 23.

Fig. 3. Active sites of *B. napus* acyl-ACP reductases. (**a**) The positions of key residues in the active site of 3-ketoacyl-ACP reductase relative to the nicotinamide ring of the bound cofactor are shown, depicted in stick representation. Note the location and separation of the Lys171 and Tyr167 residues. (**b**) The active site of enoyl-ACP reductase is depicted as for 3-ketoacyl-ACP reductase. The relative location of the critical Lys206 residue is maintained when compared to 3-ketoacyl-ACP reductase but the Tyr198 has moved in correlation with the different substrate groups that must be reduced by the two enzymes. See Chapter 2, p. 24.

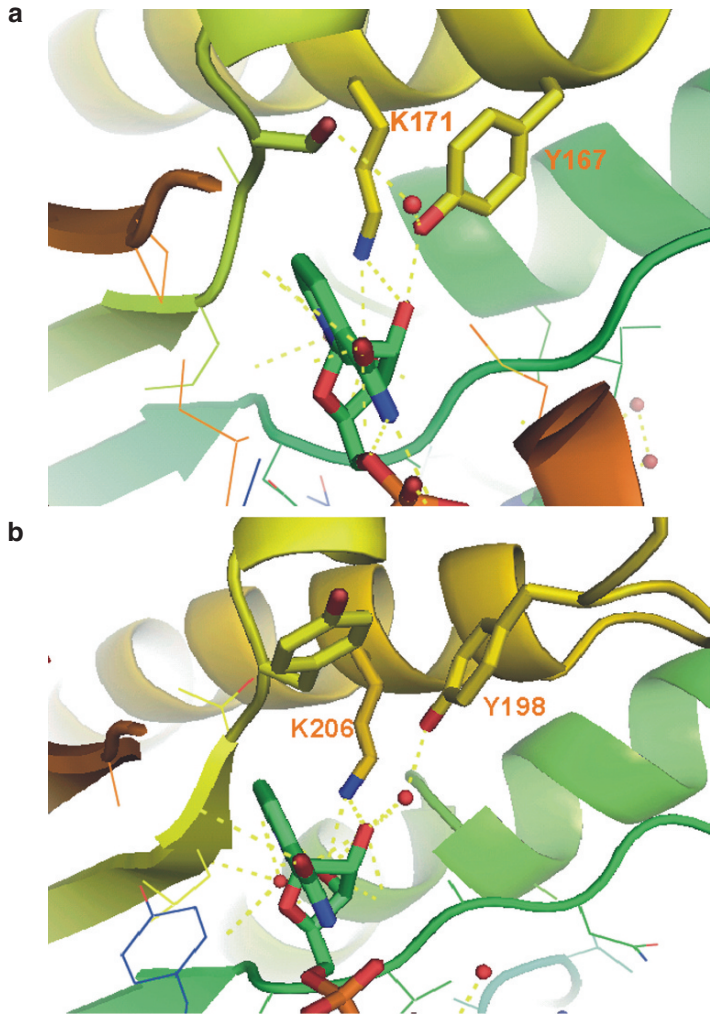
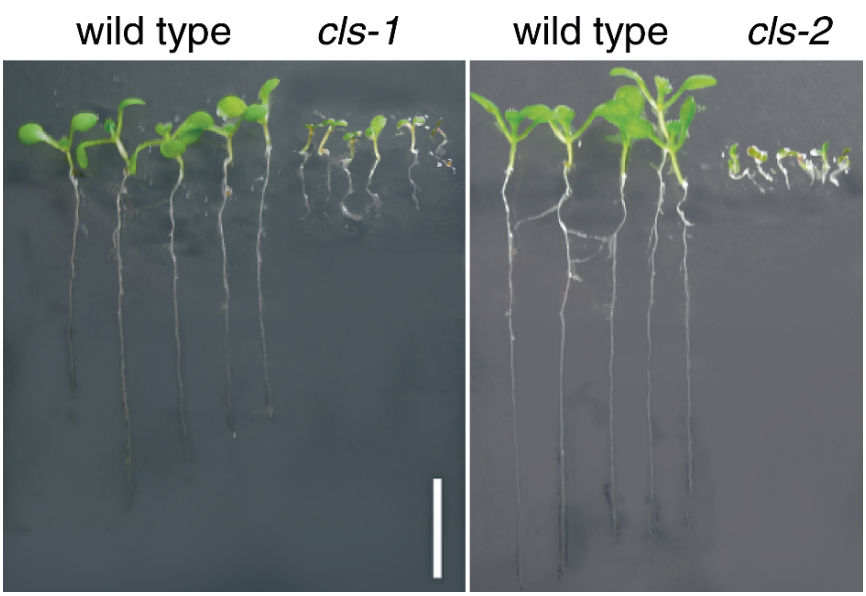


Fig. 4. Phenotype of homozygous *cls-1* and *cls-2* mutant seedlings from *Arabidopsis*, as well as wild-type controls cultivated for 7 days on sugar-containing agar plates. The mutant lines are affected in growth in comparison to the wild type, especially with regard to root growth. The *cls-2* mutant displays more severe defects than the *cls-1* mutant. The *white bar* corresponds to 1 cm (the figure was provided by K. Katayama and H. Wada (unpublished data). See Chapter 4, p. 69.



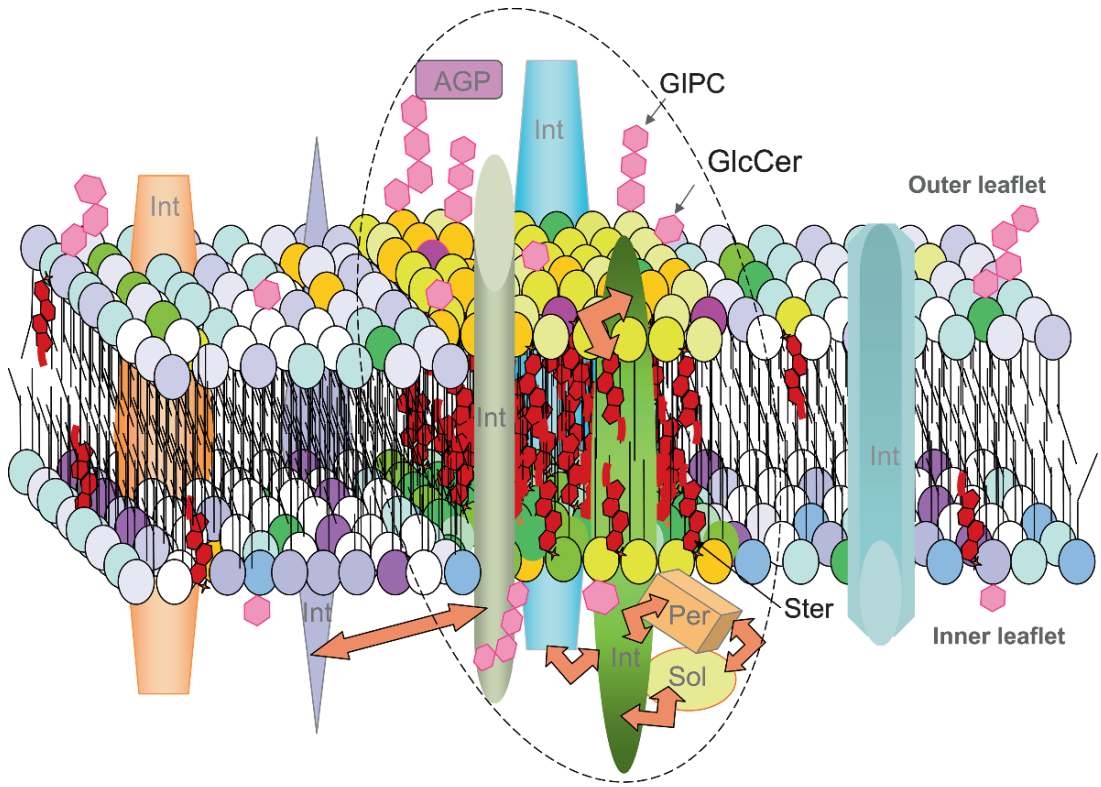


Fig. 5. Sphingolipids as structural building blocks of membrane microdomains or lipid rafts. Sphingolipids, including GlcCers and glycosyl inositolphosphoceramides (GIPC), can spontaneously associate with high affinity to sterols (Ster) in cell membranes, forming transient domains that provide a site where specific interactions between lipid and/or proteins are favored (the microdomain is indicated by circled area of membrane). However, these assembly sites may function to exclude proteins in order to avoid undesirable functional collisions as well. *Arrows* indicate favored or unfavored interactions of proteins. Integral membrane proteins (Int), arabinogalactan proteins (AGP), peripheral (Per) and soluble (Sol) proteins are representative proteins associated to these regions (courtesy of Laura Carmona-Salazar). See Chapter 5, p. 100.

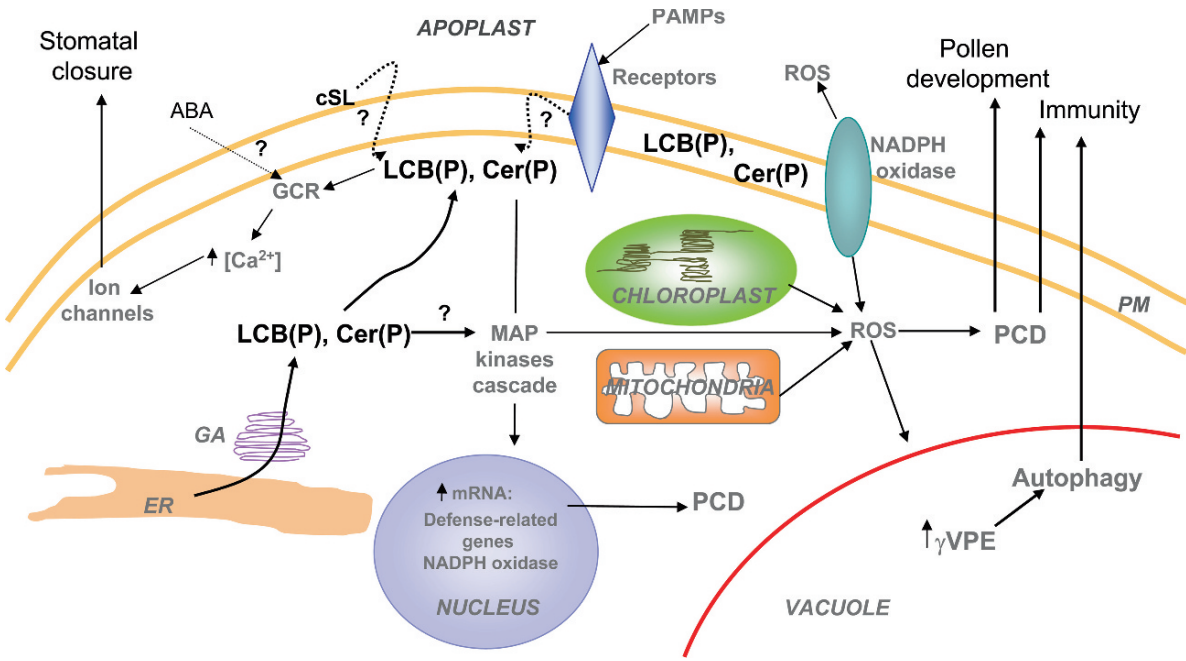


Fig. 6. Bioactive roles of sphingolipid metabolites. Free or phosphorylated long chain bases (LCB(P)) and ceramides (Cer(P)) are intracellular mediators in transduction pathways that lead to control of stomata closure and programmed cell death. Given their demonstrated role in the generation of reactive oxygen species (ROS) and the initiation of programmed cell death (PCD), it is possible that these sphingolipid metabolites also contribute to pollen development and immunity to pathogens. With regard to plant–pathogen interactions, sphingolipid metabolites may be associated with pathogen-induced triggering of MAP kinases cascades and may serve as downstream components of signal transduction pathways mediated by pathogen-associated molecular patterns. It is unknown what the relative contributions of de novo sphingolipid synthesis and sphingolipid degradation are to the production of bioactive LCBs and ceramides. Abbreviatons: cSL, complex sphingolipids; ER, endoplasmic reticulum; GA, Golgi apparatus; PM, plasma membrane; GCR, G-protein coupled receptor; PAMPs, pathogen-associated molecular patterns; γVPE, vacuolar processing enzyme. See Chapter 5, p. 102.

Fig. 7. (continued) shown. (**d**) One monomer of cyanobacterial PS I (pdb: 1jb0), view is along the membrane plane. The three cytoplasmic subunits PsaC (magenta), PsaD (blue) and PsaE (green) are shown at the top, other subunits are indicated in panel e, Fe_4S_4 clusters are shown as red and yellow spheres, other cofactors are colored as in panel b. (**e**) Trimeric PS I, view is from the cytoplasmic side onto the membrane, membrane extrinsic parts are omitted. In monomer I all cofactors are shown, in monomer II only Chls are given and in monomer III subunit assignments and positions of Car, lipids and phylloquinones (light blue) are given and TMH of PsaA and PsaB are labeled a–k. (**f**) Protein and cofactor assignment in the cyt b_6f dimer from *M. laminosus* (pdb: 2e74). Cofactors are colored as in previous panels; subunits are given in yellow (ISP), cyan (cyt b_6), red (cyt *f*), green (subunit 8), and magenta (subunit 4). (**g**) Top view (from the cytoplasmic side) of the trimeric LHCII complex from pea (pdb: 2bhw). Cofactors are colored as in other panels, protein in grey, TMH a-c are labeled in one monomer. See Chapter 10, p. 207.

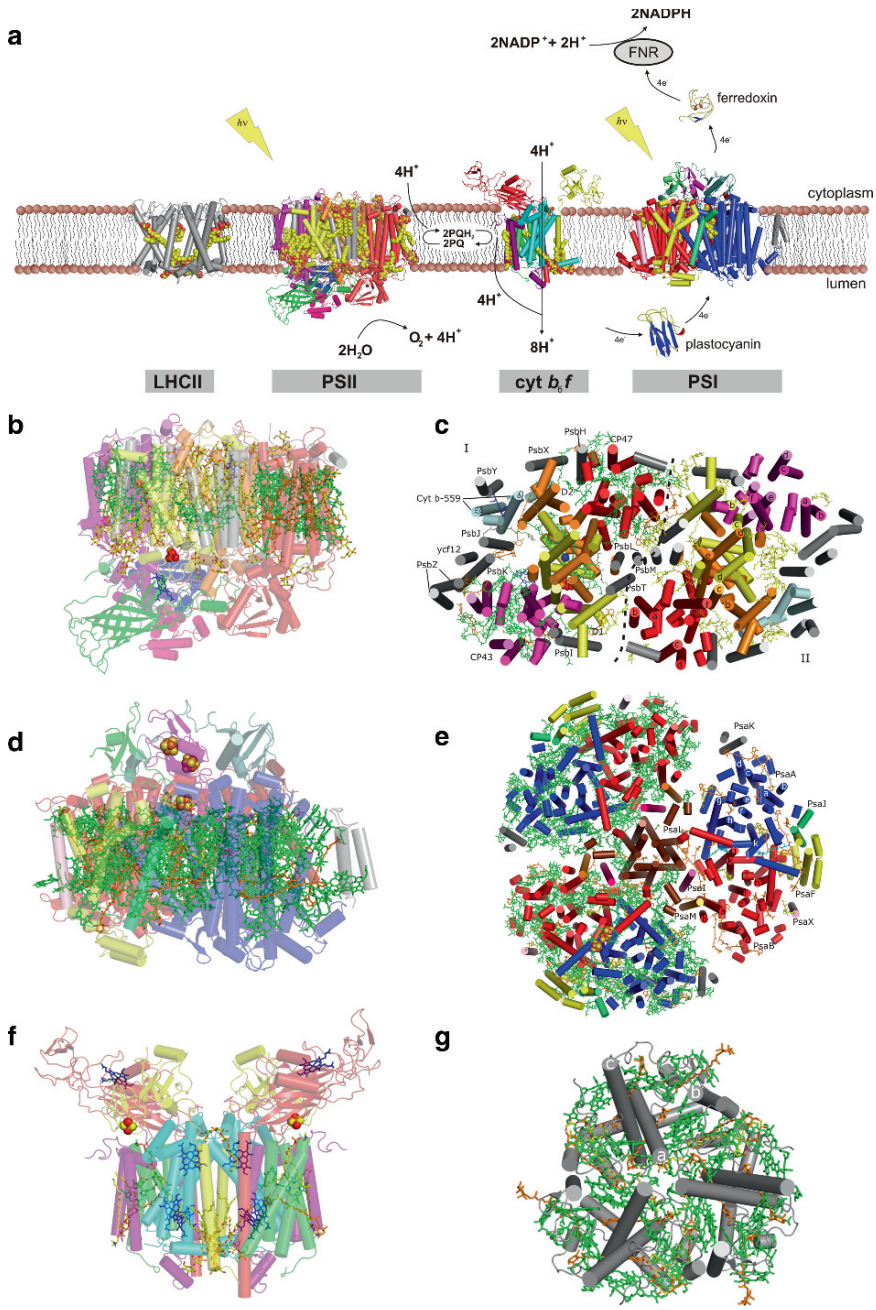


Fig. 7. Lipids in protein complexes of oxygenic photosynthesis. (**a**) Schematic view of the thylakoid membrane with PS I, PS II, LHCII and cyt b_6f embedded. For simplicity only a monomer of each complex is shown, TMHs are shown as cylinders, all cofactors except lipids are omitted. The lipids intrinsic to the structures are shown in space filling representation with yellow spheres for carbon and red spheres for oxygen. Proton as well as electron fluxes are indicated by *arrows*. (**b**) One monomer of cyanobacterial PS II (pdb: 3bz1), view is along the membrane plane. Chls are shown in green, Car in orange, lipids in yellow, heme in blue. Mn (red) and Ca (orange) of the Mn_4Ca cluster and the non-heme Fe^{2+} (blue) are shown as spheres. Membrane intrinsic subunits are assigned in panel c; the three lumenal membrane extrinsic subunits PsbO (green), PsbU (pink) and PsbV (blue) are visible at the *bottom*. (**c**) Dimeric PS II, view is onto the membrane plane from the cytoplasmic side, membrane extrinsic parts are omitted. The monomer–monomer interface is indicated by a black dotted line, the non-crystallographic C_2 axis relating the two monomers by a black ellipse. In monomer I (*left*) protein subunits are indicated and Chl, Car, Heme and PQ (olive) cofactors shown. In monomer II (*right*) TMH of D1, D2, CP43 and CP47 are named a-e/f and lipid and detergent molecules (yellow) are

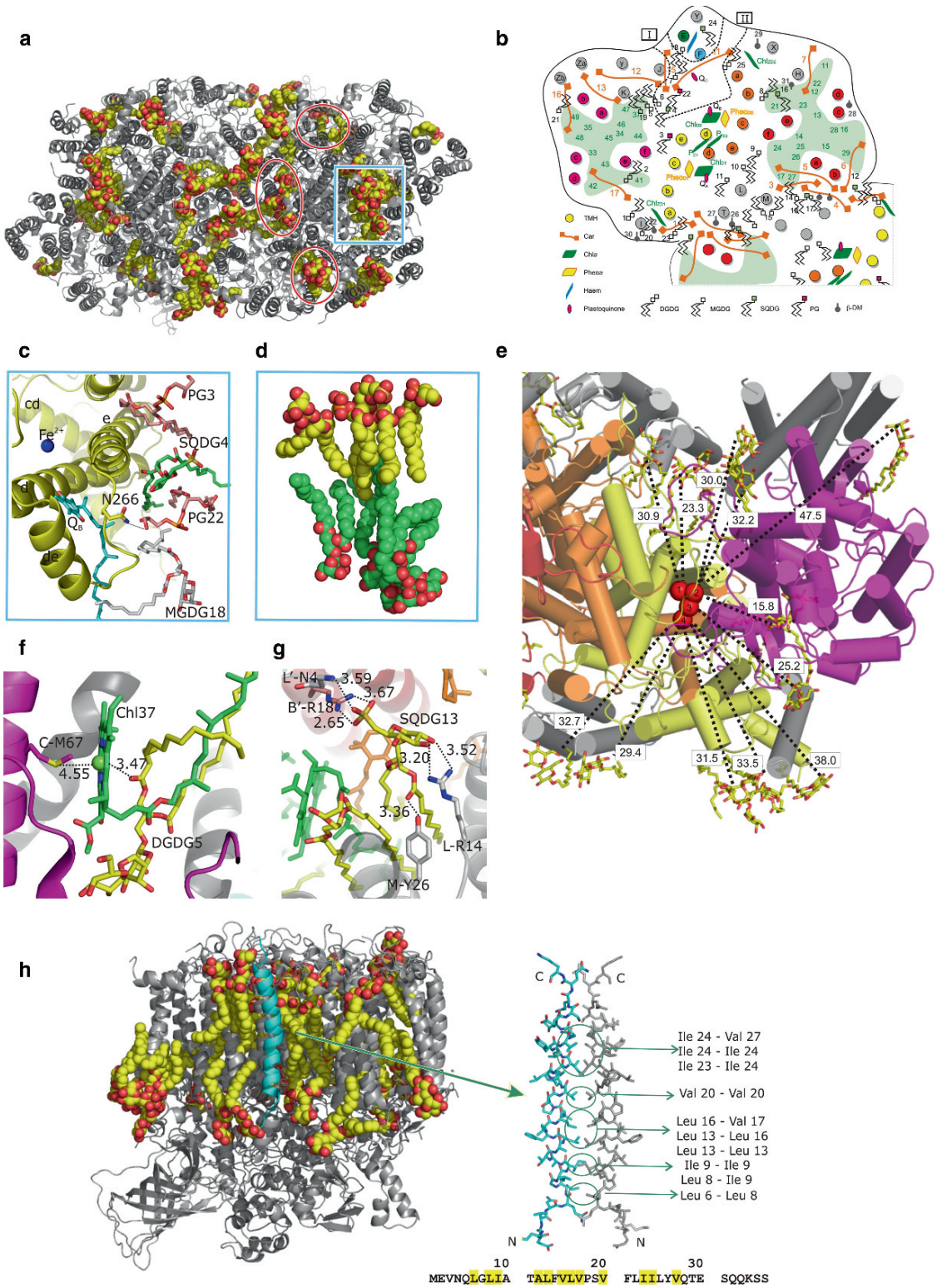

a
b
c
d
e
f
g
h
cd
e
PG3
Fe2+
SQDG4
d
N266
QB
PG22
de
MGDG18
L'-N4
3.59
3.67
B'-R18
2.65
SQDG13
3.20
3.52
3.36
L-R14
M-Y26
Chl37
C-M67
4.55
3.47
DGDG5
30.0
30.9
23.3
32.2
47.5
15.8
25.2
32.7
29.4
31.5
33.5
38.0
TMH
Car
Chla
Pheoa
Haem
Plastoquinone
DGDG
MGDG
SQDG
PG
β-DM
Ile 24 - Val 27
Ile 24 - Ile 24
Ile 23 - Ile 24
Val 20 - Val 20
Leu 16 - Val 17
Leu 13 - Leu 16
Leu 13 - Leu 13
Ile 9 - Ile 9
Leu 8 - Ile 9
Leu 6 - Leu 8
C
C
N
N
10
20
30
MEVNQLGLIA TALFVLVPSV FLIILYVQTE SQQKSS

Fig. 8. Lipids in PS II. (**a**) The membrane intrinsic part of the dimeric PS II complex, viewed from the cytoplasmic side, protein is shown in cartoon mode in grey, lipid and detergent molecules in space filling representation with carbons in yellow and oxygens in red. Lipid clusters between D1/D2 and CP43/CP47 are indicated by *red ellipses*, the region around the Q_B site is marked by a *blue rectangle*. (**b**) Schematic view of cofactor positions in PS II, shown is one monomer and part of the second monomer; the quinone exchange cavity is indicated by a *broken line* and the two portals by roman numerals. TMHs are shown as *circles* with coloring according to subunits (D1 yellow, D2, orange, CP47 red, CP43 magenta, PsbE green, PsbF blue). Other subunits are indicated by letters, for example, PsbE by "E", whereas ycf12 is labeled "y". Symbols for lipids with head upwards or downwards indicate location of the head group at the cytoplasmic or lumenal side, respectively. Chl positions in CP43 and CP47 are given by green numbers. (**c**) Lipids positioned around the Q_B binding site formed by subunit D1 (yellow). The non heme Fe^{2+} is shown as blue sphere, the PQ9 bound to the Q_B-site in light blue, PG in salmon, SQDG in green and MGDG in grey. (**d**) Bilayer like arrangement of eight lipids in the quinone exchange cavity, lipids with head group at the cytoplasmic side are shown with carbons in yellow, lipids with head group at the lumenal side with carbons in green. (**e**) Lipids on the lumenal side in vicinity of the Mn_4Ca cluster (*red spheres*). Coloring of subunit is as in panel (b), view is from the lumenal side onto the membrane plane. Distances from lipid head groups to the Mn_4Ca cluster in Å are indicated. (**f**) Coordination of the central Mg^{2+} of Chl 37 (green) by the glycerol moiety of DGDG5 (yellow). Surrounding protein is shown in magenta (CP43) and grey (other subunits). (**g**) Interactions of the polar head group of SQDG13 (yellow) with amino acid side chains from PsbL, PsbM, PsbL′ and CP47′. Possible hydrogen bond interactions are shown by *dotted lines* and distances are given in Å. (**h**) Lipids in the monomer-monomer interface, shown is one monomer looking onto the monomer–monomer interface along the membrane plane, cytoplasm at the *top*, lumen at the *bottom*. Proteins are shown in cartoon presentation in grey; lipid and detergent molecules in space filling representation, with carbons in yellow, oxygens in red, subunit PsbM is highlighted in cyan. Interactions between subunits PsbM (cyan) of monomer I and of monomer II (PsbM' [grey]) are shown on the *right*. N- and C-termini are labeled; specific protein–protein interactions are indicated by *circles* and involve residues highlighted (yellow) in the amino acid sequence of PsbM given at the *bottom.* Panels a-d and h are adapted from Guskov et al. (2009). See Chapter 10, p. 216.

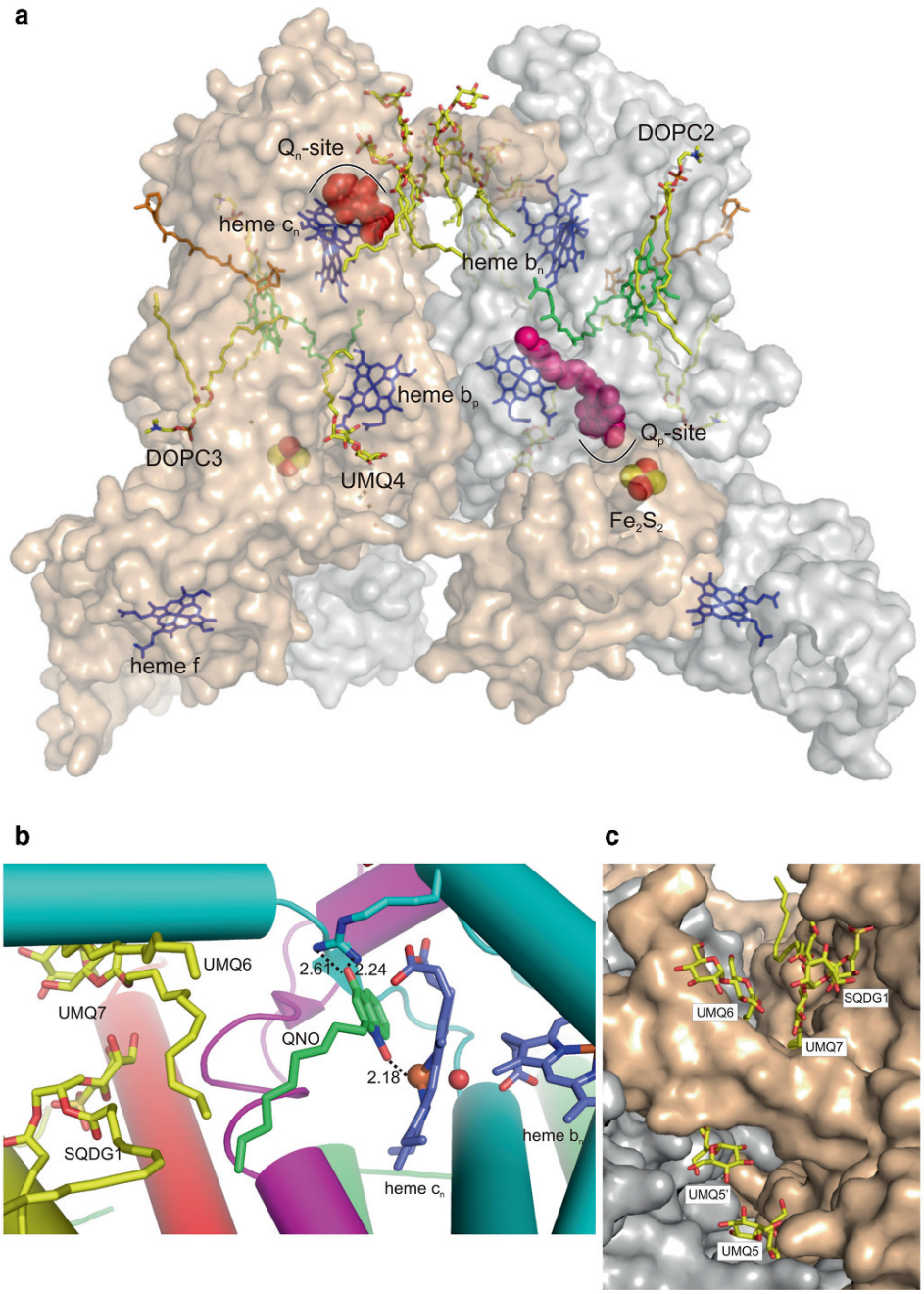


Fig. 9. The cyt b_6f complex from *M. laminosus*. (**a**) Side view of the dimeric cyt b_6f complex from the membrane interior into the quinone exchange cavity. Molecular surface as defined only by the polypeptide subunits of one monomer from the crystal structure of the complex with QNO (pdb 2E75) is shown in grey, corresponding surface of the second monomer from structure of the complex with TDS (pdb 2E76) in brown. The two inhibitors TDS (magenta), bound to the Q_p side, and QNO (red), bound to the Q_n side, as well as the Fe_2S_2 clusters (orange and red) are shown in sphere representation. Lipids and detergent molecules are shown in yellow, other cofactors in green (Chl), blue (Heme) and orange (Car). (**b**) The cytoplasmic quinone binding side Q_n and its surroundings (pdb code 2E76). Protein is shown in cartoon mode with cyt b_6 in cyan, cyt f in red, subunit 8 in green, and subunit 4 in magenta. The Q_n side inhibitor QNO (green), replacing PQ at the Q_n side, binds as an axial ligand to the central Fe of heme c_n (blue). Lipid SQDG1 and the two detergent molecules UMQ6 and UMQ7 (yellow) are in the direct vicinity of the Q_n side and their fatty acids are partially interacting with the aliphatic tail of QNO/PQ. (**c**) Top view onto a part of the monomer–monomer interface from the cytoplasmic side, surface of one monomer is shown in grey the other in brown. The lipid and detergent molecules in the monomer-monomer interface are shown in yellow (pdb code 2E74). See Chapter 10, p. 219.

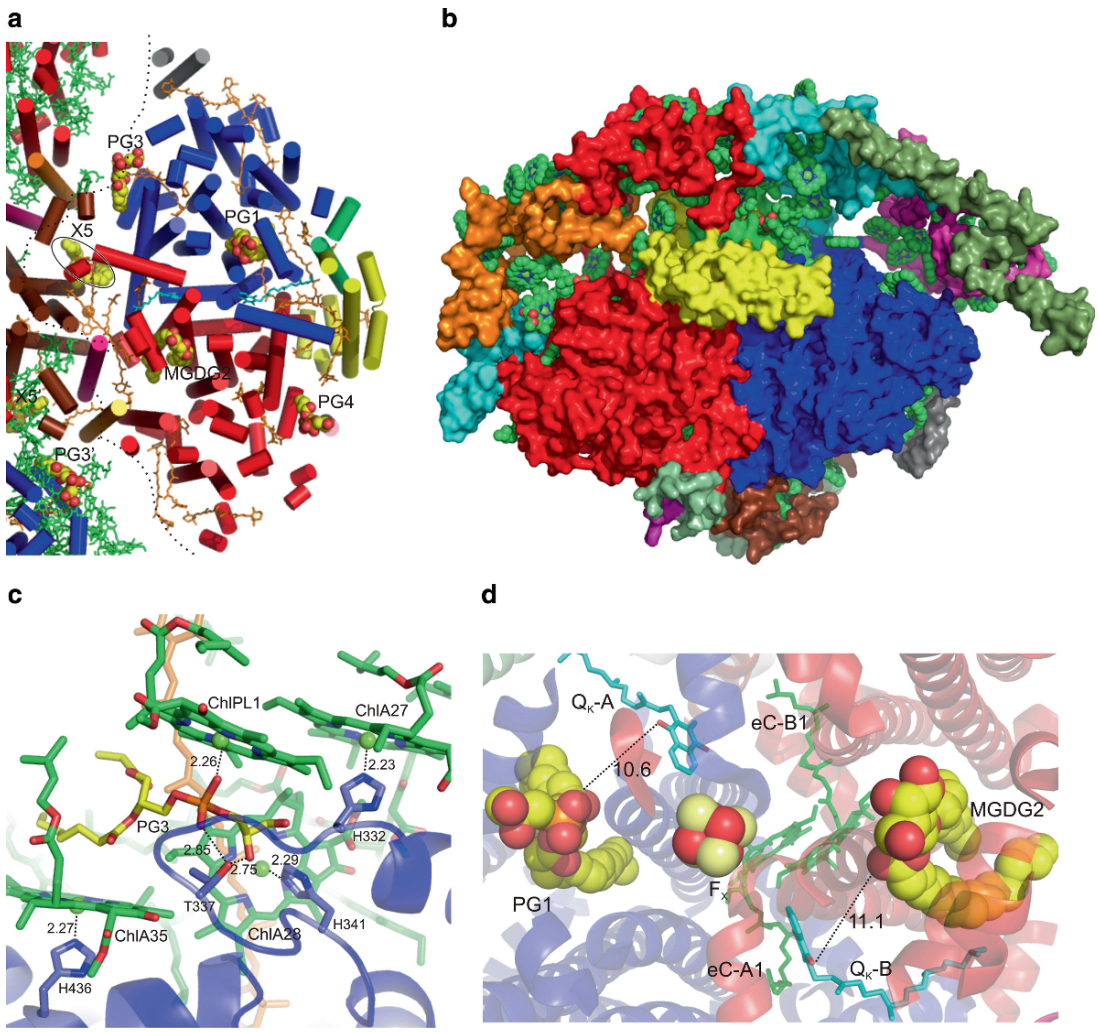


Fig. 10. Lipids in PS I. (**a**) Lipid positions in cyanobacterial PS I (pdb 1jb0), view is from the cytoplasm onto the membrane plane, protein is shown in cartoon mode with coloring of subunits and cofactors as in Fig. 1e, Chapter 10. The four lipids PG1, MGDG2, PG3 and PG4, as well as the glycerol moiety of the putative fifth lipid X5, are shown in space filling representation. The position of the fifth lipid is also indicated by a *black ellipse*. (**b**) Molecular surface of the plant PS I complex (pdb 2o01) as defined by the polypeptide subunits, view is from the lumenal side onto the membrane plane. The subunits common with the cyanobacterial system are colored as in Fig. 1e, the array of LHCI complexes arranged in a half moon shape around one side of the PS I core is shown in magenta (Lhca3), cyan (Lhca2), red (Lhca4) and orange (Lhca1) with the lumenal subunit PsaN (olive) partly connecting Lhca3 and Lhca2. The Chls are shown in space filling representation with carbons in green, nitrogens in blue, oxygens in red. Clearly visible is the void region between LHCI and the PS I core, which is most likely filled by ca. 30 lipids. (**c**) Coordination of Chl PL1 (green) by lipid PG3 in cyanobacterial PS I. Possible hydrogen bond interaction between the lipid head group and the protein and ligation of the central Mg^{2+} of the Chl are indicated by black dotted lines, distances are given in Å. (**d**) Arrangement of lipids PG1 and MGDG2 (in space filling representation, carbons yellow) symmetrically to the Fe_4S_4 cluster F_X (shown as *spheres*) and next to phylloquinones Q_K-A and Q_K-B (in stick representation, carbons in cyan) with distances given in Å. Subunits PsaA (blue) and PsaB (red) are shown in cartoon mode. See Chapter 10, p. 221.

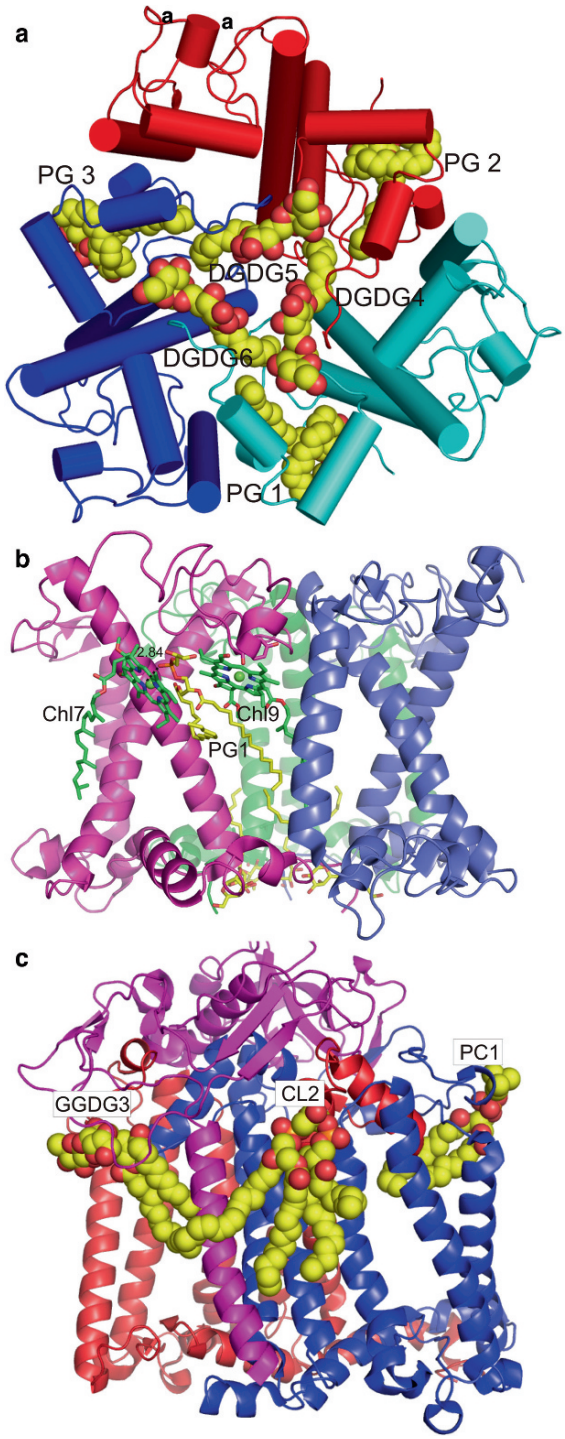


Fig. 11. Lipids in other membrane protein complexes. (**a**) Structure of trimeric LHCII from pea (pdb 2bwh), view is from the lumenal side onto the membrane. Protein from each monomer is shown in different color, lipids are shown in space filling mode with carbons in yellow and oxygen in red. The central position of DGDG in the middle of the trimer is easily visible. (**b**) View along the membrane plane, cytoplasm at top of trimeric LHCII. Visible is the ligation of the central Mg^{2+} of Chl 7 (green) by the oxygen of PG (yellow) and the interaction of PG with Chl 9, maybe providing indirect stabilization to the LHCII trimer. (**c**) Structure of PBRC from *Rb. sphaeroides* (pdb 2j8c) with subunits H (magenta), L (red) and M (blue) in cartoon mode. The three lipids identified in the structure are shown in space filling representation with carbons in yellow and oxygen in red, view is along the membrane plane with cytoplasm at *top.* See Chapter 10, p. 223.

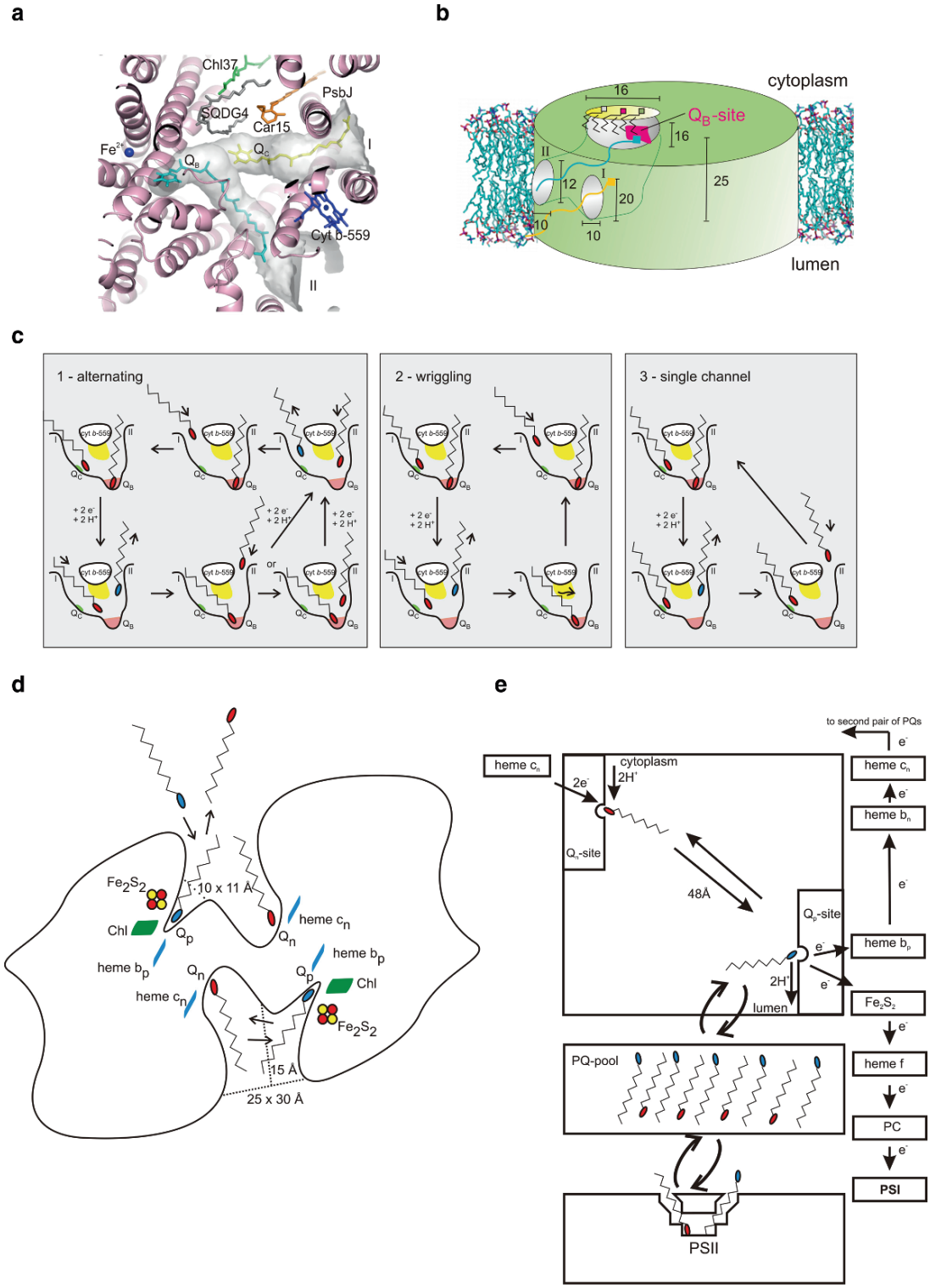


Fig. 12. Quinone exchange in PS II and cyt b_6f. For explanation and details. See Chapter 10, p. 233.

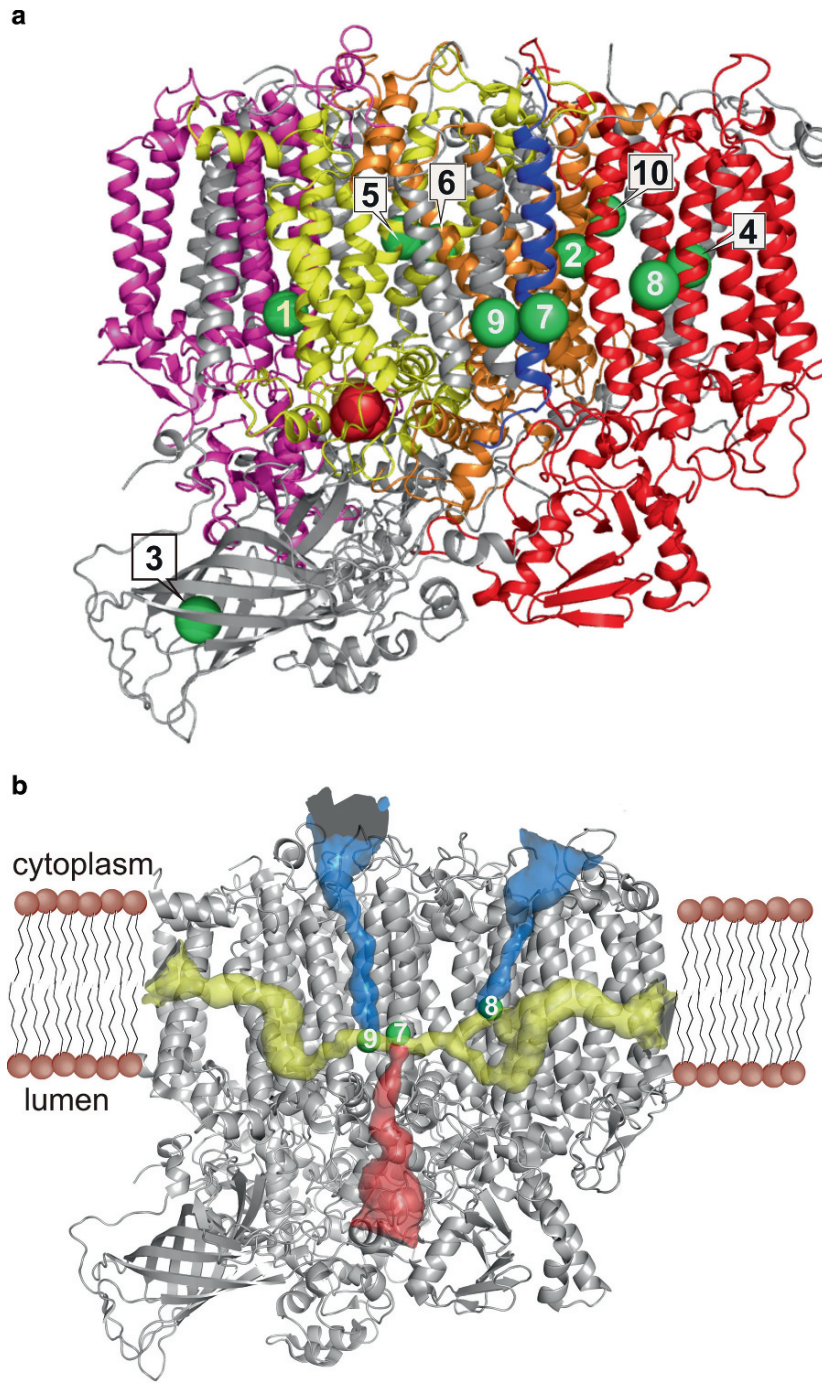


Fig. 13. Xe positions and possible oxygen diffusion in PS II. (**a**) Location of Xe sites (*green spheres* labeled 1–10) in the PS II monomer looking onto the monomer–monomer interface along the membrane plane, cytoplasm at the top, lumen at the bottom. All 20 protein subunits are shown in cartoon mode with D1 in yellow, D2 in orange, CP47 in red, CP43 in magenta and PsbM in blue, the remaining subunits in grey, cofactors, apart from the Mn_4Ca cluster (*red* and *yellow spheres*), are omitted. (**b**) Possible diffusion channels for Xe (and maybe oxygen) connecting Xe sites 7, 8 and 9 with the lumen (red), the cytoplasm (blue) and the membrane interior (yellow). See Chapter 10, p. 236.

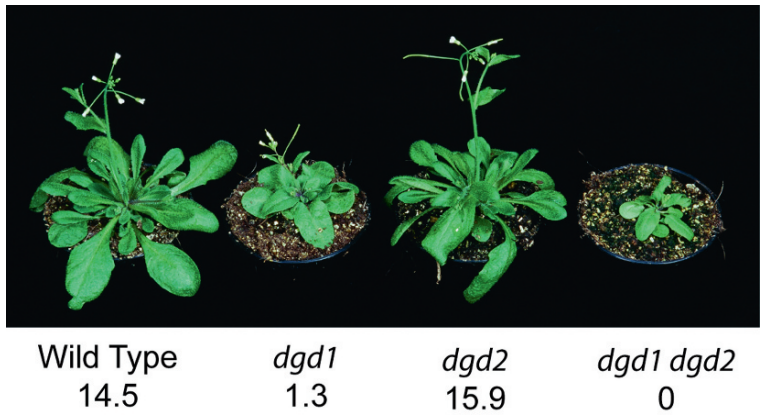


Fig. 14. Galactolipid deficient mutants of *Arabidopsis thaliana*. The lines *dgd1* and *dgd2* and the double mutant *dgd1 dgd2* carrying mutations in the *DGD1* and/or *DGD2* genes show different degrees of galactolipid reduction and growth retardation. The numbers represent the levels of DGDG in mole percent of total leaf lipids. See Chapter 12, p. 274.

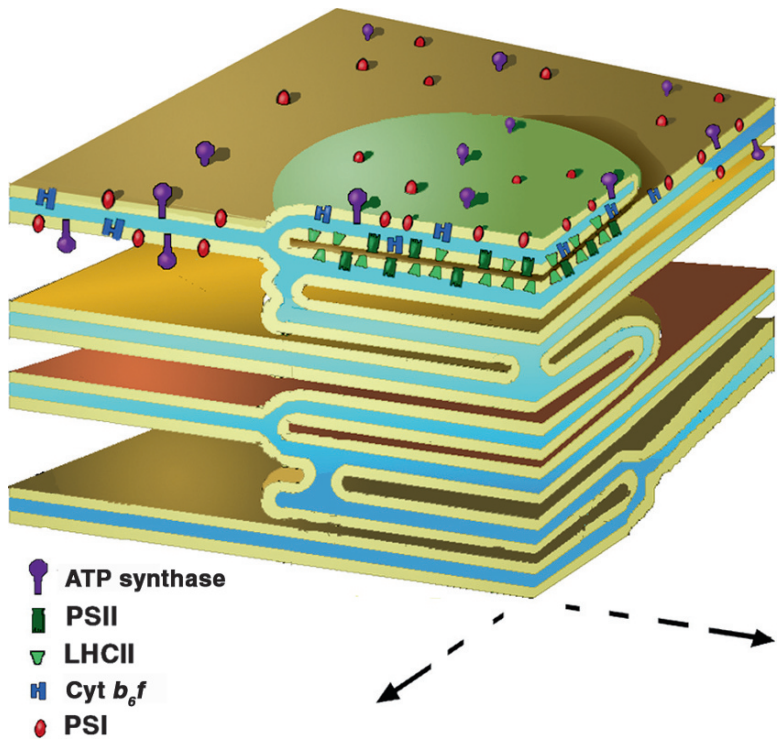


Fig. 15. Distribution of the major photosynthetic complexes within the thylakoid network of higher plants. PS II and its cognate light-harvesting complex, LHCII, are concentrated mainly in the appressed granal domains whereas PS I and ATP synthase populate non-appressed regions, which include the stroma lamellae and grana end membranes and margins. The other major photosynthetic complex, cytochrome b_6f, is distributed roughly evenly between the two membrane domains. (Note that, for clarity, only representatives of each type of protein complex are drawn; the actual protein density is much higher.). See Chapter 14, p. 308.

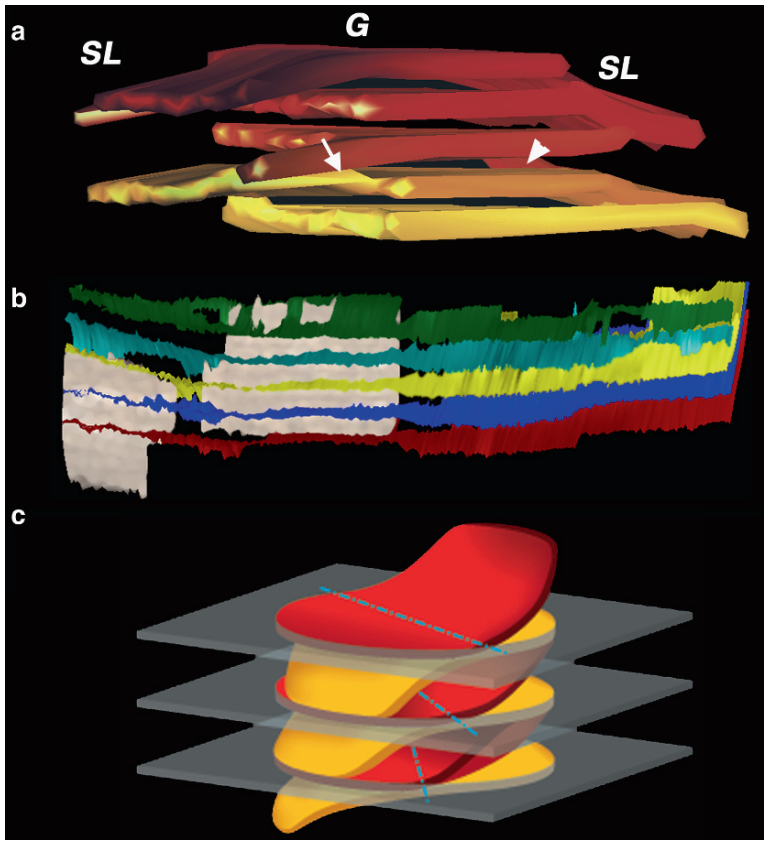


Fig. 16. Architecture of higher-plant thylakoid networks as derived from dual-axis tomographic data. (**a**) Three-dimensional structure of a granum–stroma assembly of a dark-adapted lettuce chloroplast. The granum layers (G) are contiguous with the stroma thylakoids (SL) that bifurcate at the granum–stroma interface. Adjacent layers in the granum are joined by internal connections (*arrows*). (**b**) An ensemble consisting of two grana interconnected by multiple stroma lamellae. The stroma lamellae (colored) form wide, slightly curved sheets that run parallel to each other and intersect the grana (white/gray objects) at an angle roughly perpendicular to the axis of the granum cylinder. The grana act as defined regions of sheet consolidation and increased connectivity. (**c**) A topological model of the granum–stroma assembly. The granum is made of repeating units that consist of two layers (red and yellow), which are formed by bifurcations of the stroma lamellae (gray). In each unit, part of the top layer (red) bends upwards and fuses with the layer above it whereas the other layer (yellow) bends downwards at the opposite side and fuses with the layer below. As indicated by the *blue dashed lines*, the units are rotated (counterclockwise) relative to each other (adapted from Shimoni et al., 2005, www.plantcell.org, copyright American Society of Plant Biologists). See Chapter 14, p. 312.

Fig. 18. Metabolic grid of ketocarotenoid biosynthesis. The metabolic grid of possible reactions that are part of astaxanthin biosynthesis is created by the combinatorial action of bacterial (CRTW) or algal (CRTO) ketolase enzymes and endogenous or recombinatly produced β-carotene hydoxylase enzymes (CRTZ, B1, B2). The figure shows intermediates/by-products that are frequently observed when ketolase genes are expressed alone or in combination with bacterial β-carotene hydoxylase enzymes (CRTZ) in transgenic plants. See Chapter 19, p. 416.

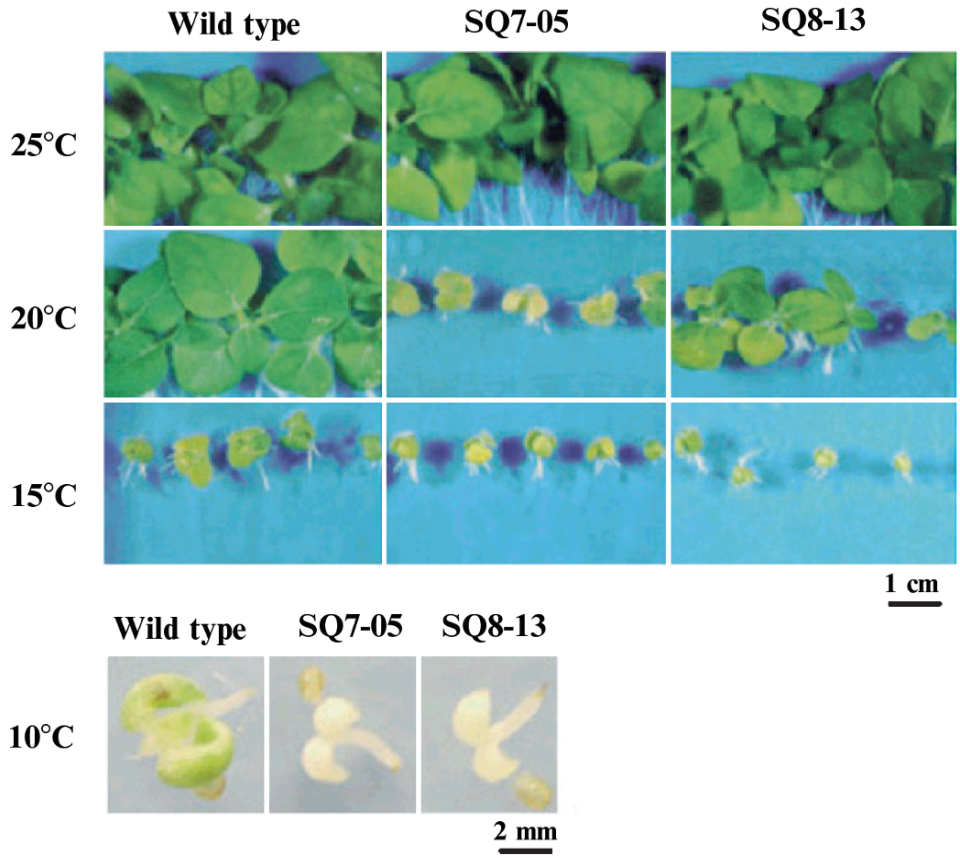
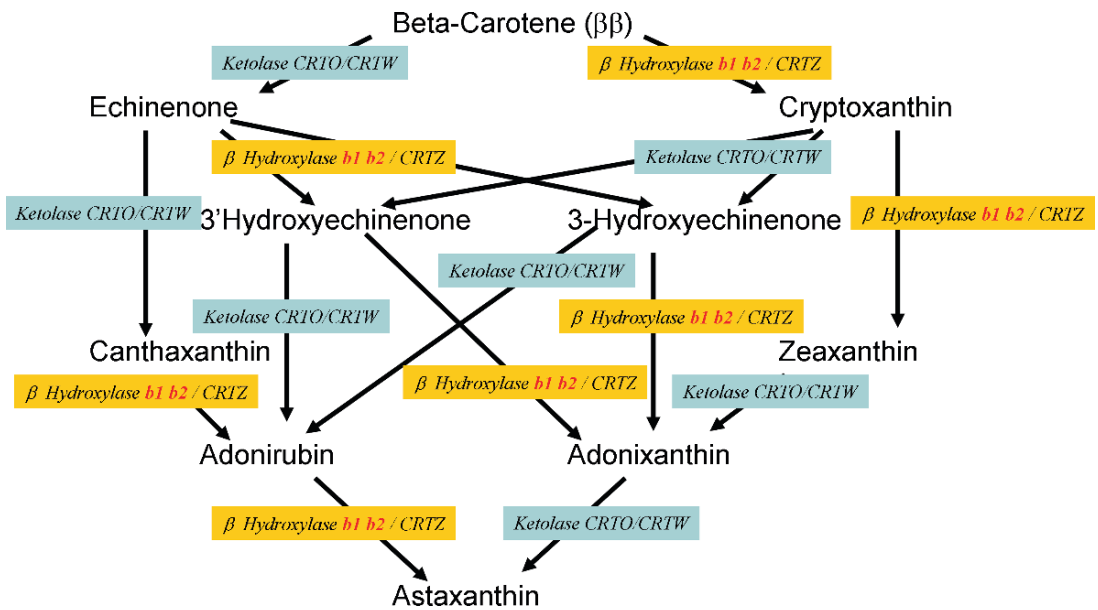


Fig. 17. A decrease in the unsaturation of fatty acids in chloroplastic PG in transgenic tobacco plants suppressed growth at 20°C and inhibited the greening of cotyledons at 10°C. Seeds were allowed to germinate and seedlings were allowed to grow for 1 month at designated temperatures under a 16-h light/8-h dark cycle. SQ7–05 and SQ8–13 were two independent lines of transgenic tobacco (reproduced with permission from Sakamoto et al., 2003). See Chapter 17, p. 384.



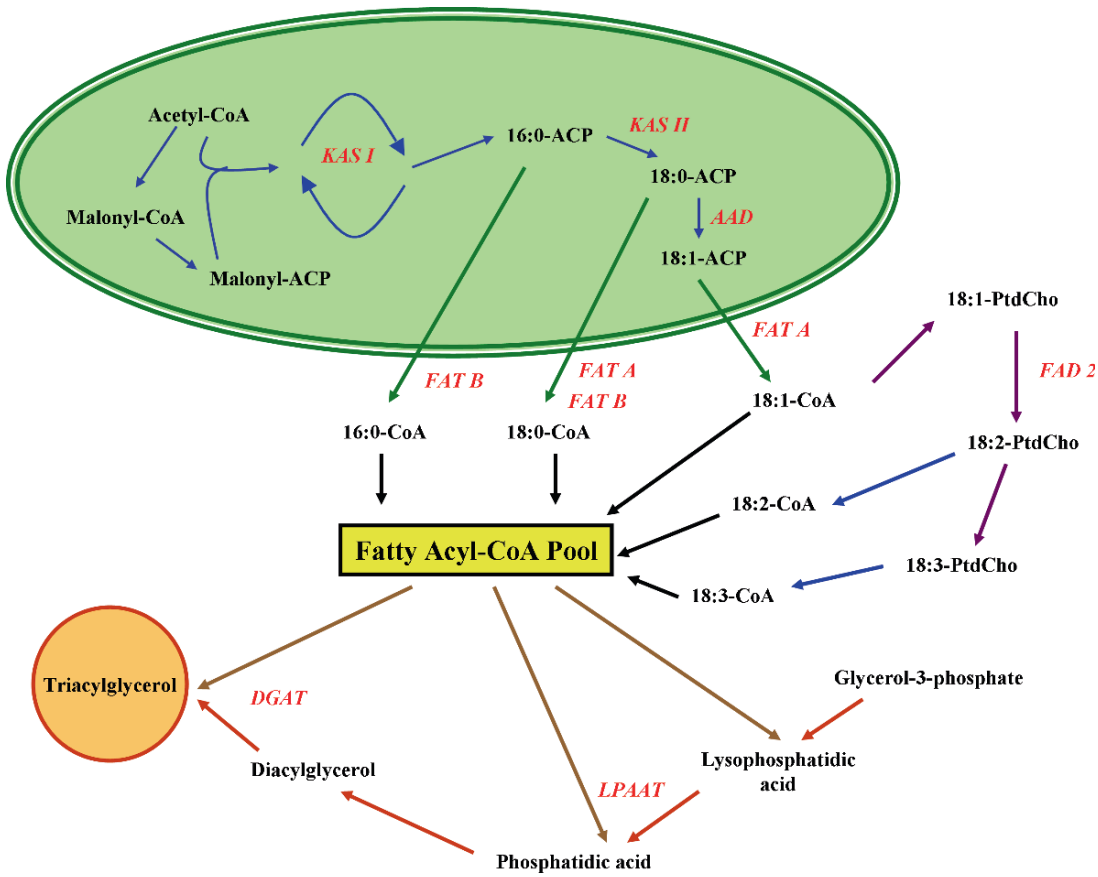


Fig. 19. Simplified pathway of primary metabolism in a developing oil seed and its relationship to production of acetyl-CoA for acyl lipid biosynthesis. Reactions discussed in Section V, Chapter 19 are highlighted in red. Abbreviations: cytACL, cytosolic ATP:citrate lyase; DHAP, dihydroxyacetone-3-phospate; G6PT, glucose-6-phosphate transporter; GAP, glyceraldehyde-3-phosphate; GK, glucokinase; INV, invertase; mtIDH, mitochondrial isocitrate dehydrogenase; mtME, mitochondrial malic enzyme; mtPDH, mitochondrial pyruvate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; PRK, phosphoribulokinase; ptPDH, plastidic pyruvatedehydrogenase; ptPGM, plastidic phosphoglucomutase; ptPK, plastidic pyruvate kinase; RUBISCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SUSY, sucrose synthase. See Chapter 19, p. 423.

Chapter 1

Lipids in Thylakoid Membranes and Photosynthetic Cells

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Summary

The goal of this book is to provide a comprehensive overview of recent advances in plant-lipid research, with special emphasis on the lipids in thylakoid membranes. The determination of the genome sequences of photosynthetic organisms, such as cyanobacteria, algae and higher plants; the development of mutational and transgenic techniques; and the development of other analytical methods have had a major impact on plant-lipid research and have advanced our understanding of the functions of individual species of lipids in photosynthesis. In this introductory chapter, we provide a brief summary of the material in subsequent chapters, in which specific topics are covered in greater depth.

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I Introduction

The early events in photosynthesis, such as the absorption of light, the photochemical reactions, the transport of electrons, and the synthesis of ATP, all occur in the thylakoid membranes of eukaryotic chloroplasts and cyanobacterial cells. Thus, obviously, the characteristics of these membranes are important for many aspects of photosynthesis. For basic information on the above topics (see Ke 2001). Thylakoid membranes are composed mainly of glycerolipids and proteins. There are four major glycerolipid components in thylakoid membranes, namely, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG). Since the discovery of these glycerolipids, many researchers have studied and debated the functions of the various glycerolipids in photosynthesis.

Photosynthesis involves the coordinated activities of chloroplasts and other organelles in plant cells and, therefore, the membrane lipids of all the cellular organelles influence photosynthetic activity. Thus, studies of the lipids in mitochondria, oxylipins (products of the oxygenation of unsaturated fatty acids), sphingolipids, and storage lipids (end products of photosynthesis) are also important for a full understanding and appreciation of photosynthesis.

The book "Lipids in Photosynthesis: Structure, Function and Genetics," which was edited by Siegenthaler and Murata (1998), summarized all available information about lipids and their relationship to thylakoid membranes and photosynthetic cells. However, genome sequences, the development of mutational and transgenic techniques, and the availability of new analytical methods have accelerated progress in the clarification of the roles of individual species of lipids in photosynthesis. Thus, it seems appropriate, at this time, to present a new book, "Lipids in Photosynthesis: Essential and Regulatory Functions," which summarizes the past decade's research on the synthesis and functions of lipids in chloroplasts and photosynthetic cells.

Abbreviations: ACP – Acyl-carrier protein; CoA – Coenzyme A; DGDG – Digalactosyldiacylglycerol; ER – Endoplasmic reticulum; FTIR – Fourier transform infrared; MGDG – Monogalactosyldiacylglycerol; PG – Phosphatidylglycerol; PGP – Phosphatidylglycerol phosphate; PS I – Photosystem I; PS II – Photosystem II; SQDG – Sulfoquinovosyldiacylglycerol; X:Y(Z) – Fatty acid in which X and Y indicate numbers of carbon atoms and double bonds, respectively, and Z, in parenthesis, indicates the position(s) of the double bond(s), counted from the carboxyl terminus of the fatty-acyl chain

II Genome Sequences Reveal Genes Involved in Lipid Synthesis

The sequencing of genomes of photosynthetic organisms has provided new and powerful tools for plant-lipid research and, in particular, for the identification of the genes and enzymes that are involved in lipid synthesis in chloroplasts and photosynthetic cells.

A Cyanobacterial Genomes

The determination of the sequence of the genome of the cyanobacterium *Synechocystis* sp. PCC 6803 (Kaneko et al., 1996) allowed the first genome-wide identification of genes in a photosynthetic organism. Since then, the genome sequences of many strains of cyanobacteria have been determined, for example, *Anabaena* sp. PCC 7120 (Kaneko et al., 2001), and *Thermosynechococcus elongatus* (Nakamura et al., 2002). Sequences of cyanobacterial genomes are freely available from the Genome Database for Cyanobacteria (CyanoBase; http://genome.kazusa.or.jp/cyanobase/).

Genome sequences revealed a number of previously unidentified genes for lipid synthesis in cyanobacteria, for example, genes for lysophosphatidic acid acyltransferase (Okazaki et al., 2006), for phosphatidic acid phosphatase (Nakamura et al., 2007), for monoglucosyldiacylglycerol synthase (Awai et al., 2006), for DGDG synthase (Awai et al., 2007; Sakurai et al., 2007), and for the synthesis of sulfolipids and PG (see Chapters 8 and 11). Comparison of genome sequences revealed the presence of two *desC* genes (*desC1* and *desC2*) for Δ9 desaturases in cyanobacterial species in so-called Group 2 (Chintalapati et al., 2006) but of only one *desC* gene in species in the other groups. This observation reflects the fact that only species in Group 2 have a Δ9-unsaturated fatty acid at the *sn*-2 position of the glycerol moiety of MGDG (see Chapter 8).

The genome sequence of *Synechocystis* predicts the presence of close to 40 putative genes for lipoproteins. Three of them are genes for PsbP, PsbQ, and Psb27, which are known to regulate the activity of photosystem II (PS II) (see, Chapter 11).

Cyanobacteria, such as *Anabaena* sp. PCC 7120, form specialized cells called heterocysts under nitrogen-deficient conditions. Heterocysts can fix N_2 as ammonia via the activity of a nitrogenase that is rapidly inactivated by O_2. They produce heterocyst-specific glycolipids that are used for construction of an envelope barrier that slows the rate of entry of O_2. The genome sequence of *Anabaena* sp. PCC 7120 revealed a number of genes that are involved in the biosynthesis and deposition of heterocyst-specific glycolipids (Chapter 9).

B Lower-Plant Genomes

The genome sequences of five lower plants have been determined, namely, those of a red alga, two diatoms, a green alga and a moss. The genome sequence of the red alga *Cyanidioschyzon merolae* (Matsuzaki et al., 2004) suggests the presence of three putative genes for a stearoyl-CoA desaturase and for two acyl-lipid desaturases that may act on Δ9 and Δ12 positions of fatty acids (Sato and Moriyama, 2007). Moreover, sequence homology and biochemical analysis suggest that these desaturases might be similar to desaturases of marine cyanobacteria but not to typical cyanobacterial desaturases and that fatty acids might be desaturated in the endoplasmic reticulum (ER) and then transported to chloroplasts in the red alga (Sato and Moriyama, 2007). This scenario is distinctly different from the synthesis of chloroplast lipids in other algae and higher plants.

Diatoms are unusual in terms of their fatty acid composition since they contain eicosapentaenoic acid [20:5(5,8,11,14,17)] and docosahexaenoic acid [22:6(4,7,10,13,16,19)] (see Chapter 6). Analysis of the sequence of the nuclear genome of the diatom *Thalassiosira pseudonana* (Armbrust et al., 2004) revealed the presence of eleven open reading frames whose putative products exhibited significant similarity to functionally characterized fatty-acid desaturases. Among the open reading frames, Tonon et al. (2005) identified putative genes for Δ6-, Δ5- and Δ4-desaturases of fatty acids, as well as a putative gene for a Δ11-desaturase that is active on palmitic acid and Δ8-sphingolipids. The genome sequence of another diatom, *Phaeodactylum tricornutum*, has recently determined (Bowler et al., 2008). The genome sequences of the green alga *Chlamydomonas reinhardtii* (Karpowicz et al., 2007) and the moss *Physcomitrella patens* (Rensing et al., 2008) have been also determined. Analysis of these genome sequences will stimulate productive research on the lipids of algae and other lower plants. For information on molecular biology of the green alga *Chlamydomonas reinhardtii*, see Rochaix et al. (1998).

C Higher-Plant Genomes

The genome sequence of *Arabidopsis thaliana* was determined in 2000 (The Arabidopsis Genome Initiative) and was the first for a higher plant. Since then, the genome sequences of many higher plants have been determined, namely, *Glycine max* (soybean), *Medicago truncatula* (barrel clover), *Oryza sativa* (rice), *Populus trichocarpa* (black cottonwood), *Sorghum bicolor* (sorghum), and *Zea mays* (corn). Sequencing of the genomes of *Brachypodium distachyon* (purple false brome), *Lotus japonicus* (lotus), *Manihot esculenta* (cassava), *Solanum lycopersicum* (tomato), and *Solanum tuberosum* (potato) is underway and complete genome sequences should be available in the near future. Sequence data can be obtained from the database of the National Center for Biotechnology Information (NCBI, USA; http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html).

The above-mentioned genome sequences have revealed a number of genes that are involved in the biosynthesis of fatty acids and lipids in higher plants, for example, genes for MGDG synthase (see Chapter 3), for DGDG synthase (see Chapter 12), and for the synthesis of sulfolipids and PG (see Chapters 11 and 12). In *A. thaliana*, there appear to be three genes (*MGD1, MGD2* and *MGD3*) for MGDG synthase (Awai et al., 2001), two genes for DGDG synthase (Dörmann et al., 1999; Kelly et al., 2003), and two genes for phosphatidylglycerol phosphate (PGP) synthase (Müller and Frentzen, 2001; Xu et al., 2002). The MGDG synthase encoded by *MGD1* is localized in the inner envelope membrane, whereas the synthases

encoded by *MGD2* and *MGD3* are localized in the outer envelope membrane. The expression of the latter MGDG synthases is strongly induced under phosphate-limiting conditions. The DGDG synthases encoded by *DGD1* and *DGD2* are localized in the outer envelope membrane and the expression of both genes is induced by phosphate starvation. These findings indicate that MGDG synthesized by MGD2 and MGD3 in the outer envelope membrane is used for the biosynthesis of DGDG by DGD1 and DGD2, and they are consistent with the evidence that phospholipids in plasma membranes and mitochondrial membranes are replaced by DGDG that has been transported from plastids under phosphate-limiting conditions (Jouhet et al., 2004; Andersson et al., 2005). The PGP synthase encoded by *PGP1* is localized in plastids and mitochondria (Babiychuk et al., 2003), whereas the PGP synthase encoded by *PGP2* is localized in the ER (Müller and Frentzen, 2001), suggesting that PG might be synthesized in three compartments in plant cells. This scenario is different from that in yeast and mammalian cells, in which PG is synthesized mainly in mitochondria as a precursor for biosynthesis of cardiolipin, except cells of fetal lung tissues where PG that is required for the synthesis of surfactants is synthesized in both mitochondria and ER (Batenburg et al., 1985).

III Mutagenesis and Transgenic Techniques

A Targeted Mutagenesis in Cyanobacteria

In some strains of naturally transformable cyanobacteria, such as *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942 and *Synechococcus* sp. PCC 7002, a target gene can be mutated by double homologous recombination (Williams, 1988). *Anabaena* sp. PCC 7120 can be transformed more efficiently by conjugation (Elhai and Wolk, 1988). These techniques have been used for the functional characterization of fatty acid desaturases (Wada and Murata, 1998) and their use led to the demonstration that the extent of unsaturation of fatty acids in membrane lipids influences the tolerance to cold stress and salt stress (Chapter 17).

Once the genes for the synthesis of specific lipid classes, such as PG, SQDG and DGDG, had been identified, they were mutated by targeted mutagenesis. The resultant mutants were devoid of the respective lipids, and their properties suggested that PG (Chapter 11) might be essential for photosynthetic activity, whereas DGDG (Chapter 12) might not essential for photosynthetic activity but might stabilize the PS II complex. The role of SQDG in photosynthesis appears to be dependent on cyanobacterial species (Chapters 8 and 12). In *Synechococcus* sp. PCC 7942, SQDG might be unessential for photosynthesis and could be replaced by PG, in terms of function, when supplies of phosphate were adequate. By contrast, in *Synechocystis* sp. PCC 6803, SQDG is essential for photosynthesis. *Anabaena* sp. PCC 7120 produces heterocyst-specific glycolipids in addition to MGDG, DGDG, SQDG, and PG, and a number of genes that might be involved in the biosynthesis and deposition of heterocyst-specific glycolipids have been identified (Chapter 9). Their functions in the biosynthesis and deposition of heterocyst-specific glycolipids have been studied by targeted mutagenesis (Chapter 9).

B T-DNA Insertional Mutagenesis

Mutagenesis via the insertion of T-DNA is a powerful method that can be exploited in higher plants for the identification of specific genes and for the characterization of the products of such genes and their specific functions. An analysis of T-DNA mutants of *Arabidopsis thaliana* revealed the three isoforms of MGDG synthase, namely, MGD1, MGD2 and MGD3, as well as their respective localizations and functions (Chapter 3). Studies of T-DNA mutants of genes involved in the synthesis of DGDG, SQDG and PG have suggested important functions for DGDG and PG in photosynthesis and other physiological activities in plants, whereas SQDG appears to be unessential for photosynthesis, even though it is required for growth when the availability of phosphate is limited (Chapters 11 and 12). T-DNA insertional mutagenesis has been used to identify genes involved in the biosynthesis of sphingolipids (Chapter 5) and carotenoids (Chapter 19), as well as the functions of these lipids.

C Repression of Gene Expression by Antisense RNA and RNA Interference

The expression of antisense RNAs in plants represses the expression of the corresponding genes. This method has been exploited to repress the expression of the *ATS1* gene for glycerol-3-phosphate acyltransferase in tomato and, thereby, to demonstrate that the unsaturation of fatty acids in lipids of thylakoid membranes is important for male fertility and other physiological activities (Chapter 17). A similar method was used to repress the expression of a gene that is involved in the synthesis of systemin (Chapter 18) and of the gene for geranylgeranyl reductase, which is important in the synthesis of chlorophylls, carotenoids and tocopherols (Chapter 19).

RNA interference can also be used to limit or repress the expression of target genes. For example, levels of sphingolipids and tocopherols can be lowered by RNA interference that represses the expression of genes for enzymes that are involved in the synthesis of respective compounds (Chapters 5 and 19).

IV Crystallographic Analysis of Soluble and Membrane-Bound Proteins

Crystallographic techniques and the x-ray diffraction analysis of crystals have allowed the detailed characterization of proteins in chloroplasts and cyanobacterial cells. These proteins can be classified into two groups, one that includes water-soluble proteins, such as enzymes of the Calvin–Benson cycle and fatty acid-synthesizing enzymes, and one that includes water-insoluble membrane-spanning proteins, of which typical example in photosynthetic cells are the photosystem I (PS I) and PS II supercomplexes (for details on PS I, see Golbeck, 2006; and for PS II, see Wydrzynski and Satoh, 2006). In general, crystallization of the latter group of proteins is difficult. However, recent progress in crystallization techniques has allowed high-resolution analysis of these proteins.

A Soluble Proteins

The first successful crystallographic analysis of a lipid synthesis-related protein from plants was that of enoyl-ACP reductase from *Brassica napus* (Rafferty et al., 1995). Next, complete crystallographic analysis was reported for stearoyl-ACP desaturase from castor bean (*Ricinus communis*; Lindqvist et al., 1996) and for 3-ketoacyl-ACP reductase from rapeseed (*Brassica napus*; Fisher et al., 2000), glycerol-3-phosphate acyltransferase from squash (*Cucurbita moschata*; Turnbull et al., 2001), and 3-ketoacyl-ACP synthase from *A. thaliana* (Olsen et al., 2004). To date, no other enzymes involved in fatty acid synthesis in plants have been analyzed crystallographically. However, homologous proteins from bacteria and eukaryotes have been analyzed crystallographically and, thus, some information is available for extrapolated considerations of their respective functions in plant cells (see Chapter 2).

B Membrane-Bound Proteins

The first success in this field was the crystallographic analysis of the photochemical reaction center that mediates bacterial photosynthesis (Deisenhofer et al., 1985). This achievement led to the successful analysis of the structures of other photosynthetic supercomplexes, such as the PS I complex, the PS II complex, the cytochrome b_6f complex and the light-harvesting complex II. The resolution in early studies was not high enough to reveal details of the lipid molecules in the complexes but more recent analysis, at high resolution, has revealed the numbers of individual lipid molecules and their exact locations, as well as their interactions with specific amino acid residues (see Chapter 10). For example, analysis of the PS II complex from *Thermosynechococcus elongatus* at 2.9 Å resolution indicated that this complex contains 25 integral lipids, namely, eleven molecules of MGDG, seven molecules of DGDG, five molecules of SQDG and two molecules of PG (Guskov et al., 2009). Analysis of PS I from *Thermosynechococcus elongatus* at 2.5 Å resolution indicated that this complex contains one molecule of MGDG and three molecules of PG (Jordan et al., 2001); Analysis of the cytochrome b_6f complex from *Chlamydomonas reinhardtii* indicated that this complex contains two molecules of MGDG and one molecule of SQDG (Stroebel et al., 2003).

V Electron Microscopy

The structures of thylakoid membranes in cyanobacterial cells and plant chloroplasts have been studied by electron microscopy, which allows visualization of thylakoid and other membranes with a resolution two orders of magnitude higher than that attainable by light microscopy. Cross-sectional electron-microscopic analysis of cryo-fixed materials and electron-microscopic tomography has revealed the structures of thylakoid membranes in three dimensions at molecular or near-molecular resolution. These techniques have advanced our understanding of the distribution and organization of the major photosynthetic protein complexes within the thylakoid membranes, as described in detail in Chapter 14.

VI Improvements in Analytical Techniques

Recent years have seen the development of very sensitive methods for the analysis of lipids, and several methods for the separation of lipid molecules have been greatly improved. Combinations of such methods have allowed detailed analyses of lipids from very small amounts of test materials derived, for example, from chloroplasts and photosynthetic cells. Thus, each individual molecular species of lipids in thylakoid membranes can now be identified by combination of high-performance liquid chromatography/mass spectrometry, as discussed in Chapter 20.

VII Physical Methods for Assessing the Fluidity of Membrane Lipids

The importance of membrane fluidity in the regulation of photosynthetic activity has been discussed for many years, and changes in fluidity have been predicted from the extent of unsaturation of the fatty acids in membrane lipids. In some earlier studies, the fluorescence anisotropy of diphenylhexatriene was used to monitor the changes in membrane fluidity. However, this method does not yield a quantitative assessment of the fluidity or rigidity of membrane lipids, and there has been a need for methods that provide quantitative measures of membrane fluidity. Recently, two appropriate methods were developed and applied to thylakoid membranes from cyanobacterial cells and plant chloroplasts.

Szalontai et al. (2000) used Fourier transform infrared (FTIR) spectroscopy for the quantitative analysis of the physical state of membrane lipids. FTIR spectroscopy allows an assessment of the disorder of the acyl chains of lipids and the interactions between lipids and membrane proteins in terms of the frequency of the symmetric CH_2 stretching mode near 2851 cm^{-1}. This frequency decreases, by approximately 2 to 5 cm^{-1}, upon the transition of membrane lipids from an ordered to a disordered state. Thus, low and high frequencies of the CH_2 stretching mode correspond to the rigidified and fluid states of membrane lipids, respectively, as discussed in Chapters 13 and 15.

Mullinaux and his colleagues used a method of fluorescence recovery after photobleaching and the single-particle tracking to determine the rate of diffusion of proteins along thylakoid membranes, as discussed in Chapter 13. They observed that, when the unsaturation of fatty acids in membrane lipids was increased by expressing the *desA* gene for Δ12 desaturase from *Synechocystis* sp. PCC 6803 in *Synechococcus* sp. PCC 7942 cells, the rate of diffusion of membrane proteins fell significantly.

VIII DNA Microarrays

In early studies of the effects of membrane fluidity on the cold- and heat-induced expression of genes, only a limited number of genes could be examined because of practical limitations to the number of transcripts that can easily be subjected to Northern blotting analysis (see Chapter 15).

DNA microarrays, which have recently become available, are powerful tool with which to examine the genome-wide expression of genes that respond to various kinds of perturbation, such as forms of various biotic and abiotic stress and genetic mutations. Such microarrays have been used successfully to investigate the effects of membrane fluidity/rigidity and of stresses on genome-wide expression of genes. When membranes of *Synechocystis* sp. PCC 6803 were rigidified by mutation of fatty acid desaturases, with elimination of polyunsaturated fatty acids from the membrane lipids, the cold-induced expression of many genes was markedly altered

(see Chapter 15; Inaba et al., 2003). These findings suggested that the cold signal might be perceived by cells via the rigidification of membrane lipids, with resultant activation of the expression of many genes. Hyperosmotic stress seems to be perceived, in part, via similar rigidification of membranes, as also discussed in Chapter 15.

IX Perspective

Recent advances in the identification of genes involved in the biosynthesis of lipids in photosynthetic organisms and in the gene manipulation of genes by, for example, targeted mutagenesis in cyanobacteria and T-DNA insertional mutagenesis in higher plants, have provided very important information on the biosynthesis of lipids and its regulation, and they continue to serve as powerful tools in efforts to understand the functions of lipids in photosynthesis. However, changes in lipids in genetically modified cells and plants affect not only photosynthesis but also many other processes, inducing a variety of phenotypic alterations. Although changes in phenotype can suggest important roles for individual lipids in photosynthesis, they do not necessarily reveal the specific function of each lipid because changes in the phenotype can be induced indirectly as well as directly. In the future, we need to develop methods for manipulating lipid molecules at specific sites in individual membranes and membrane-bound proteins.

Methods for the analysis of plant lipids have greatly improved in recent years and detailed analysis of lipids can now be performed with very small amounts of material. Improvements in analytical methods allow us to monitor even a tiny change in the lipid composition of thylakoid membranes. However, analytical methods that reveal the distribution and topology of lipid molecules in thylakoid membranes remain rudimentary, and there is an urgent need for molecular probes that allow us to monitor the location of individual lipid molecules in these and other membranes.

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Chapter 2

Fatty Acid Biosynthesis in Plants – Metabolic Pathways, Structure and Organization

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Summary

Progress in the elucidation of metabolic pathways for fatty acid and triacylglycerol biosynthesis in plants is reviewed, together with evidence for gene function. Research in this area is being driven by the importance of storage lipids as potential new raw materials to replace petrochemicals. Significant advances have been made in the structural analysis of a number of the soluble enzymes in these pathways but progress still has to be made on membrane-bound enzymes. Many of the enzymes of triacylglycerol biosynthesis have been identified but the relative importance of the acyl-CoA dependent and independent pathways remains to be determined. The role of particular isoenzymes in specific triacylglycerol assembly remains a major challenge together with determining higher orders of enzyme interaction and metabolic channeling.

I Introduction

Fatty acids serve a number of diverse biological roles. Free fatty acids are rarely found in nature but instead are often esterified to a phospholipid, glycerolipid or sterol backbone. Fatty acids are important constituents of membrane structural li-pids, signaling molecules (Lerouge et al., 1990; Drøbak and Heras, 2002; Feussner and Wasternack, 2002), major energy-storage reserves (Murphy, 1990), protective outer layers of animals and plants (von Wettstein-Knowles, 1993) and are integral components of response/protection strategies for low temperature tolerance in photosynthetic organisms (Wada et al., 1990; Gibson et al., 1994). As components of membrane lipids, fatty acids are essential to provide the correct molecular environment for a number of enzymes (Larson et al., 1980; Green and Bell, 1984) and to allow partitioning and channeling of metabolism, as exemplified by specific complexes involved in both photosynthetic and mitochondrial electron transport (Mullineaux et al., 1997; Sun et al., 2003; Wittig and Schägger, 2008). Several covalent modifications of proteins with fatty acids occur, including direct palmitoylation and myristoylation as well as more complex linkage to glycosylated phospholipid anchors. These influence the activity and location of proteins in the cell and provide a way, in addition to the presence of amino acid hydrophobic domains, to direct proteins to certain subcompartments and faces of membrane systems (Lodish et al., 2000).

There is a large variety of fatty acids which exist in plants (Somerville and Browse, 1991). They differ in chain length, degree of unsaturation and elaboration of chemical structure by inclusion of substituents, such as epoxy, acetylenic, hydroxy, cyclopropene and furan rings. Interest in elucidating the biosynthesis of fatty acids and their incorporation into storage lipids has been driven by the importance which they have as a raw material for the oleochemical industry, as well as their role in a number of fundamental biological processes (Kaufman, 1990).

In this chapter we focus on the biosynthesis of saturated and unsaturated fatty acids in plants, structural knowledge on the component enzymes of this pathway, mechanisms for introduction of substituents onto fatty acyl chains and incorporation of fatty acids into storage and membrane lipids. Reactions leading to glycerolipid synthesis in the endoplasmic reticulum are described here and additional information on lipid synthesis in chloroplasts and mitochondria can be found in Chapters 3 and 4.

II Biosynthesis of Fatty Acids

The de novo biosynthesis of fatty acids, up to a chain length of C18, from acetyl-CoA involves two enzyme systems: (i) acetyl-CoA carboxylase (ACCase) and (ii) fatty acid synthase (FAS). In higher plants both of these systems are located in the chloroplast and as the majority of the component polypeptides are nuclear-encoded, they require a transit sequence for entry into the chloroplast. In seeds de novo fatty acid biosynthesis

Abbreviations: ACCase – Acetyl-CoA carboxylase; ACP – Acyl-carrier protein; CoA – Coenzyme A; DGAT – Diacylglycerol acyltransferase; FAS – Fatty acid synthase; GPAT – Glycerol-3-phosphate acyltransferase; PDAT – Phospholipid: diacylglycerol acyltransferase; LPAT – 1-Acyl *sn*-glycerol-3-phosphate acyltransferase

occurs in proplastids, which might have fundamentally different properties from chloroplasts – so care should be taken in directly extrapolating results obtained with photosynthetically active chloroplasts from leaves.

A Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase (ACCase) catalyzes the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, an essential substrate for FAS. There are two different types of ACCase in plant plastids. Plants outside the family Poaceae (Gramineae) contain a type II dissociable ACCase in their plastids, which is structurally similar to that found in the majority of bacteria (Sasaki et al., 1993, 1995). This consists of four separate polypeptides: biotin-carboxyl-carrier-protein, biotin-carboxylase, α-carboxyltransferase and β-carboxyltransferase. The first three are encoded by nuclear genes but the latter is present on the plastid genome (Sasaki and Nagano, 2004). In Poaceae species, such as wheat, the plastids instead contain a type I ACCase, which has all the necessary domains on one polypeptide, a structure which is similar to that encountered in animals and yeast. The difference in plastidial ACCase types is the basis of differential herbicide sensitivity of fatty acid synthesis between grass and dicotyledonous plant species. Type II ACCase is insensitive to inhibition by aryloxyphenoxypropionates and cyclohexanediones, whilst plastidial type I ACCase is sensitive (Price et al., 2003; Liu et al., 2007). ACCase is also present in the cytoplasm of plant cells, where it is responsible for generating malonyl-CoA for the synthesis of very long chain fatty acids and flavonoids. This type I cytoplasmic ACCase is insensitive to aryloxyphenoxypropionates and cyclohexanediones.

B Fatty Acid Synthase

Plants contain a type II dissociable fatty acid synthase (FAS), which is similar in structure to that present in most bacterial species and synthesizes saturated fatty acids of generally 16 or 18 carbons. Plant FAS consists of seven separate polypeptides, of which six are catalytic and the seventh is a low molecular weight acyl-carrier protein (ACP). This protein contains a thiol group that is essential for fatty acid biosynthesis as it forms thioesters with the growing acyl chain. The active thiol is present on a 4′ phosphopantetheine prosthetic group that is transferred from CoA to a highly conserved serine residue during conversion of apo- to holo-ACP by holo-ACP synthase. Synthesis of saturated fatty acids up to C18 occurs on ACP-linked substrates via a series of reactions catalyzed by the other six proteins of plant FAS (Harwood, 1996). These add two carbon units to the nascent acyl chain in a cyclical fashion that is shown in Fig. 1 and outlined below.

Malonyl-CoA: ACP transacylase transfers the 3-carbon malonyl-group from malonyl-CoA onto the essential thiol of ACP, thus providing the source of carbon atoms for fatty acid elongation. The initial condensation reaction is catalyzed by 3-ketoacyl synthase III, which covalently links the acetyl group from one of its substrates, acetyl-CoA, to a cysteine-thiol moiety within it and then uses malonyl-ACP to form a four-carbon 3-ketoacyl-ACP, concomitantly releasing carbon dioxide. The ketoacyl-ACP is subsequently reduced by NADPH in a reaction catalyzed by 3-ketoacyl-ACP reductase to generate a 3-hydroxyacyl-ACP. 3-Hydroxyacyl-ACP dehydratase removes a molecule of water from this to generate an enoyl-ACP, which is finally reduced by NADH to butyryl-ACP in a reaction catalyzed by enoyl-ACP reductase. The product of this first synthetic cycle, butyryl-ACP, is the substrate for further elongation rounds, each of which uses one molecule of malonyl-ACP and releases carbon dioxide. Elongation is catalyzed by the same enzymes used for generation of butyryl-ACP from acetyl-CoA and malonyl-ACP with the exception of subsequent condensation reactions. These occur in a similar way to the initial 3-ketoacyl-ACP synthase III reaction, with transfer of the acyl-primer onto cysteine and reaction with malonyl-ACP, but condensations from C4 to C16 are carried out by 3-ketoactyl-ACP synthase I and from C16 to C18 by 3-ketoactyl-ACP synthase II. An additional ketoacyl-ACP synthase enzyme specific for medium-chain length fatty acid synthesis, 3-ketoacyl-ACP synthase IV, has been isolated from *Cuphea* species (Dehesh et al., 1998).

Genes encoding all plant FAS proteins have been identified and function of the majority of gene products proven in a number of different plant species. The exception was 3-hydroxyacyl-ACP dehydratase, for which no confirmed amino acid sequence data is available, although putative

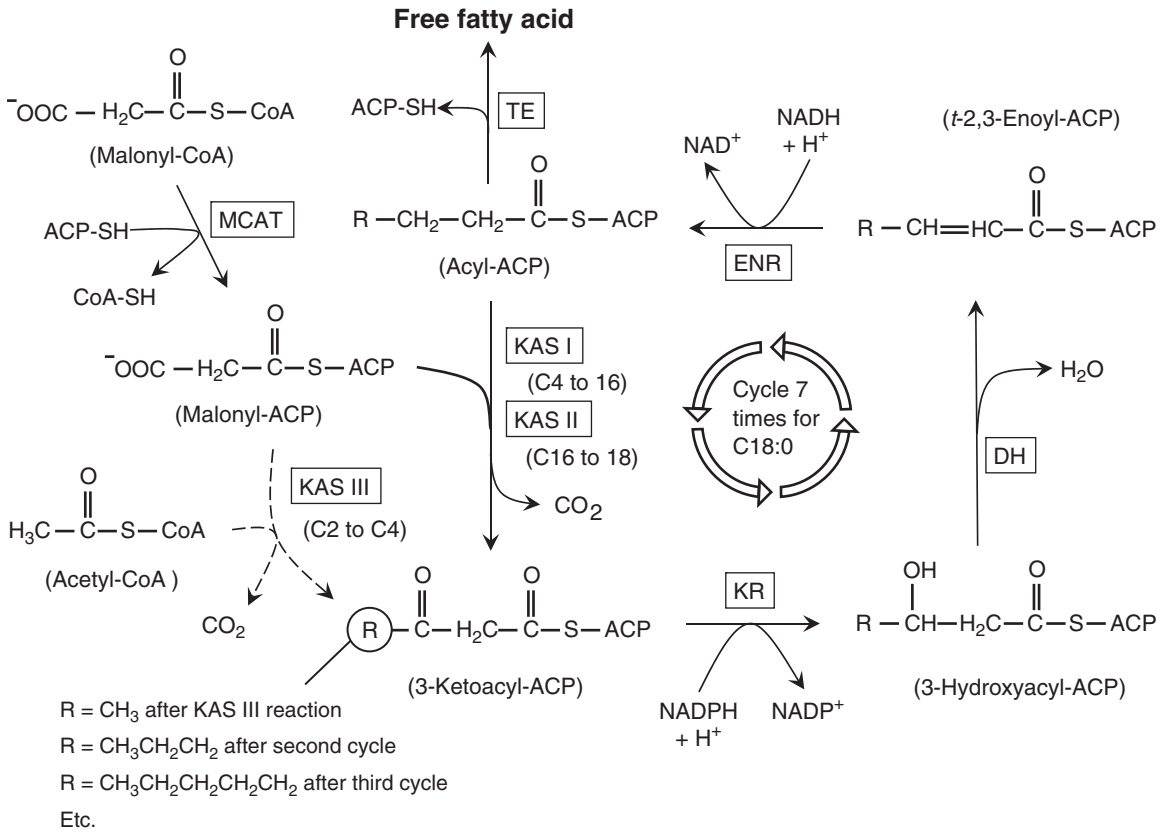

Fig. 1. Core reactions of fatty acid synthase. The cyclical reactions adding two-carbon units during fatty acid synthesis in plant plastids are shown. The *dashed arrows* indicate the reaction catalyzed by 3-ketoacyl-ACP synthase III, which is specific for the synthesis of butyryl-ACP. Thereafter the four-step cycle is repeated until the appropriate chain-length is reached and a thioesterase releases the fatty acid from ACP. Enzyme abbreviations: MCAT, malonyl-CoA: ACP transacylase; KAS, 3-ketoacyl-ACP synthase; KR, 3-ketoacyl-ACP reductase; DH, 3-hydroxyacyl-ACP dehydratase; ENR, enoyl-ACP reductase and TE, acyl-ACP thioesterase.

plant sequences had been assigned in databases by homology with known bacterial genes. However assignment of gene function by homology alone might lead to incorrect conclusions. An example of this was the discovery of the gene encoding for acyl-CoA: diacylglycerol acyltransferase (DGAT). This was identified by analysis of homologs of the acyl-CoA: cholesterol acyltransferase family. Expression of one of the candidate genes did not result in increased cholesterol acyltransferase activity but instead it turned out to encode a DGAT (Cases et al., 1998). In order to prove the function of a putative *Arabidopsis* 3-hydroxyacyl-ACP dehydratase gene we have expressed the encoded protein in *E. coli*, which required growth at low temperature to obtain a soluble protein. The purified protein was demonstrated to catalyse the dehydratase reaction using mass spectrometry (Brown et al., 2009). Table 1 shows how candidate genes for plant FAS enzymes were isolated and how experimental confirmation of assigned function was obtained.

Once a saturated acyl-ACP has been through an appropriate number of FAS cycles (generally leading to the formation of C16 and mainly C18 fatty acids although alternative chain lengths accumulate in some plant seeds) three alternative reactions are possible (Joyard et al., 1998): (1) a double bond can be introduced, commonly at the Δ9 position, to give mono-unsaturated 18:1-ACP, a reaction catalyzed by stearoyl-ACP desaturase; (2) the acyl group can be incorporated into plastid glycerolipids by sequential transfer to the sn-1 and sn-2 positions of glycerol-3-phosphate

Table 1. Functional proof of assigned plant FAS genes. Representative literature detailing FAS protein purification, amino acid sequencing and gene isolation are listed. Some functions were proved by heterologous complementation of mutants. The number of sequenced peptides is shown and NT signifies data was obtained from the protein N-terminus. Enzyme abbreviations: ACP, acyl-carrier protein; MCAT, malonyl-CoA: ACP transacylase; KAS, 3-ketoacyl-ACP synthase; KR, 3-ketoacyl-ACP reductase; DH, 3-hydroxyacyl-ACP dehydratase and ENR, enoyl-ACP reductase.

	Source of pure protein	Amino acid sequence determined	Gene identified/cloned	Evidence of function
ACP	*B. napus* seed	N-terminal 48 amino acids; Slabas et al., 1988	Safford et al., 1988	Substrate for various FAS enzyme assays Enzyme assay during purification
	Spinach leaf	Complete; Kuo and Ohlrogge, 1984	Synthesized; Beremand et al., 1987	
MCAT		–	Simon and Slabas, 1998	Complementation of *E. coli* mutant
KAS I	Barley leaf	Two peptides; Siggard-Andersen et al., 1991	Siggard-Andersen et al., 1991	Enzyme assay, binding of labeled enzyme inhibitor
KAS II		–	Carlsson et al., 2002	Complementation of *Arabidopsis* mutant
			Slabaugh et al., 1998	Correlation of expression and fatty acid composition in seeds
KAS III	Spinach leaf	Ten peptides; Tai and Jaworski, 1993	Tai and Jaworski, 1993	Expressed and assayed
KR	*B. napus* seed	Two peptides; Sheldon et al., 1992	Slabas et al., 1992 (*Arabidopsis* and *B. napus*)	Direct assay during protein purification
	Avocado	NT plus two peptides; Sheldon et al., 1990		
DH		–	(Putative genes assigned by homology to prokaryotic genes)	Direct assay with mass-spec analysis of ACP substrates (Brown et al., 2009)
ENR	*B. napus*	NT; Cottingham et al., 1988	Kater et al., 1991	Enzyme assay

– catalyzed by a soluble glycerol-3-phosphate-1-acyltransferase (GPAT) and membrane bound 1-acyl-sn-glycerol-3-phosphate acyltransferase (LPAT) respectively, or (3) the acyl group can be cleaved to form a free fatty acid and ACP – catalyzed by acyl-ACP thioesterase.

Enzymes in different plant species can utilise alternative chain length ACPs for these reactions. For example, Δ4 16:0-ACP and Δ9 14:0-ACP desaturases exist in coriander and geranium (Cahoon and Ohlrogge, 1994; Schultz et al., 1996). The structure of Δ9 18:0-ACP desaturase from castor seed has been determined (Lindqvist et al., 1996) and detailed analysis of the structure function relationships of such plastidial acyl-ACP desaturases performed (Shanklin and Cahoon, 1998). Soluble GPATs with different selectivity for 16:0 and 18:1 fatty acids (Nishida and Murata, 1996) and acyl-ACP thioesterases with different chain length preferences (Voelker et al., 1992; Dehesh et al., 1996) have also been discovered. Differences in the substrate preferences of enzymes that utilize newly synthesized acyl-ACPs have been exploited in transgenic studies to modify the acyl-lipid composition of plants (Cahoon et al., 1992; Voelker et al., 1996) or alter their chilling sensitivity (Murata et al., 1992).

III Export of Fatty Acids from the Plastid

The export of fatty acids from the plastid to other organelles is not as well defined biochemically as their synthesis. In the cytoplasm acyl-CoAs can be utilized in further fatty acid elongation reactions and are the substrates for acyltransferase enzymes, which incorporate the acyl groups into membrane and storage lipids, such as triacylglycerol. It is clear that acyl-CoA synthetases exist in the chloroplast envelope (Schnurr et al., 2002) and likely convert the free fatty acids generated from acyl-ACPs by acyl-ACP thioesterase activity into acyl-CoAs. Channeling of fatty acids from the plastid stroma to outer plastid envelope

is thought to occur by an ABC transporter protein (Koo et al., 2004; Jouhet et al., 2007).

Free acyl-CoAs are presumed to be deleterious to cells because of their detergent-like properties and once outside the plastid it is thought that they are bound to acyl-CoA binding proteins (Fox et al., 2000). Movement of acyl-CoA and acyl-CoA binding protein complexes within cells and delivery of acyl-CoAs to correct organelles and enzyme systems is a poorly understood topic. Acyl-CoA binding proteins do increase the solubility of acyl-CoAs and can promote their transfer between membranes (Rasmussen et al., 1994), but how they are involved in the coordinated movement of fatty acids within cells is unclear. Separate systems for fatty acid metabolism and incorporation into lipids are indicated by the occurrence of several acyl-CoA synthetase isoforms present in different sub-cellular compartments (Shockey et al., 2002, 2003).

In addition to transfer of synthesized acyl-CoAs between different organelles, movement of glycerolipids between the endoplasmic reticulum and plastid occurs (Somerville and Browse, 1991; Ohlrogge and Browse, 1995). The nature of the fatty acids incorporated at the *sn*-1 and *sn*-2 positions of glycerolipids is dependent on the site of synthesis, since acyltransferases in the plastid and endoplasmic reticulum have different substrate selectivity. Plastidial acylation reactions (the prokaryotic pathway) lead to glycerolipids with a fatty acid distribution similar to that found in bacteria, whereas extra-plastidial pathways, especially in the endoplasmic reticulum (the eukaryotic pathway), result in lipids with profiles commonly found in higher organisms. Determination of the fatty acids at the *sn*-1 and *sn*-2 positions of a glycerolipid is, therefore, indicative of where acylation reactions occurred. The proportion of plastid fatty acids derived from prokaryotic and eukaryotic pathways varies in different species, but in some plants the majority of fatty acids within plastid glycerolipids cycle through the endoplasmic reticulum. It is thought that the diacylglycerol moiety of membrane phospholipids is the molecular species transferred to plastids, but this has not been proved and the detailed mechanism of transfer between organelles is unclear at present. Firm attachment sites between the endoplasmic reticulum and plastids have been described recently and it is proposed that these may provide sites of transfer for lipid species, including newly synthesized acyl chains, between these organelles (Andersson et al., 2007). Lipid trafficking in plant photosynthetic cells is reviewed in Chapter 16 by Jouhet et al.

IV Biosynthesis of Polyunsaturated Fatty Acids, Unusual Fatty Acids and Chain Elongation

Initial fatty acid desaturation is catalyzed by soluble acyl-ACP desaturases that occur in plastids. Following transport out of the plastid, additional desaturation and other modifications, such as elongation, are carried out by membrane-bound enzymes in the endoplasmic reticulum (Fig. 2). The archetypal enzymes of this type are the Δ12 and Δ15 desaturases which sequentially convert 18:1Δ9 to 18:2Δ9,12 and 18:3Δ9,12,15. Desaturation occurs on an acyl group at the *sn*-2 position of phosphatidylcholine and an associated electron transport chain containing cytochrome b_5 is required (Smith et al., 1990). Desaturase enzymes contain conserved histidine-motifs, associated with coordination of non-haem iron ligands central to their catalytic reaction mechanisms (Shanklin and Cahoon, 1998). Analysis of the conserved histidine residues in desaturases and their probable conservation in enzymes catalyzing related reactions, such as hydroxylation, epoxidation and acetylenation, has been used to clone and prove function of related enzymes involved in generation of alternative substituted fatty acids (van de Loo et al., 1995; Lee et al., 1998).

Several elongated fatty acids with chain lengths longer than C18 are found in plants and very long chain fatty acids are vital components of plant surfaces (Samuels et al., 2008) Extra-plastidial elongation reactions use acyl-CoA and malonyl-CoA substrates and are mechanistically similar to de novo synthetic reactions. A four-step cycle involving condensation with concomitant decarboxylation, reduction, dehydration and a further reduction is catalyzed by a membrane-bound enzyme complex. Genes encoding 3-ketoacyl-CoA synthase enzymes, which carry out condensation steps, have been identified for several years as they are linked to changes in seed oil fatty acid profiles or alteration of external wax composition

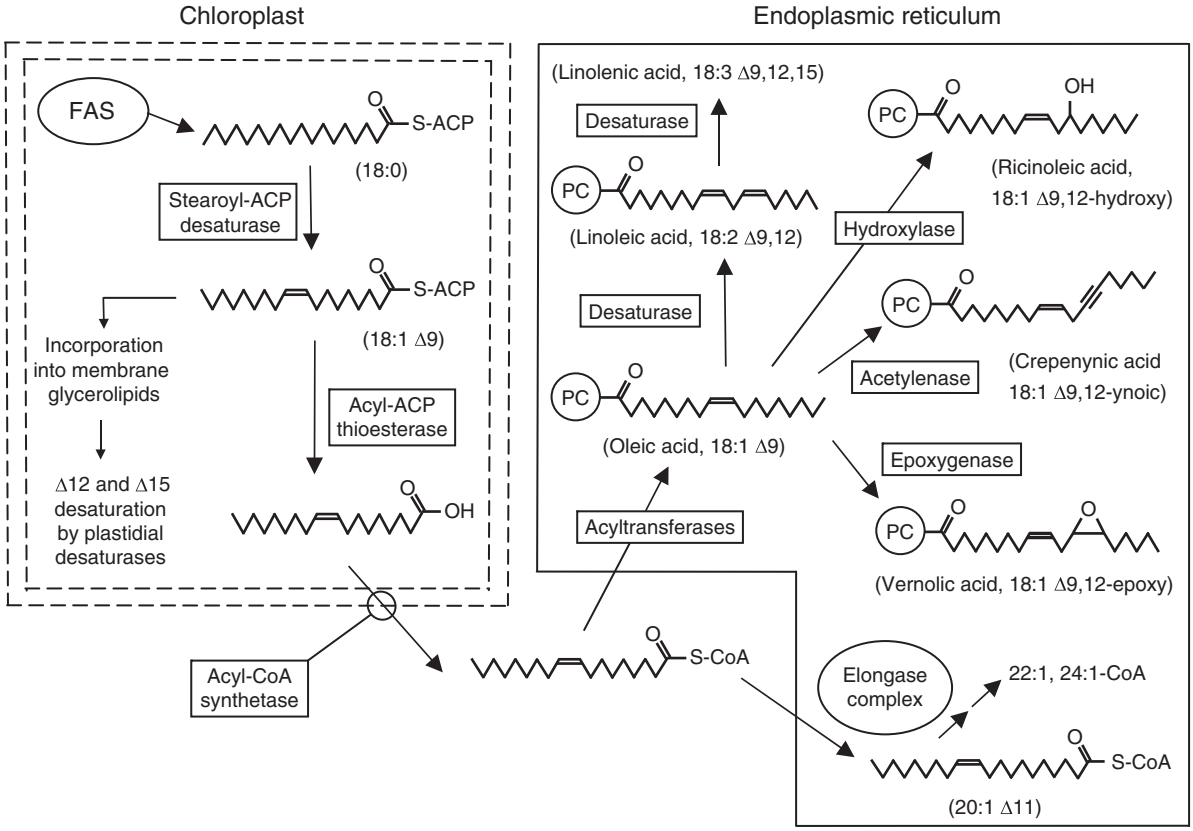

Fig. 2. Modification of stearic acid (18:0) in developing plant seeds. Pathways for the synthesis of economically important fatty acids in different species are shown. Initial desaturation in the plastid (*dashed box*) utilizes acyl carrier protein (ACP) substrates. A proportion of the resulting oleic acid is incorporated into plastid membrane lipids, where additional desaturation reactions occur. Following export of oleic acid out of the plastid, desaturation and related reactions in the endoplasmic reticulum (*solid line*) occur on acyl chains at the *sn*-2 carbon of phosphatidylcholine (PC). FAS represents fatty acid synthase and CoA, Coenzyme A.

(Millar and Kunst, 1997; Millar et al., 1999; Todd et al., 1999). *Arabidopsis* contains 21 genes for 3-ketoacyl-CoA synthase, reflecting the variety of long chain fatty acids and alcohols synthesized in different tissues and a 3-ketoacyl-CoA synthase specific for elongation of hydroxylated fatty acids has also been described in *Lesquerella* (Moon et al., 2001). Condensation reactions performed by 3-ketoacyl-CoA synthase enzymes are the site of action of K_3 class herbicides (Trenkamp et al., 2004). The remaining three components in the endoplasmic reticulum elongation complex have been characterized recently in plants, following pioneering work in yeast (Beaudoin et al., 2002; Xu et al., 2002; Gable et al., 2004; Bach et al., 2008).

Engineering of fatty acid desaturation and elongation pathways in plants, specifically to get essential fatty acids such as eicosapentaenoic (20:5Δ5,8,11,14,17) and docosahexaenoic acid (22:6Δ4,7,10,13,16,19), is an important research goal. We will not consider this in detail here but refer readers to recent articles (Qi et al., 2004; Wu et al., 2005; Napier, 2007)

V Acylation Reactions and the Biosynthesis of Triacylglycerol

Incorporation of fatty acids into membrane structural- and storage-lipids is catalyzed by acyltransferase enzymes (Fig. 3). There are common

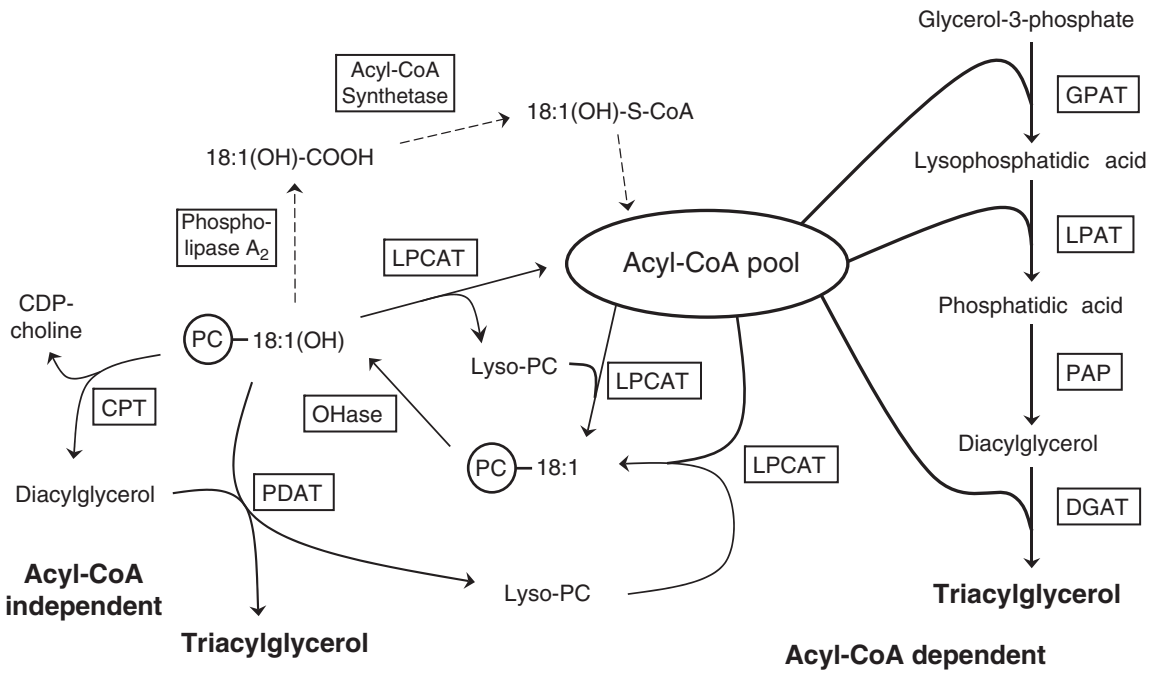

Fig. 3. Acylation reactions leading to triacylglycerol formation. Acyl-CoA dependent and independent pathways for synthesis of triacylglycerol containing ricinoleic acid are shown. Reactions indicated have been detected in different plants but the relative pathway importance for individual species is not known. Abbreviations: PC, phosphatidylcholine; CPT, diacylglycerol cholinephosphotransferase; OHase, oleate 12-hydroxylase; LPCAT, lysophosphatidylcholine:acyl-CoA acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; LPAT, 1-acyl *sn*-glycerol-3-phosphate acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol acyltransferase and PDAT, phospholipid:diacylglycerol acyltransferase. *Dashed arrows* indicate an alternative pathway, in addition to LPCAT, that can return ricinoleic acid, 18:1(OH), from phosphatidylcholine to the acyl-CoA pool.

intermediates for both glycero-phospholipid and storage triacylglycerol synthesis but it is unknown how the flux of fatty acids into these different products is organized and regulated. Acyltransferase activity is detectable in several different organelles, such as the mitochondria, endoplasmic reticulum and plastid, but the most-biochemically characterized is the series of reactions leading to triacylglycerol biosynthesis in the endoplasmic reticulum of developing seeds (Frentzen, 1998; Slabas et al., 2001). This is partly due to the potential for engineering seed oil composition and progress in this field is reviewed by Meyer and Kinney in Chapter 19.

The classical pathway for triacylglycerol biosynthesis is the Kennedy or acyl-CoA dependent pathway. There also exists an acyl-CoA independent pathway leading to triacylglycerol formation (discussed below) and several other transacylation reactions have been assayed in developing oilseeds (Mancha and Stymne, 1997; Stobart et al., 1997). It is generally accepted that triacylglycerol biosynthesis in most species occurs predominantly in the endoplasmic reticulum, but a soluble triacylglycerol-synthetic system in peanut has been reported (Tumaney et al., 2001; Saha et al., 2006).

In the Kennedy pathway, first described in animals, the initial substrates are acyl-CoA and glycerol-3-phosphate. Some organisms can use dihydroxyacetone phosphate as an acceptor for the first acylation, but this reaction is not thought to be important for the majority of plant species and no dihydroxyacetone phosphate acyltransferase gene has been identified from plants. Acylation of glycerol-3-phosphate at the *sn*-1 position is catalyzed by glycerol-3-phosphate acyltransferase (GPAT) to give rise to 1-acyl *sn*-glycerol-3-phosphate (alternatively called lysophosphatidic acid). The second fatty acid is

added at the *sn*-2 position by 1-acyl *sn*-glycerol-3-phosphate acyltransferase (lysophosphatidic acid acyltransferase – LPAT) to produce 1,2-diacylglycerol-*sn*-3-phosphate (phosphatidic acid). Two fates are possible for this phosphatidic acid: either biosynthesis proceeds towards phospholipids by further derivatization of the phosphate group or alternatively it is dephosphorylated by phosphatidic acid phosphatase to diacylglycerol, which contains an OH-group at position 3. Diacylglycerol can be the substrate for synthesis of certain phospholipids but the final step of the Kennedy pathway, the only committed step in triacylglycerol synthesis, is acylation at the *sn*-3 carbon by diacylglycerol acyltransferase (DGAT). In oilseeds, synthesized triacylglycerol is sequestered into oil bodies, which are ~1 μm organelles surrounded by a single layer of phospholipids containing amphipathic oleosin proteins (Hsieh and Huang, 2004).

Storage triacylglycerol biosynthesis in seeds occurs in cells that are synthesizing both membrane phospholipids and neutral storage lipid. How these two processes are spatially organized and perhaps separated in the endoplasmic reticulum is unknown at present and whilst we have information on some of the enzymes involved, there is still a lot to be discovered. One of the current areas of research is elucidation of enzymatic components which allow for the assembly of triacylglycerols with unusual fatty acids in them. Several fatty acids, such as ricinoleic (18:1Δ9,12-OH) or petroselinic (18:1Δ6) acid, are found in storage lipid of some plant seeds but are notably absent from the membrane lipids. These unusual fatty acids can accumulate to high levels in plants such as castor, where ricinoleic acid constitutes up to 90% of the fatty acid in seed triacylglycerols. Production of such unusual fatty acids in transgenic plants, however, is limited to levels below those found in native species (Thelen and Ohlrogge, 2002a; Jaworski and Cahoon, 2003; Napier, 2007). For example, expression of hydroxylase genes in *Arabidopsis* leads to the synthesis of ricinoleic acid in seeds but the amounts are limited to <20%, even in mutants that lack desaturase or elongase enzymes, which might compete for substrates against the hydroxylase (Smith et al., 2003). This is substantially lower than the 90% ricinoleate present in castor bean oil and identification of additional enzymes that might raise the amounts of unusual fatty acids in triacylglycerol is of great interest.

This area is complicated by the fact that most Kennedy pathway enzymes are coded for by gene families. For example, seven membrane-bound GPAT isoforms have been identified in *Arabidopsis* (Zheng et al., 2003), but it is unclear if any of them are involved in a triacylglycerol-specific pathway as opposed to one that might be shared with phospholipid synthesis. One of these GPATs has recently been shown to be involved with suberin biosynthesis and two others in the formation of cutin (Beisson et al., 2007; Li et al., 2007).

Five membrane-bound LPATs can be assigned in the *Arabidopsis* genome (Kim and Huang, 2004; Kim et al., 2005) and three different types of plant LPAT sequences have been isolated using complementation of *E. coli* JC201, which contains a temperature sensitive LPAT mutation (Brown et al., 1994; Hanke et al., 1995; Bourgis et al., 1999). One such LPAT class is targeted to the plastid while the other two are thought to be present in the endoplasmic reticulum. Expression of the latter has been analyzed in *Limnanthes douglasii* and while the originally-defined LPAT type I gene is constitutively expressed, the type 2 LPAT is seed specific and likely involved in triacylglycerol biosynthesis (Brown et al., 1995). This idea was strengthened by assay of the fatty acid selectivity of *Limnanthes* LPAT-2 expressed in *E. coli*. However, caution should be exercised with drawing firm conclusions from data on the substrate selectivity of membrane-bound enzymes, as in vitro assays may bear little relationship to the in vivo situation. The *Limnanthes douglasii* LPAT-2 significantly increased the level of erucic acid at the *sn*-2 position of triacylglycerol when introduced into *B. napus* (Brough et al., 1996), from 0% to 28.3%, which is indicative of the substrate preference of the enzyme and illustrates the potential for engineering oil compositions with acyltransferase enzymes. Another LPAT with significant effects on transgenic seed oil composition, increasing the incorporation of medium-chain length fatty acids, has been isolated from coconut (Knutzon et al., 1995, 1999).

A phosphatidic acid phosphatase has been purified from *Avocado* mesocarp – a tissue which is highly active in triacylglycerol biosynthesis (Pearce and Slabas, 1998). It would appear, however, that this type II phosphatidic acid phosphatase can utilize

a number of different lipids and is unlikely to be the main triacylglycerol biosynthetic enzyme. A membrane-associated phosphatidic acid phosphatase has recently been identified in *S. cerevisiae* (Han et al., 2006) which is linked to triacylglycerol biosynthesis and its functional knockout results in decreased triacylglycerol accumulation. In our lab we have isolated homologs of this type I phosphatidic acid phosphatase and are studying their function following expression.

A DGAT gene was first identified on the basis of homology to acyl-CoA: cholesterol acyltransferase and two families of DGAT have now been described in plants. DGAT-1 sequences were the first to be identified and have been reported from several species including *Arabidopsis*, *Brassica* and *Ricinus* (Hobbs et al., 1999; Routaboul et al., 1999; Nykiforuk et al., 2002; He et al., 2004). Genes homologous to the DGAT-2 class of acyltransferases (Lardizabal et al., 2001) have been identified and their function proven from both tung tree *Vernicia fordii* (Shockey et al., 2006) and *Ricinus* (Kroon et al., 2006). In both of these species, DGAT-2 is the most highly expressed DGAT in developing seeds and it is believed to be the enzyme required for incorporation of unusual fatty acids into triacylglycerol. Transformation of hydroxylase-expressing *Arabidopsis* has confirmed that introduction of *Ricinus* DGAT-2 increases the level of ricinoleate in seed triacylglycerol (Burgal et al., 2008) and expression of heterologous DGAT-1 or DGAT-2 has also been reported to increase seed oil content (Jako et al., 2001; Lardizabal et al., 2008).

An acyl-CoA independent pathway leading to triacylglycerol synthesis is also found in plants (Fig. 3). In pioneering work Dahlqvist et al. (2000) identified a phospholipid: diacylglycerol acyltransferase (PDAT), which catalyzes the transfer of an acyl group from the *sn*-2 position of phosphatidylcholine to diacylglycerol, generating triacylglycerol and lysophosphatidylcholine. The latter can be re-acylated with a simple fatty acid, such as 18:1, which can undergo further modifications (e.g., desaturation and hydroxylation) before PDAT-mediated transfer to triacylglycerol. This enzyme provides a direct link between fatty acid modification and incorporation into triacylglycerol without intermediate conversion to a CoA thioester. High PDAT activity is found in developing seeds which contain unusual fatty acids, such as hydroxylated or epoxidated, and this activity could well be a critical last step in triacylglycerol biosynthesis in these species. *Arabidopsis* has one confirmed PDAT gene (Ståhl et al., 2004) but knockout of this locus shows it is not essential for triacylglycerol biosynthesis (Mhaske et al., 2005). Since there are related sequences of unproven function in *Arabidopsis* that may be active PDATs in this mutant, these could take over the role of the deleted gene as a consequence of functional redundancy. An important consideration which has to be made when evaluating the role of PDATs in triacylglycerol biosynthesis is the possibility that they may be critical in plants containing unusual fatty acids, such as castor, but are of less importance in plants which have conventional fatty acids. The triacylglycerol biosynthetic machinery could be different in different species, particularly the relative importance of acyl-CoA dependent and independent pathways, and whilst PDAT activity may not be significant in *Arabidopsis* that does not mean that it does not serve an important role in other developing seeds.

In the last decade, great progress has been made in defining membrane-bound enzymes that can contribute to glycerolipid biosynthesis in plants. The challenge ahead is to discover which are important for determining the triacylglycerol composition in different plant species and how they can be used for manipulation of transgenic seed oils. The organization of triacylglycerol biosynthetic machinery within the cell and how unusual fatty acids, such as ricinoleate, are prevented from accumulating in membrane lipids are also important research targets. One approach to try and identify relevant genes is in-depth sequencing of ESTs from tissues accumulating unusual fatty acids and recently 4,902 EST sequences have been deposited by our laboratory from a normalized developing castor bean cDNA library http://www.ncbi.nlm.nih.gov/sites/entrez?db = nucest&term = Castor (November 28, 2008).

VI Structural Models for Fatty Acid Biosynthetic Pathway Components

There are several levels to the understanding of biochemical pathways. The first resides in knowledge of the role of individual enzymes and their

structures and the next is how these enzymes associate and interact with each other. Above that is the integration of a pathway with other components of the organism's metabolic machinery and the dynamics and interactions of the various facets of metabolism – how things are balanced, optimized and regulated. In this section we wish to consider what our state of knowledge is regarding structural aspects of storage lipid synthesis in plants.

A Individual Enzymes and Their Structure

A thorough review of the structural biology of type II FAS was recently published (White et al., 2005), which focused on the situation in bacterial systems known at that time. Some of the enzymes of the type II FAS found in plants have also been structurally characterized but not all of them. The absence of structures of plant enzymes is also mirrored in the small number of structures of plant proteins in the Protein Data Bank (PDB) where of the approximately 55,000 structures (November 2008) there are less than 1,000 structures of non-redundant sequence from plants. Possible reasons for past difficulties in obtaining these structures may lie in poor identification of the termini of plant genes for recombinant protein expression and problems associated with amino acid modifications in plant systems. Both of these will have consequences for proper protein folding and the propensity to crystallize. Alternatively it might simply be that the amino acid sequence for a specific protein from a particular source seriously and adversely affects the ability of that protein to crystallize. In attempts at structural determinations it is always advisable to over-express and try to crystallize the target protein from a variety of plant sources in order to find the best sequence variant. Thus, a number of the key plant enzymes have only models derived from structures of homologous enzymes in prokaryotes or other eukaryotes. In a number of cases the enzymes have also had structures determined with ligands bound and this has greatly increased our ability to understand the underlying biochemistry.

The structural landscape can be viewed by considering the individual protein structures as below but it is likely that they associate into larger non-covalently linked complex structures to facilitate the passage of substrates/products between the enzyme active sites (Roughan and Ohlrogge, 1996; Roughan, 1997). The elegance of the association of active sites in a multi-component catalytic processing machine is beautifully illustrated by the structures of the type I FAS from mammals and yeast (Jenni et al., 2006; Maier et al., 2006; Lomakin et al., 2007; Maier et al., 2008). The individual catalytic domains or modules have structural folds similar to those seen for their prokaryotic or plant counterparts in most cases. Although these two type I FAS share a common catalytic cycle and many common structural features, the three-dimensional organization of their active sites is quite different. Thus, it is not unreasonable to presume that any association of enzymes within a large FAS II complex may adopt a variety of combinations or conformations – little knowledge is currently available in this area. In the following sections, all structure cartoons have been produced with PyMOL (DeLano, 2002) and examples of each enzyme structure can be viewed in PDB [http://www.rcsb.org/pdb/home/home.do (November 29, 2008)]. The entry codes for each protein in this database (e.g., 1w2x) are given in parentheses.

1 Acyl-Carrier Protein

Acyl-carrier protein (ACP) is a ubiquitous protein with a simple four helical bundle structure. Many structures of ACP have been determined and many fundamental NMR studies were carried out using the protein. The crystal structure determinations of *E. coli* ACP with bound butyryl, hexanoyl, heptanoyl and decanoyl fatty acid chains (1loi, 2fac, 2fad, 2fae) showed the details of how a central cavity in the helical bundle opens up to accommodate the growing chain for transport in the cytosol (Roujeinikova et al., 2002; Roujeinikova et al., 2007) (Fig. 4). However, there is only one ACP structure from plants in the database and that is the spinach ACP structure (2ava) determined by NMR. Its structure resembles that seen for the other ACP proteins and analysis of acyl-loaded forms confirms the acyl chain is carried in the central cavity (Zornetzer et al., 2006).

2 Holo-ACP Synthase

The loading of the pantotheine arm onto ACP is carried out by holo-ACP synthase and requires CoA as the donor. The structures of bacterial

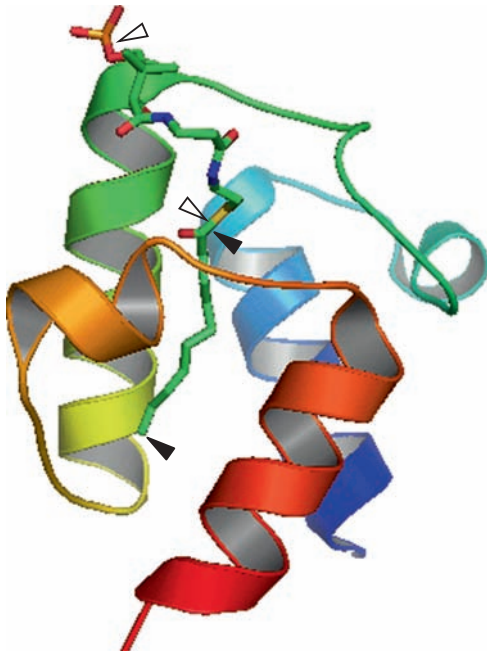

Fig. 4. Acylated *E. coli* ACP. A cartoon of ACP main chain, colored in rainbow depiction from N- to C-terminus, is shown. A C10 acyl chain (*solid arrows*) attached via a pantotheine arm (*open arrows*) to Ser36 is inserted into a central cavity of the four helix bundle (See Color Fig. 1 on Color Plate 1).

and fungal holo-ACP synthase have been determined but there is no equivalent structure from a plant source. The bacterial holo-ACP synthase structure (1f7l) reveals a trimeric arrangement wherein the catalytic centres are located on the inter-monomer interfaces (Parris et al., 2000).

3 Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase (ACCase) catalyzes the first committed step in fatty acid biosynthesis and is found both in a hetero-tetrameric form and a large monomeric form in most plants. There are no structures for any of the subunits of a plant ACCase currently in the PDB. However, structures for the carboxyltransferase (1w2x), biotin carboxylase (1bnc) and biotin carboxyl carrier protein (1bdo) subunits of the heteromeric form have been determined from prokaryotic and yeast sources. These models have some limitations for analysis of equivalent plant proteins as, for example, the yeast carboxyltransferase enzyme has two identical subunits rather than the α and β subunits found in plants (Sasaki and Nagano, 2004). Ligand complex structures are available for some of the homologs.

4 Malonyl CoA: ACP Transacylase

Malonyl-CoA: ACP transacylase catalyzes the formation of malonyl ACP, the key substrate for fatty acid biosynthesis. There are currently no structures of malonyl-CoA: ACP transacylase from a plant source but structures of homologs in prokaryotes and human have been determined (1mla) including those with substrates (2g2z) (Serre et al., 1995; Oefner, 2006).

5 FAS Elongation Cycle Enzymes: 3-Ketoacyl-ACP Synthases, 3-Ketoacyl-ACP Reductase, 3-Hydroxyacyl-ACP Dehydratase and Enoyl-ACP Reductase

The 3-ketoacyl-ACP synthase enzymes are involved in the condensation stage of fatty acid biosynthesis wherein malonyl-ACP is added to a growing acyl-ACP (or initially acetyl-CoA) and are of three varieties, depending upon the extent of the growing substrate chain. The 3-ketoacyl-ACP synthase I and II enzymes share relatively high sequence identities of typically greater than 35% whereas they are noticeably lower with the 3-ketoacyl-ACP synthase III enzymes, which initiate fatty acid synthesis. From plants there is currently only a structure for a 3-ketoacyl-ACP synthase type II ligand-free enzyme (1w0i) from *Arabidopsis thaliana* mitochondria (Olsen et al., 2004) but there are large number of other 3-ketoacyl-ACP synthase I, II and III structures from prokaryotes and human. Many of these structures also have ligands bound, which helps in interpretation of the biochemical data. The plant mitochondrial enzyme has a two domain structure with a large αβαβα or thiolase fold domain plus an alpha helical 'cap' domain. Like the other 3-ketoacyl-ACP synthase enzymes it dimerizes, which helps to establish a tunnel for binding the fatty acid chain but the dimerization interface is notably larger than that seen in other 3-ketoacyl-ACP synthase enzymes. At the active site it has a Cys-His-His catalytic triad found in 3-ketoacyl-ACP synthase I and II enzymes.

Structures for the 3-ketoacyl-ACP reductase (1edo) and enoyl reductase (1enp) enzymes are available from a number of sources but in the plant kingdom there are only those from

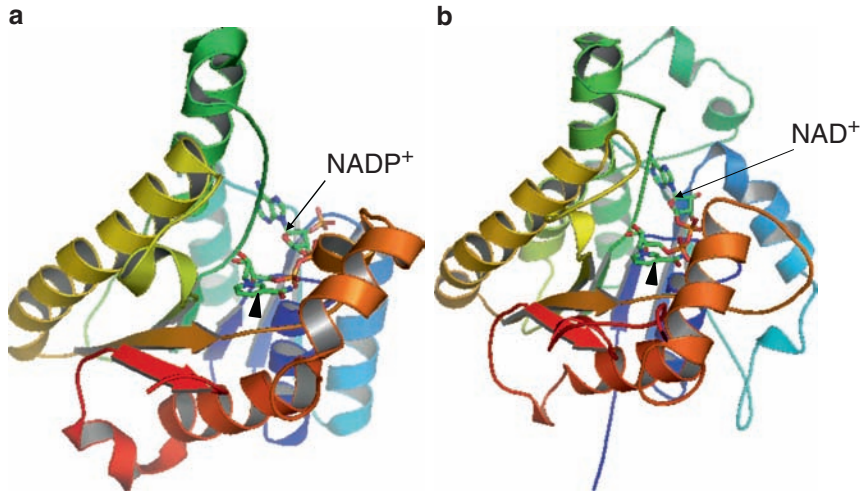

Fig. 5. Structure of *B. napus* acyl-ACP reductases. (**a**) A monomer of *B. napus* 3-ketoacyl-ACP reductase is shown, with the enzyme depicted and colored as for ACP in Fig. 4. The view is into the active site from the nicotinamide ring (solid arrow) end of a bound $NADP^+$ cofactor. (**b**) A monomer of *B. napus* enoyl-ACP reductase, depicted, colored and viewed as for 3-ketoacyl-ACP reductase, but this time with a bound NAD^+ cofactor (See Color Fig. 2 on Color Plate 1).

Brassica napus plastids (Rafferty et al., 1995; Fisher et al., 2000). The two enzymes are structurally quite similar (Fig. 5), are members of the short chain dehydrogenase family and possess a single domain consisting of a standard Rossman fold that binds the reducing cofactor NAD(P)H. They are both tetrameric enzymes and share a reasonable level of sequence identity (25%). Despite the similar overall fold, they have evolved to act upon related but distinct substrates and the enzymes have subtle but important differences in the locations of key residues at the active site (Fig. 6). These structural differences allow reduction of distinctly different chemical groups, i.e., a keto group versus a carbon–carbon double bond. Plant enoyl-ACP reductase has been shown also to bind drug molecules such as triclosan, which is a broad range bactericide (Roujeinikova et al., 1999).

The dehydratase enzyme forms the link between the two reductive enzymes in the fatty acid elongation cycle and two forms have been identified in prokaryotes, FabZ and FabA. The latter has an isomerase function in addition to dehydratase activity and a more limited distribution. Structures of FabA (1mka) and FabZ (1u1z) have been determined from prokaryotes but as yet only a homolog of FabZ can be clearly identified in plants and there are no plant enzyme structures. The FabZ has a $\alpha + \beta$ roll composition and the structure of the *P. aeruginosa* enzyme shows a deep channel on the dimer interface between the monomers where substrate is believed to bind near critical Glu and His residues at the active site (Kimber et al., 2004). Further oligomerization generates an overall hexameric structure.

6 Acyl-ACP Thioesterase

The terminal step in the fatty acid biosynthetic pathway is catalyzed by thioesterase, which cleaves the fatty acid from the acyl-ACP. There are currently no structures of plant thioesterases. Early studies suggested that the plant enzymes were likely to be quite distinct from their bacterial and animal counterparts but models of the plant enzymes have been constructed. Using bioinformatic analysis of relatively low-level sequence similarities, models of the plant thioesterase comprising a double 'hot dog roll' structure have been proposed and tested by mutagenesis studies on putative critical residues (Mayer and Shanklin, 2005).

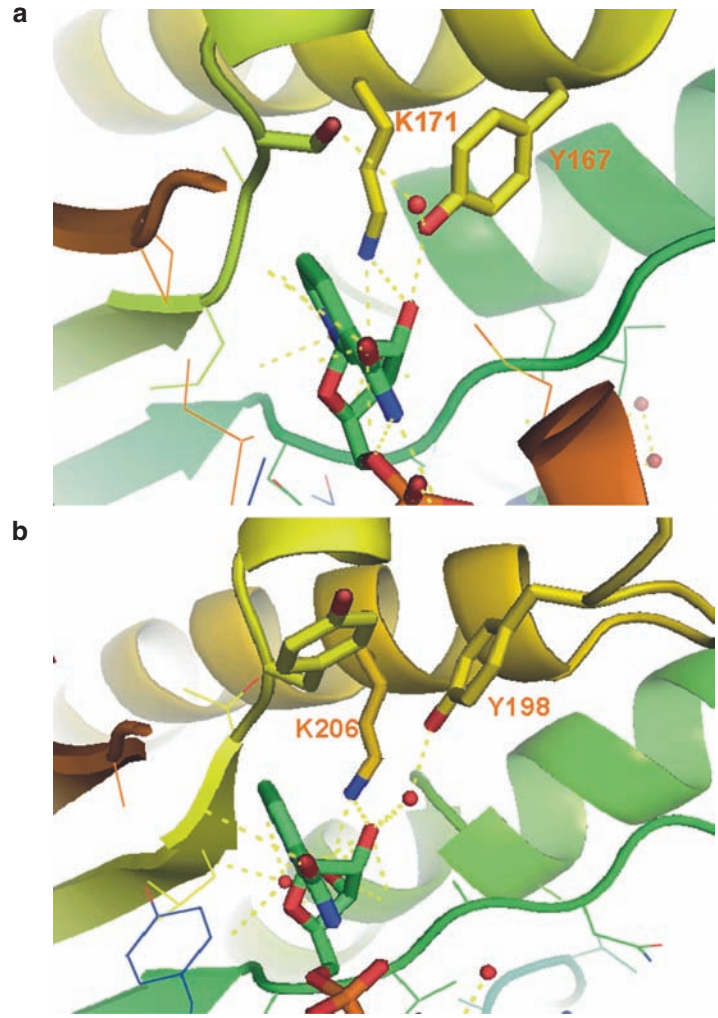

Fig. 6. Active sites of *B. napus* acyl-ACP reductases. (**a**) The positions of key residues in the active site of 3-ketoacyl-ACP reductase relative to the nicotinamide ring of the bound cofactor are shown, depicted in stick representation. Note the location and separation of the Lys171 and Tyr167 residues. (**b**) The active site of enoyl-ACP reductase is depicted as for 3-ketoacyl-ACP reductase. The relative location of the critical Lys206 residue is maintained when compared to 3-ketoacyl-ACP reductase but the Tyr198 has moved in correlation with the different substrate groups that must be reduced by the two enzymes (See Color Fig. 3 on Color Plate 2).

7 Acyltransferase

Subsequent to synthesis of the fatty acid, the acyl chain can be added to glycerol during lipid formation. There are two structures (1k30, 1iuq) of a plant acyltransferase, glycerol-3-phosphate acyl transferase from squash (Turnbull et al., 2001; Tamada et al., 2004). It has a unique overall fold consisting of two domains that comprise an N-terminal four helix bundle and a C-terminal mixed α/β structure with a central 9 stranded anti-parallel β-sheet. The structure reveals a long largely hydrophobic cleft in the surface with a conserved $H(X)_4D$ motif at one end, believed to be critical for catalytic function. It has enabled the construction of a model for the binding of substrates. It should be noted that these structures are ones of the soluble plastid acyltransferase and no structural information is available for the membrane bound acyltransferases which are involved in triacylglycerol and glycerolipid biosynthesis.

8 Desaturase

The introduction of the first double bond into fatty acid chains can be catalyzed by plastidial soluble desaturases and structures of these from castor seeds (1oq4) and ivy (2uw1) have been determined (Moche et al., 2003; Guy et al., 2007). They reveal an enzyme that is composed of a large helix bundle, which binds its substrate plus a di-iron centre and NADPH cofactor. The structures permit a molecular model for ligand chain binding into a long channel extending inwards from the surface, which places the Δ9 carbon near the active site di-iron centre. Again as with the acyltransferases no structures are available for membrane bound desaturases. The castor desaturase referred to above is the soluble acyl-ACP desaturase. Even in the absence of a structure, considerable advances have been made in identifying key residues in the membrane bound enzymes which determine the product an enzyme makes. For example consideration of the conserved residues in Δ12 desaturases and their variance in the highly related Δ12 hydroxylase has allowed site directed mutagenesis to convert a desaturase to a hydroxylase and vice versa (Broun et al., 1998).

B Interaction of Individual Proteins

Knowledge of the association of individual components of fatty acid biosynthesis has come from a variety of studies. We will consider the current status with regards to individual components.

1 Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase (ACCase) consists of three functional domains (biotin carboxyl carrier protein, biotin carboxylase and transcarboxylase). In all there are a total of four polypeptides in the multisubunit (type II) and one in the multi-functional (type I) enzymes (Nikolau et al., 2003). Type I ACCase has an observed mass of ~500 kDa and consists of a dimer of a 210–250 kDa polypeptide. It may be that dimerization of this enzyme correctly orientates sub-domains and allows the enzyme to function biochemically, as has been described for animal type I FAS (Maier et al., 2008).

Type II ACCase consists of four proteins, which are coordinately regulated (Ke et al., 2000). This enzyme system has been studied from a number of plant species but to date there is no definitive indication of the stoichiometry of its subunits or how they interact with each other. This ACCase is labile, and readily dissociates during purification to produce two subcomplexes: (i) biotin carboxyl carrier protein (BCCP) together with biotin-carboxylase (BC) and (ii) carboxyltransferase, containing the α- and β-carboxyltransferase (CT) subunits. These carboxyltransferase proteins are tightly associated and co-purify under a number of different conditions. In pea leaf, the multi-subunit ACCase has an apparent mass > 600 kDa (Sasaki et al., 1993; Alban et al., 1994), larger than the calculated value (506 kDa) of a $(BCCP)_4(BC)_2(\alpha CT)_2(\beta CT)_2$ complex, which is thought to exist in *E. coli*. The reason for this discrepancy is not known.

2 Fatty Acid Synthase Components

ACP is a monomeric protein, which has been purified from a number of plant sources including *B. napus* seed and spinach leaf. Interaction between ACP and other proteins might be expected, since acyl-ACPs are substrates for a variety of lipid biosynthetic reactions. Chromatography using columns containing immobilized ACP or non-hydrolysable acyl-ACP analogues has indeed proved effective for purification of enoyl-ACP reductase, acyl-ACP thioesterase, stearoyl-ACP desaturase and 3-hydroxyacyl-ACP dehydratase (Slabas et al., 1990; Kater et al., 1991; Shanklin and Somerville, 1991; Hellyer et al., 1992). Some caution needs to be exercised in consideration of these affinity experiments, however, as ACP is a highly acidic protein and could to a certain extent behave like an ion-exchange matrix. More recently ACP has been tagged for tandem affinity purifications from *E. coli* and interactions demonstrated with 3-ketoacyl-ACP synthase I and II, 3-hydroxyacyl-ACP dehydratase and 3-ketoacyl-ACP reductase (Butland et al., 2005). Detailed modeling and analysis of interacting sites between *E. coli* ACP and FAS enzymes 3-ketoacyl-ACP synthase III and 3-ketoacyl-ACP reductase has also been reported (Zhang et al., 2001, 2003).

Aside from ACP and malonyl-CoA: ACP transacylase, all of the type II FAS components are multimeric, forming homo-polymers as active enzymes. Table 2 shows experimentally-determined

Table 2. Experimental analysis of the relative molecular masses of FAS components. A representative summary of experimentally determined masses for monomeric and native forms of proteins is shown. Calculated masses for processed *Arabidopsis* leaf proteins and the structure of *E. coli* homologs are also listed. Enzyme abbreviations are as in Table 1 and TE, acyl-ACP thioesterase; SD, stearoyl-ACP desaturase.

		Determined mass of monomeric; and native protein[a] (kDa)	Likely association	Reference	Arabidopsis protein mass[b] (kDa)	*E. coli* protein	*E. coli* protein structure[c]
MCAT	Soybean	43; –	Monomer	Guerra and Ohlrogge, 1986	34.0	FabD	Monomer
	Avocado	–; 40.5		Caughey and Kekwick, 1982			
KAS III	Spinach	40.5; 63	Dimer	Clough et al., 1992	35.5	FabH	Dimer
KAS I	*B. napus*	43; 86.7	Dimer	Mackintosh et al., 1989	44.9	FabB	Dimer
KAS II	*B. napus*	–; 87.8	Dimer	Mackintosh et al., 1989	46.2	FabF	Dimer
	B. napus	45; –		Page and Harwood, 1992			
KR	Avocado	28; 130	Tetramer[d]	Sheldon et al., 1990	27.3	FabG	Tetramer
	Spinach	24.2; 97		Shimakata and Stumpf, 1982			
DH	Spinach	19; 85	Tetramer	Shimakata and Stumpf, 1982	17.8	FabZ	3× dimer (Hexamer)
ENR	*B. napus*	34.8; 140	Tetramer[d]	Slabas et al., 1986	33.2	FabI	Tetramer
	Spinach	32.5; 115		Shimakata and Stumpf, 1982			
TE	*B. napus*	38; 70	Dimer	Hellyer et al., 1992	40.9	N/A	–
	Safflower	41; 74		McKeon and Stumpf, 1982			
SD	Safflower	36; 68	Dimer	McKeon and Stumpf, 1982	41.8	N/A	–

[a] Approximate monomeric mass from SDS-PAGE and native mass from gel-filtration.

[b] Mass of predicted mature polypeptides in A. thaliana leaf. The most likely chloroplast-localised sequence from any gene family was analysed with chloroplast-signal-peptide predictive programs and the mass of the mature polypeptide calculated.

[c] Taken from White et al., 2005.

[d] Confirmed by crystallization studies; N/A, not applicable – no direct *E. coli* homolog.

masses for plant FAS components and their likely polymerization state. All three 3-ketoacyl-ACP synthase enzymes in plants are apparently dimeric, as is the case in *E. coli*. Enoyl-ACP reductase, 3-hydroxyacyl-ACP dehydratase and 3-ketoacyl-ACP reductase have been purified from spinach, avocado or oilseed rape and all three determined to be tetrameric (Shimakata and Stumpf, 1982; Slabas et al., 1986; Sheldon et al., 1990). Crystal structures for enoyl-ACP reductase and 3-ketoacyl-ACP reductase have confirmed this finding. Both stearoyl-ACP desaturase and acyl-ACP thioesterase are thought to be dimeric (McKeon and Stumpf, 1982; Hellyer et al., 1992).

With information about the association of each of FAS subunit and its Mr, it should be possible to make an estimate of the size of a fatty acid complex if it exists in vivo. Indeed Paul Stumpf did this many years ago (Stumpf and Shimakata, 1983) and estimated a value of 488,500. With current knowledge and assuming 1:1 stoichiometries between enzyme oligomers, this value can now be calculated as 764,000, which is considerably larger. Just how the constituents of such a complex in vivo might be arranged and interact is open to speculation. What might be especially important is where the active sites are and how the various acyl-ACPs fit into them. In an attempt to pave the way to look at such interactions we have expressed and purified all the FAS components from *Arabidopsis* and raised antibodies against most of them. These reagents will be used to undertake studies on the stoichiometry of FAS components and identification of potentially interacting partners.

Interactions between different catalytic FAS enzymes, leading to a higher order of structure above the formation of active enzyme homopolymers, have not been proven in plants to date. Experimental results consistent with substrate-channeling during fatty acid synthesis have been reported (Roughan and Ohlrogge, 1996; Roughan, 1997) but any FAS complex, if it exists, is apparently labile. One has to bear in mind that only one FAS component needs to be lost for FAS activity to become compromised. Association of at least some of the proteins required for oleic acid (18:1Δ9) biosynthesis has been suggested however, as enoyl-ACP reductase, 3-hydroxyacyl-ACP dehydratase and acyl-ACP thioesterase co-purified through several chromatography steps (Hellyer et al., 1992).

C Pathways of Fatty Acid Metabolism

The structural biology of lipid biosynthesis following FAS is poorly understood. This is linked to the fact that, apart from the soluble plastidial GPAT, the enzymes of subsequent acyl metabolism are membrane-bound. For example, the structure of the export machinery that transfers fatty acids out of the plastids is unclear. Until recently this would have been modeled on the analogous vectorial uptake of fatty acids by FadD in *E. coli*, but the reported link between plastids and endoplasmic reticulum (Andersson et al., 2007) might be important for fatty acid movement. The connection between these organelles is broken by protease treatment and further analysis of the proteins at these contact sites may provide information about the mechanism of lipid transfer between organelles.

Components of the endoplasmic reticulum complex that elongates fatty acids are known and attention is now turning towards structural analysis of this important enzyme system. The membrane topology of enoyl-CoA reductase and hydroxyacyl-CoA dehydratase enzymes have been mapped (Paul et al., 2007; Kihara et al., 2008) but resolution of the structure of a membrane-bound complex is not easy. Similarly, although many acyltransferases have been identified and some limited analysis of functionally important residues carried out, there is no structural information about the enzymes incorporating fatty acids into membrane and storage triacylglycerol. Acquisition of such information may require the development of new technologies, such as cryo-electron microscopy, which has recently been used to investigate large soluble complexes (Marles-Wright et al., 2008).

An intriguing question is whether there are enzyme complexes in the endoplasmic reticulum that channel fatty acids into triacylglycerol, or sub-domains of the endoplasmic reticulum specific for triacylglycerol biosynthesis. The current model of oil body biogenesis is that they bud off from the endoplasmic reticulum (Hsieh and Huang, 2004) and this may entail localization of acyltransferases to these sites, but finding strong evidence of such sub-domains is difficult. A low density membrane fraction that has high acyltransferase-specific activity has been described from *B. napus* seed homogenates (Lacey and Hills, 1996) and this may be enriched in parts of the endoplasmic reticulum that are actively synthesizing oil, but no data on its protein composition has been reported. Fluorescent localization of the two different DGAT enzymes from tung did suggest that they were localized in different parts of the endoplasmic reticulum after transient expression in tobacco suspension-cultured cells (Shockey et al., 2006), but this type of work needs to be performed with additional enzymes before firm conclusions can be drawn. Some caution needs to be exercised in extrapolation from such experiments due to the heterologous nature of the expression studies. Investigation of the organization and structure of membrane-bound systems that metabolize fatty acids, though difficult, is of key importance in understanding how unusual fatty acids are excluded from membrane phospholipids and what limits engineering of plant oil composition.

VII Integration of Fatty Acid Biosynthesis with Other Metabolic Machinery

The incorporation of acetate into fatty acids by plastids, brought about by ACCase and FAS, has historically been thought of as located in the stroma. It now appears, however, that at least some of the components of these systems are membrane-associated. A substantial proportion of the α- and β-carboxyltransferase proteins of multi-subunit ACCase are strongly bound to the plastid envelope (Shorrosh et al., 1996; Thelen and Ohlrogge, 2002b) and early immunogold labeling of ACP demonstrated localization to thylakoids (Slabas and Smith, 1988). It should be remembered that a number of FAS reactions use reduced dinucleotides arising from photosynthesis, so it is not perhaps surprising that some of them may be occurring near photosynthetic membranes.

Acetyl-CoA is the substrate of ACCase and the source of it within plastids depends on the tissue studied and its developmental stage. In non-photosynthetic plastids, metabolites are imported to provide carbon and also probably reductant and some ATP (via the glycolytic and oxidative pentose phosphate pathways) for fatty acid biosynthesis (Rawsthorne, 2002). Compounds such as glucose-6-phosphate, pyruvate, malate and phosphoenol pyruvate are incorporated into fatty acids

by plastids from various developing oilseeds and plastidial protein transporters provide a route for their uptake, together with additional ATP required by ACCase (Rawsthorne, 2002). ATP and reduced cofactors are in abundance in chloroplasts, by contrast, but the link from carbon fixation to fatty acid synthesis is not well understood at present. One possibility is that triose phosphates produced by the Calvin cycle directly undergo glycolytic and pyruvate dehydrogenase reactions to form acetyl-CoA within the plastid. Alternatively, the triose phosphates may be exported into the cytosol and converted into another compound, such as pyruvate, before re-uptake and breakdown to acetyl-CoA.

It is clear, however, that synthesis of fatty acids in photosynthetic tissues is light-dependent and a key control point is the regulation of ACCase. In leaves, this is affected by pH, Mg^{2+} and redox potential. ACCase activity increases at pH 8.0 and 2–5 mM Mg^{2+}, conditions that occur in illuminated plastids, but in addition there is also a light-activated redox pathway that activates ACCase by reducing a disulphide bond that forms between α- and β-carboxyltransferase (Sasaki and Nagano, 2004). There is no information available to date on other covalent modifications to FAS components, but with the development of protein mass spectrometry methodology this issue can now be addressed. It could well be that some of the proteins are modified in the light, potentially facilitating interactions and increasing flux through FAS. There is report that a serine residue in the β-carboxyltransferase subunit of plastidial ACCase is phosphorylated in the light in pea (Savage and Ohlrogge, 1999) but the exact residue and significance of phosphorylation has still to be determined.

VIII Conclusions

All the genes of de novo fatty acid biosynthesis have been identified, cloned and their function proven from plant species. Recently advances have been made in the identification and isolation of key genes involved in glycerolipid biosynthesis but the relative importance of the acyl-CoA dependent and independent pathways has still to be established. There is little direct evidence for the mechanism of export of fatty acids from the plastid and this area warrants further investigations. Knowledge of the specific isoforms of enzymes involved in the synthesis of particular triacylglycerol species is being addressed in a number of laboratories and this is central to obtaining high yields of unusual fatty acids in transgenic plants. Advances have been made in structural biology of lipid biosynthetic enzymes but this is limited to soluble components and greater effort will be required to elucidate the structure of membrane-bound enzymes. A fascinating area for future research will be higher orders of interactions of lipid biosynthetic enzymes with each other and other facets of metabolism, the isolation of enzyme complexes and global regulation of phospholipids and triacylglycerol biosynthesis.

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Chapter 3

Biosynthesis and Function of Chloroplast Lipids

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Summary

Chloroplast membranes are composed of four unique lipids, including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG). These lipids are crucial for maintaining the function of chloroplasts not simply because they account for a large fraction of the photosynthetic membranes, but because they are assembled into the photosynthetic machinery and are, therefore, directly involved in photosynthetic processes. Indeed, Arabidopsis mutants of these lipids possess some photosynthetic defects. Diacylglycerol (DAG), a common precursor of the glycolipids, is produced by both prokaryotic and eukaryotic pathways but the detailed mechanism of DAG supply to chloroplasts remains ambiguous. Because most of the genes encoding the lipid-synthesizing enzymes have been identified in this decade, significant progress delineating the physiological functions and regulatory mechanisms of lipid biosynthesis in chloroplasts has been achieved.

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In Arabidopsis, two types of MGDG synthases, Type A (AtMGD1) and Type B (AtMGD2, AtMGD3), were identified and their distinct functions in chloroplasts have been unveiled. Type A MGDG synthase is involved in the bulk of MGDG synthesis whereas Type B MGDG synthase is induced under phosphate (Pi)-limited conditions. Two genes, *DGD1* and *DGD2*, for DGDG synthases, which are involved in DGDG synthesis, were identified. DGD1 is the predominant DGDG synthase whereas DGD2 is induced under Pi-limited growth conditions. SQDG synthesis is mediated by two enzymes, SQD1 and SQD2. The key enzyme for PG synthesis is PG phosphate synthase, which is encoded by two genes, *PGP1* and *PGP2*. Plants have homeostatic mechanisms to balance the amount of these lipids by regulating their biosyntheses under various environmental conditions, such as limiting Pi, which stimulates replacement of phospholipids with glycolipids through regulation of enzymes involved in lipid biosynthesis.

I Introduction

Among all organelles, the membrane lipid constituents of chloroplasts are very unique. In many eukaryotic and prokaryotic species, cell membranes are mainly composed of phospholipids. However, in plant chloroplasts, phospholipids constitute a minor portion of the membrane lipids. The following four lipids comprise the major proportion of photosynthetic tissues: two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which contain a galactose moiety; a sulfolipid, sulfoquinovosyldiacylglycerol (SQDG); and a phospholipid, phosphatidylglycerol (PG). The three glycolipids comprise more than 90% of chloroplast membrane lipids, as shown in Table 1 (Block et al., 1983) and support photosynthetic activity by maintaining thylakoid membrane structure or via assembly into the photosynthetic machinery (Jones, 2007). PG is the only phospholipid in thylakoid membranes and has also been reported to be essential for the photosynthetic ability of higher plants and cyanobacteria (Hagio et al., 2000, 2002; Sato et al., 2000a) as will be discussed in Chapter 11. In addition to these lipids, the diacylglycerol (DAG) synthesis pathway is crucial for lipid regulation, because DAG is a substrate for synthesis of all chloroplast glycolipids. DAG is supplied by multiple metabolic pathways, allowing fatty acid diversity in the glycerol backbone of chloroplast lipids (Murphy, 1986; Miège and Maréchal, 1999).

Chloroplast lipids are believed to originate from cyanobacteria, and indeed lipid compositions are very similar among plant chloroplasts and cyanobacteria (Douce and Joyard, 1980). In this chapter, we will introduce these lipids, focusing on their biosynthesis in chloroplasts, and we will also discuss their biosynthetic regulation in photosynthetic tissues, non-photosynthetic tissues, or under specific conditions, such as Pi-limited growth. For details regarding these lipids and photosynthesis, readers are advised to refer to Joyard et al. (1998) and the later chapters in this book.

II Structures of Chloroplast Lipids

All of the four lipids in chloroplasts, MGDG, DGDG, SQDG and PG, are glycerolipids, which have a glycerol backbone and two acylated fatty acids as shown in Fig. 1. For MGDG and DGDG,

Table 1. Lipid composition of chloroplast membranes in spinach (mol %) (Block et al., 1983).

		MGDG	DGDG	SQDG	PG	PC	PI
Envelope membrane	Outer	17	29	6	10	32	5
	Inner	49	30	5	8	6	1
Thylakoid membrane		52	26	6.5	9.5	4.5	1.5

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Abbreviations: CDP – Cytidyldiphosphate; DAG – *sn*-1,2-diacylglycerol; DGD – Digalactosyldiacylglycerol synthase; DGDG – Digalactosyldiacylglycerol; ER – Endoplasmic reticulum; FdGOGAT – Ferredoxin-dependent glutamate synthase; LPP – Lipid phosphate phosphatase; MGDG – Monogalactosyldiacylglycerol; MGD – Monogalactosyldiacylglycerol synthase; NPC – Non-specific phospholipase C; PA – Phosphatidic acid; PAP – Phosphatidic acid phosphatase; PC – Phosphatidylcholine; PE – Phosphatidylethanolamine; PG – Phosphatidylglycerol; PGP – Phosphatidylglycerol phosphate; Pi – Phosphate; PI – Phosphatidylinositol; PLC – Phospholipase C; PLD – Phospholipase D; SQDG – Sulfoquinovosyldiacylglycerol; SQD2 – Sulfoquinovosyldiacylglycerol synthase; UDP-Gal – Uridine diphosphate-galactose; UDP-SQ – Uridine diphosphate-sulfoquinovose; SQD1 – Uridine diphosphate-sulfoquinovose synthase

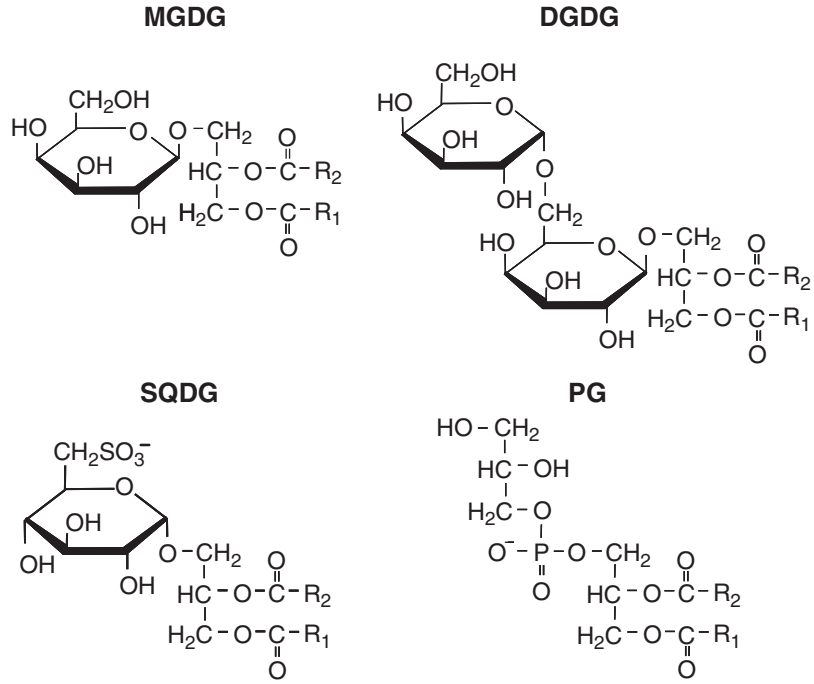

Fig. 1. Structure of the four major chloroplast glycerolipids. Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; R1 and R2, fatty acyl groups.

the head group is galactose, whereas for SQDG it is sulfoquinovose and for PG it is glycerol phosphate. DGDG is a bilayer-forming lipid similar to the phospholipid phosphatidylcholine (PC) (Murphy, 1986) and can therefore substitute for PC, the most dominant lipid in membranes other than chloroplasts, and maintain membrane structure. On the other hand, MGDG is cylindrical and thus cannot be arranged in a lamellar structure. However, it has been demonstrated in vitro that association of the two main components of pea thylakoids, the non-bilayer lipid MGDG and the chlorophyll-a/b light-harvesting antenna protein of photosystem II, leads to the formation of large, ordered lamellar structures (Simidjiev et al., 2000). In another in vitro study, the morphology of *E. coli* transformants harboring MGDG synthase was observed by atomic force microscopy (Gad et al., 2001). MGDG comprised almost 20% of the total lipid content and the transformants appeared elongated, likely due to a defect in cell division. Therefore, it was suggested that excessive accumulation of the non-bilayer lipid MGDG interfered with the translocation of proteins across the plasma membrane, including those for cell division.

In relatively recent studies, MGDG was shown to support violaxanthin de-epoxidase activity, an enzyme of the violaxanthin cycle, by efficiently solubilizing the xanthophyll cycle pigments diadinoxanthin and violaxanthin (Goss et al., 2005; Yamamoto, 2006). The hexagonal structure-forming lipids MGDG and phosphatidylethanolamine (PE) are able to solubilize diadinoxanthin and violaxanthin at much lower lipid concentrations than the bilayer-forming lipids DGDG and PC. Recently, it was reported that leaves of a MGDG knock-down mutant *mgd1-1* contained less zeaxanthin and more violaxanthin than WT after high-light exposure and suffered from increased inhibition of photosystem II (Aronsson et al., 2008). In the mutants, conductivity of the thylakoid membranes was increased, causing impairment of pH-dependent activation of violaxanthin de-epoxidase and the protein of photosystem II subunit, PsbS. These results suggest that MGDG does not simply support photosynthetic complexes by providing an amenable membrane structure but also contributes directly to various photosynthesis-related processes.

SQDG has been called "Nature's Finest Surfactant Molecule" by A. A. Benson, the discoverer of the plant sulfolipid (Benson, 2002). However, detailed analysis of the biophysical properties of SQDG were not performed until the discovery of its antiviral properties (Gustafson et al., 1989). To date, SQDG and its derivatives are known to have many valuable medical and physiological characteristics due to their structure, such as anti-AIDS or anti-tumor properties. A summary of these reports is tabulated in a recent review (Benning et al., 2008).

III Biosynthesis of Chloroplast Lipids

A Monogalactosyldiacylglycerol

In seed plants, the biosynthesis of MGDG occurs exclusively in plastids and is catalyzed by MGDG synthase (UDP-galactose: 1,2-*sn*-diacylglycerol 3-β-D-galactosyltransferase) (Douce and Joyard, 1980). MGDG synthase transfers galactose from UDP-galactose (UDP-Gal) to DAG and produces MGDG as shown in Fig. 2. Because MGDG is the most abundant polar lipid in plants, many researchers have sought to identify the enzymes involved in its synthesis. However, because plant membranes contain only trace amounts of MGDG synthase (MGD), purification of the enzyme from plants was initially unsuccessful (Slabas, 1997). Subcellular localization of MGD had also been discussed by many researchers. In *Spinacia oleracea*, the activity of MGD was detected in the inner envelope membrane of chloroplasts (Block et al., 1983), whereas in *Pisum sativum*, the activity was identified in the outer envelope membranes (Cline and Keegstra, 1983). As a result, two groups in the 1980s were the first to achieve partial purification of MGD by isolation of the enzyme from the envelope fraction of spinach chloroplasts. Both groups reported that the molecular mass of MGD was ~20 kDa (Maréchal et al., 1991; Teucher and Heinz, 1991). However, limiting amounts of the purified protein prevented them from identifying the protein as MGD. Later, Shimojima et al. (1997) made the breakthrough by purifying the enzyme from the microsomal fraction of cucumber cotyledons. Although purification to homogeneity was not achieved, the molecular mass of MGD was established as 47 kDa (Ohta et al., 1995a; Shimojima et al., 1997). Because a sufficient amount of protein was obtained for internal amino acid sequence determination, it was possible to clone the gene corresponding to the 47 kDa protein from cucumber. The recombinant protein was expressed in *E. coli* and was demonstrated to synthesize MGDG from DAG and UDP-Gal in vitro and thus cucumber MGD (CsMGD) had finally been identified. After detailed analyses of the amino acid sequence, CsMGD was revealed to be a homolog of *E. coli* MurG, a glycosyltransferase involved in the formation of peptidoglycan.

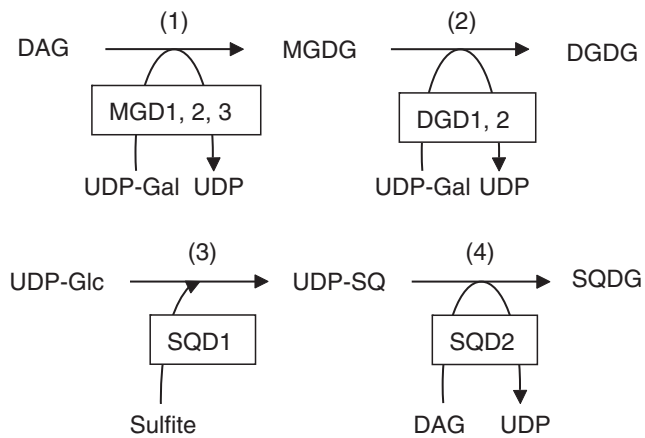

Fig. 2. Glycolipid biosynthesis pathways in Arabidopsis. (1) MGDG synthase (MGD1, 2, or 3), (2) DGDG synthase (DGD1 or 2), (3) UDP-SQ synthase (SQD1), (4) SQDG synthase (SQD2). Abbreviations: DAG, diacylglycerol; UDP-Gal, UDP-galactose; UDP-Glc, UDP-glucose; UDP-SQ, UDP-6-deoxy-6-sulfoglucose (sulfoquinovose).

Moreover, CsMGD was predicted to have a transit peptide for targeting to chloroplasts.

Shortly after the cloning of CsMGD, the 45 kDa spinach MGD (SoMGD1) was identified by screening of spinach cDNA library based on the sequence of CsMGD (Miège et al., 1999). Because the amino acid sequence of the two enzymes was very similar, the MGD enzyme was thought to be ubiquitous among seed plants.

In 2001, three isoforms of MGD in *Arabidopsis thaliana*, AtMGD1, AtMGD2 and AtMGD3, were isolated (Awai et al., 2001). These isoforms were classified into two subgroups based on sequence similarity: Type A (AtMGD1) and Type B (AtMGD2, AtMGD3). AtMGD1 is an ortholog of CsMGD and SoMGD and contains a transit peptide for targeting to chloroplasts, whereas the amino termini of AtMGD2 and AtMGD3 are less similar to AtMGD1 and do not contain transit peptides. In chloroplasts, AtMGD1 localized to the inner envelope membrane and AtMGD2 and AtMGD3 localized to the outer envelope membrane (Awai et al., 2001). AtMGD1 was also shown to face the envelope inter-membrane space (Xu et al., 2005). All recombinant AtMGDs expressed in *E. coli* showed MGD activity from DAG and UDP-Gal, but their preferences were different for DAG molecular species (Awai et al., 2001). Type A enzymes utilized 18:2/18:2-DAG and 18:1/16:0-DAG equally, whereas Type B enzymes demonstrated specificity for 18:2/18:2-DAG but not for 18:1/16:0-DAG. From these results, it was suggested that Type A enzymes utilize both prokaryotic and eukaryotic DAG substrates and Type B enzymes are more specialized for DAG derived from the eukaryotic pathway. Similarly, the substrate preference for recombinant SoMGD, an ortholog of AtMGD1, is not molecular-species specific for DAG substrates (Miège et al., 1999). This result corresponded to the DAG selectivity of native SoMGD in envelope membranes (Maréchal et al., 1994a, b), suggesting that the DAG substrate preference of Types A and B MGDG synthases was common in seed plants.

B Digalactosyldiacylglycerol

The first DGDG synthase (DGD) gene was identified from Arabidopsis after the isolation of a DGDG knock-down mutant (Dörmann et al., 1995). The mutant, *dgd1*, was screened by lipid analysis of Arabidopsis plants mutagenized using ethyl methanesulfonate. In 1999, the DGD gene, *DGD1*, was identified by map-based cloning and complementation of the mutant *dgd1* (Dörmann et al., 1999). Compared to WT, the amount of DGDG in *dgd1* was reduced by 90%, and it was therefore suggested that DGD1 is responsible for synthesizing the bulk of DGDG in Arabidopsis (Dörmann et al., 1995). On the other hand, because the chloroplasts isolated from *dgd1* mutants still possessed DGDG synthase activity and contained some DGDG, as well as the oligogalactolipids, such as trigalactosyldiacylglycerol and tetragalactosyldiacylglycerol, it was suggested that another enzyme with processive galactosylation activity was present (Dörmann et al., 1995; Klaus et al., 2002). Although an apparent paralog of *DGD1* was difficult to find in the Arabidopsis genome, the cDNA of a *DGD1*-like gene with weak similarity to *DGD1* was finally isolated and confirmed to be DGD2 by measuring the activity of the corresponding protein expressed in *E. coli* (Kelly and Dörmann, 2002).

In previous biochemical studies, the DGD1 protein was associated with the outer envelope membrane of chloroplasts (Froehlich et al., 2001). Later, DGD2 was also shown to be localized in the outer envelope membrane (Kelly et al., 2003). Although both enzymes utilize UDP-Gal and MGDG as substrates to produce DGDG, as shown in Fig. 2, they likely play as distinct a role in chloroplasts as the Type A and Type B MGD proteins because their N-termini and their expression profiles are very different (Kelly and Dörmann, 2002).

To elucidate the roles of the two DGDs and to identify additional factors involved in galactolipid synthesis, double knock-out mutants of *DGD1* and *DGD2* were isolated and analyzed (Kelly et al., 2003). Interestingly, comparative characterization of the mutants *dgd1*, *dgd2* and *dgd1dgd2* demonstrated that under normal growth conditions, DGD1 represented the major DGD activity in chloroplasts, whereas DGD2 produced such minor amounts of DGDG that its activity was only detected when *DGD1* was disrupted (Kelly et al., 2003). Moreover, in chloroplasts from the *dgd1dgd2* double mutant, synthetic activity for DGDG and the unusual oligogalactolipids tri- and tetragalactosyldiacylglycerol was still remained, suggesting that an additional, processive galactolipid

synthesis activity independent from DGD1 and DGD2 exists in Arabidopsis (Kelly et al., 2003). A gene encoding a processive galactosyltransferase for synthesis of oligogalactosyldiacylglycerol has yet to be identified.

C Sulfoquinovosyldiacylglycerol

From early studies, it was obvious that chloroplasts were capable of synthesizing SQDG if sulfate was supplied from outside the chloroplasts. Chloroplasts should therefore contain all of the enzymes and substrates to produce SQDG. This hypothesis was confirmed by successful measurement of SQDG synthesis in chloroplast envelopes using an artificially generated sulfur donor substrate (Heinz et al., 1989). Establishment of such an assay made it possible to characterize SQDG synthase and to determine its localization on the interior surface of the chloroplast inner envelope membrane (Seifert and Heinz, 1992; Tietje and Heinz, 1998). However, the pathway of SQDG synthesis was not completely resolved until the corresponding enzymes were identified at the molecular level and were biochemically characterized using recombinant proteins expressed in *E. coli*. As shown in Fig. 2, SQDG assembly in chloroplasts requires UDP-sulfoquinovose (UDP-SQ) synthase (SQD1) and SQDG synthase (SQD2). SQD1 is responsible for the biosynthesis of the unique head group donor and SQD2 transfers the sulfoquinovose to DAG to produce SQDG.

By genetic analyses of *Rhodobacter sphaeroides*, four genes, *sqdA*, *sqdB*, *sqdC*, and *sqdD,* were determined to be necessary for SQDG biosynthesis (Benning and Somerville, 1992a, b). Among them, the product of *sqdB* resembles enzymes that modify the sugar moiety on nucleotides and *sqdB* is the only gene that has apparent orthologs in every SQDG-producing organism, namely, in the cyanobacterium *Synechococcus* sp. PCC 7942 (Güler et al., 1996), in plants such as Arabidopsis and spinach (Essigmann et al., 1998; Shimojima and Benning, 2003) and in the alga *Chlamydomonas reinhardtii* (Riekhof et al., 2003; Sato et al., 2003).

In plants and algae, the *sqdB* ortholog was designated *SQD1*. In Arabidopsis and spinach, SQD1 is localized in the stroma of the chloroplast (Essigmann et al., 1998; Shimojima and Benning, 2003). The proposed reaction mechanism for SQDG synthesis are derived from the reaction intermediate and the structural similarity between Arabidopsis SQD1 (AtSQD1) and other sugar-nucleotide modifying enzymes (Pugh et al., 1995; Essigmann et al., 1999). This mechanism (Fig. 2) was supported by detailed structural analysis of recombinant AtSQD1 using X-ray crystallography, which demonstrated that AtSQD1 was a dimeric protein containing NAD+ and UDP-glucose, but biochemical evidence for the mechanism was lacking. In 2001, the synthesis of UDP-SQ from UDP-glucose and sulfite catalyzed by SQD1 was finally demonstrated by biochemical analysis of recombinant AtSQD1 expressed in *E. coli* (Sanda et al., 2001). However, because the reaction rate of the recombinant enzyme was much slower than expected, it was hypothesized that proteins and/or cofactors might be required, or that the actual sulfur donor in vivo was not sulfite.

SQD1 of spinach was purified from the chloroplast stroma as a 250 kDa complex (Shimojima and Benning, 2003). The affinity for sulfite was at least fourfold higher than recombinant spinach SQD1. Further analyses clearly showed that ferredoxin-dependent glutamate synthase (FdGOGAT) co-purified and was tightly associated with SQD1 (Shimojima et al., 2005). The physiological or biochemical meaning of the interaction is not yet fully understood. However, FdGOGAT is a flavin-containing protein that may bind sulfite and deliver it to the active site of SQD1. This suggestion was supported by modeling of cyanobacterial FdGOGAT and AtSQD1 into a plausible complex (Shimojima et al., 2005). Therefore, FdGOGAT may participate in UDP-SQ synthesis to efficiently deliver sulfite to SQD1. Sulfite is a cytotoxic compound and is maintained at a very low level in plant cells. The interaction of FdGOGAT with SQD1 would channel sulfite directly to SQD1 and would therefore be an efficient way to overcome this problem. Recently, in a proteome analysis of bell pepper *Capsicum annuum* L., SQD1 was detected with FdGOGAT (Siddique et al., 2006). This also supports the current working hypothesis, but further experiments will be required.

D Phosphatidylglycerol

In higher plants, the biosynthesis of PG takes place in three subcellular compartments: the

plastids, the endoplasmic reticulum (ER) and the mitochondria (Moore, 1974; Mudd and Dezacks, 1981; Ohlrogge and Browse, 1995), but the synthetic pathway shown in Fig. 3 does not vary and all of the fatty acids and acyl-acyl carrier proteins for PG synthesis are synthesized in the plastids. An acyl group of acyl-acyl carrier proteins is transferred to the *sn*-1 position of glycerol 3-phosphate by glycerol 3-phosphate acyltransferase to generate 1-acylglycerol 3-phosphate (lysophosphatidic acid). A second acylation at the *sn*-2 position is performed by lysophosphatidic acid acyltransferase to produce phosphatidic acid (PA). PA is the starting precursor for PG biosynthesis. The PA fatty acid moieties are 18:1(9) at the *sn*-1 position and 16:0 at the *sn*-2 position (Nishida and Murata, 1996). Although PA has recently been identified as a transport metabolite of eukaryotic DAG backbone molecules (see Section III.E and Fig. 4), the PA substrate for cytidyldiphosphate (CDP)-DAG synthase (see Fig. 3) does not originate from the ER because plastidial PG has a prokaryotic DAG backbone (Fritz et al., 2007). As shown in Fig. 3, CDP-DAG synthase transfers the CMP moiety of CTP to PA to produce CDP-DAG. Then PG phosphate (PGP) synthase produces PGP and CMP from CDP-DAG and glycerol 3-phosphate.

In Arabidopsis, the PGP synthase isozymes are encoded by *PGP1* and *PGP2* (Müller and Frentzen, 2001). PGP1 is localized to both plastids and mitochondria whereas PGP2 is a mitochondrial enzyme (Müller and Frentzen, 2001; Babiychuk et al., 2003). Both PGP1 and PGP2 are very similar to bacterial PGP synthases, but are different from the mitochondrial enzymes in yeast and mammals (Chang et al., 1998; Kawasaki et al., 1999; Hagio et al., 2000; Müller and Frentzen, 2001). Analyses of Arabidopsis mutants in which the PGP1 gene was partially or completely disrupted revealed that PGP1 is essential for PG synthesis in plastids (Hagio et al., 2002; Xu et al., 2002; Babiychuk et al., 2003). On the other hand, PG biosynthesis in mitochondria is not essential because mitochondria could import the PG produced in the ER (Babiychuk et al., 2003). This is a unique feature of plant mitochondria and has not been observed in other organelles from yeast and mammals (Chang et al., 1998; Kawasaki et al., 1999; Ostrander et al., 2001).

The final step of PG synthesis is dephosphorylation of PGP by PGP phosphatase (Moore, 1982; Andrews and Mudd, 1985). The 18:1(9) fatty acid at the *sn*-1 position of PG is then further desaturated by the acyl-lipid desaturases (Browse and Somerville, 1991; Somerville et al., 2000). The 16:0 fatty acid at the *sn*-2 position of PG is desaturated to 16:1(3t), which is known to be very unique to the PG in plastids (Roughan and Slack, 1984; Browse et al., 1985; Roughan et al., 1987; Ohnishi and Thompson, 1991; Nishida and Murata, 1996). In higher plants, the genes for PGP synthase, *PGP1* and *PGP2*, are the only genes for PG synthesis identified to date. In the cyanobacterium *Synechocystis* sp. PCC 6803, four genes involved in PG synthesis corresponding to three enzymes were identified from mutant analyses: namely, two genes for lysophosphatidic acid acyltransferase (Weier et al., 2005; Okazaki et al., 2006), *cdsA* encoding CDP-DAG synthase (Sato et al., 2000a), and *pgsA* encoding PGP synthase (Hagio et al., 2000; see Chapter 11).

E Diacylglycerol Supply Systems

Chloroplast DAG in plastids is provided by the prokaryotic pathway or by the ER via the eukaryotic pathway as shown in Fig. 4 (Roughan and Slack, 1982). In the prokaryotic pathway, DAG is

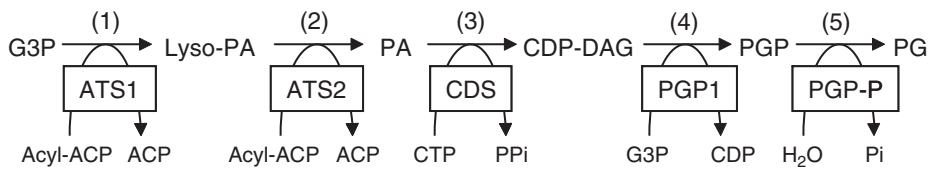

Fig. 3. Phosphatidylglycerol biosynthesis pathway in Arabidopsis plastids. (1) glycerol 3-phosphate acyl-ACP acyltransferase (ATS1), (2) lysophosphatidic acid acyltransferase (ATS2), (3) CDP-diacylglycerol synthase (CDS), (4) phosphatidylglycerophosphate synthase (PGP1), (5) phosphatidylglycerol phosphate phosphatase (PGP-P). Abbreviations: G3P, glycerol 3-phosphate; ACP, acyl carrier protein; Acyl-ACP, acyl-acyl carrier protein; Lyso-PA, lysophosphatidic acid; PA, phosphatidic acid; CDP-DAG, CDP-diacylglycerol; PGP, phosphatidylglycerol phosphate; PG, phosphatidylglycerol.

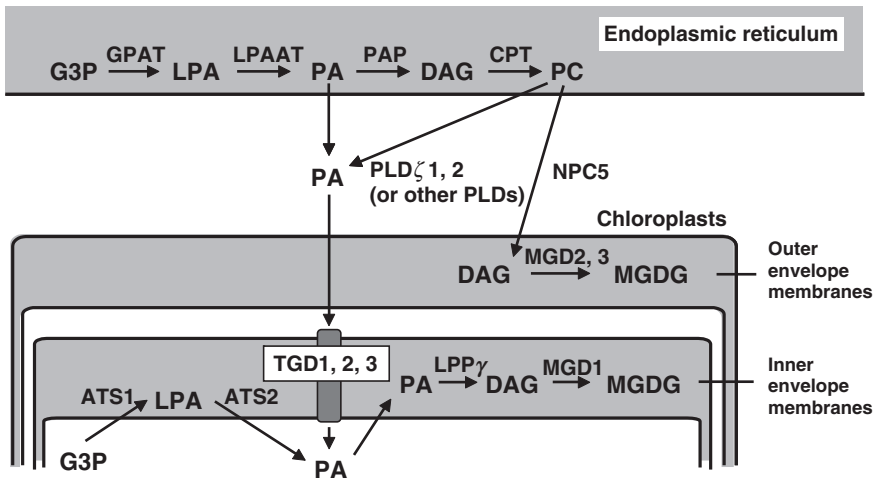

Fig. 4. Schematic diagram of DAG supply pathways in Arabidopsis plastids. Abbreviations: GPAT, glycerol 3-P acyl-ACP acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; CPT, diacylglycerol: CDP-choline choline phosphotransferase; PLDζ1, 2, phospholipase D zeta 1, 2; NPC5, non-specific phospholipase C 5; ATS1, glycerol 3-P acyltransferase; ATS2, lysophosphatidic acid acyltransferase; TGD1, 2, 3, components of the proposed phosphatidic acid transporter; LPPγ, lipid phosphate phosphatase gamma; MGD, monogalactosyldiacylglycerol synthase; G3P, glycerol 3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; MGDG, monogalactosyldiacylglycerol.

produced from PA by PA phosphatase (PAP) (Joyard and Douce, 1977). In the eukaryotic pathway, it is generally believed that DAG is derived from PC in the ER; this is known as the "PC hypothesis" (Roughan, 1970; Heinz, 1977; Douce and Joyard, 1980; Roughan and Slack, 1982) and the following lipids are suggested to be the transporting form of the lipid: lysoPC in *Allium porrum* and *Brassica napus* (Mongrand et al., 1997, 2000), DAG in *B. napus* (Williams et al., 2000) and PA in *A. thaliana* (Xu et al., 2005; Awai et al., 2006a). However, the transport mechanism and the actual lipids involved in the transport have not yet been identified.

In a suspension culture of *Acer pseudoplatanus* grown under Pi-starved conditions, PC was shown to transiently increase followed by a rapid decrease and concomitant increase in DAG (Jouhet et al., 2003). Two pathways for DAG production from PC are possible: a one-step reaction catalyzed by phospholipase C (PLC) or a combinatory two-step reaction catalyzed by phospholipase D (PLD) and PAP (Nakamura et al., 2005). However, the direct involvement of PC-hydrolyzing PLC (PC-PLC) under normal conditions is not known even though the activity has been observed in several reports (Kates, 1955; Strauss et al., 1976; Chrastil and Parrish, 1987; Rouet-Mayer et al., 1995; Scherer et al., 2002). Another group reported that cytosolic PLD is involved in the DAG supply for MGDG synthesis in pea chloroplasts (Andersson et al., 2004). Because cytosolic PLD produces PA in the cytosol, another reaction, such as DAG synthesis by PAP, is needed for subsequent MGDG synthesis.

Recently, significant progress has been made by identifying and characterizing the enzymes involved in the two-step reaction catalyzed by PLD and PAP (Nakamura et al., 2005). Arabidopsis has two mechanisms for MGDG assembly, one in the outer and the other in the inner envelope membrane of chloroplasts. Because the two pathways are independent of each other, two mechanisms for supplying DAG to chloroplasts are required (Benning and Ohta, 2005).

MGDG synthesis in the outer envelope membrane is activated under Pi-limited growth conditions in Arabidopsis (Benning and Ohta, 2005), suggesting that the metabolic pathway for DAG supply to the outer membrane may also be activated under such conditions. Consistent with this hypothesis, it was found that hydrolysis of PC to yield DAG was substantially induced upon Pi starvation (Nakamura et al., 2005). This also

suggested that the DAG supply from PC is mediated by one-step hydrolysis via PC-PLC.

There are six putative PC-PLC enzymes in Arabidopsis that are designated as non-specific phospholipase C (NPC). The expression of the PC-PLC *NPC4* was shown to be strongly induced upon Pi starvation. The NPC4 protein was localized to the plasma membranes. Recombinant NPC4 contained significant PLC activity in vitro for synthesis of PC and PE but not for phosphatidylinositol-4,5-bisphosphate or PA. In two knockout mutant alleles of *NPC4*, *npc4–1* and *npc4–2,* the total PC-PLC activity did not increase significantly upon Pi starvation, suggesting that NPC4 is responsible for PC-PLC activation under these conditions. However, it was also suggested that NPC4 is dispensable for DAG supply because the lipid composition in *npc4* was the same as that of wild type. The expression of another gene, *NPC5*, is also induced under Pi-starvation. In *npc5* mutants, significant accumulation of DGDG upon Pi starvation was not observed (Gaude et al., 2008), suggesting that NPC5 contributes to DGDG accumulation under Pi starvation. NPC5 is distinct from NPC4 in several ways although their amino acid sequences are very similar. Firstly, the level of NPC5 expressed under Pi-starved conditions and the activity of recombinant NPC5 in vitro are much lower than those of NPC4. Secondly, NPC5 is mainly a soluble protein whereas NPC4 is associated with the plasma membrane. Thirdly, NPC5 is responsible for almost half of the DGDG produced under Pi-limited conditions in leaves, whereas NPC4 is not responsible for DGDG accumulation. Whether these isoforms participate in a distinct manner in DGDG production under phosphate limitation awaits further investigation.

Recently, it was shown that PLD is also involved in DGDG accumulation during Pi starvation. In Arabidopsis, there are 12 isoforms of PLD, represented as PLDα1, α2, α3, β1, β2, γ1, γ2, γ3, δ, ε, ζ1 and ζ2 (Wang, 2005). However, most of the research on PLDs has focused on their function in production of PA, a signaling molecule in plants (Wang, 2005). PLDζ1 was first reported to be a target of transcription factor GLABRA2, which is involved in root hair formation. Indeed, the expression level of PLDζ1 was shown to affect the root hair morphology (Ohashi et al., 2003). The expression of *PLDζ2* was reported to be induced upon Pi starvation (Cruz-Ramírez et al., 2006). Knock-out mutation of *PLDζ2* resulted in a decrease in DGDG accumulation in roots with subsequent change in root architecture (Cruz-Ramírez et al., 2006). The involvement of PLDζ1 in the root hair morphology was also observed using *pldζ1pldζ2* double knock-out mutants (Li et al., 2006a, b). In the mutants, Pi starvation repressed elongation of the primary roots and induced lateral root growth (Li et al., 2006a). These morphological changes in the roots could have been caused by a decrease in PA signal and/or by a decreased amount of DGDG. Because the change in DGDG content was limited to the roots in the *pldζ1pldζ2* mutants, it is possible that upon Pi starvation, PLDs and NPCs play a distinct role in DAG supply; NPC likely controls DAG supply in shoots and both NPC and PLD likely control the supply in roots. This idea is partially supported by a report that, in the plasma membrane of Pi-starved oat roots, DAG was primarily supplied by the coupled PLD and PAP reactions (Andersson et al., 2005). Although unrelated to Pi-limited growth conditions, it is noteworthy to mention that *Petunia hybrida,* where floral organs contain higher levels of DAG than leaves, the DAG supply pathway is different among the organs. In the stamens and pistils, generation of DAG from PC via the NPC enzyme and generation of DAG from PA via the PAP enzyme occur via the Kennedy pathway. In contrast, in the petals, DAG is generated from PC by a two-step reaction catalyzed by PLD and PAP (Nakamura et al., 2003; Nakamura and Ohta, 2007). Thus DAG may be provided through a variety of pathways in floral organs.

DAG supply to the inner envelope membranes of plastids is considered to be mediated by PAP (Joyard and Douce, 1977). The optimal pH and cation sensitivity of plastidic PAP are quite different from those of PAPs in animals or yeast (Bishop and Bell, 1985; Joyard and Douce, 1987; Stymne and Stobart, 1987; Carman and Henry, 1989). The optimal pH for the plant plastid envelope PAP is alkaline, around pH 9.0, and Mg^{2+} severely inhibits the enzyme (Joyard and Douce, 1979). For animal or yeast PAPs, the optimal pH is neutral or slightly acidic and Mg^{2+} has no inhibitory effect on enzyme activity. For plant PAP, feedback inhibition of the activity by the reaction product of DAG has been reported (Malherbe et al., 1992). Importantly, PAP enzyme activity

differs among 16:3 and 18:3 plants (Gardiner and Roughan, 1983; Heinz and Roughan, 1983; Frentzen et al., 1983). The activity of PAP in plastids of 18:3 plants is lower than in 16:3 plants, in which the envelope-localized Kornberg-Pricer pathway contributes significantly to the galactolipid biosynthesis (Heinz and Roughan, 1983). This difference might explain why 18:3 plants have small amounts of galactolipid and sulfolipid containing a C16 fatty acid in the *sn*-2 position.

Although the PAP enzyme is important in membrane lipid metabolism, the corresponding protein has not yet been identified. In Arabidopsis, there are four isoforms of lipid phosphate phosphatase (LPP) that are homologs of animal and yeast LPPs. LPP1 and LPP2 were demonstrated to have PAP activity in vitro (Pierrugues et al., 2001). However, the enzymatic properties and the predicted localization of both enzymes do not correspond to that of plastidic PAP as mentioned above. Recently, a novel subfamily of LPPs with prokaryotic origin was identified and members were determined to be homologs of cyanobacterial LPP (Nakamura et al., 2007). Cyanobacteria have no mammalian or yeast LPP homologs in their genome although they have PAP activity. Based on the hypothesis that both cyanobacterial LPP and animal/yeast LPP originate from a common ancestral LPP ortholog, a putative LPP ortholog was identified in the green sulfur bacteria *Chlorobium tepidum*, which branched from other bacteria at an early evolutionary time point. Using the ortholog from *C. tepidum*, new homologs were found in cyanobacteria *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120, respectively. In Arabidopsis, there are five homologs of the cyanobacterial LPP and three of them, LPPγ, LPPε1 and LPPε2, are localized to plastids (Nakamura et al., 2007). Among them, LPPγ is considered to be the major isoform of plastidic LPPs in photosynthetic organs because its expression was the highest in shoots, its biochemical properties resembled that of native PAP in Arabidopsis chloroplasts and generation of a knock-out mutant of LPPγ failed, but the lipid composition of a *lppε1lppε2* double mutant was unchanged compared to the wild type. To date, these are the only PAP candidates in chloroplasts involved in the DAG supply for MGDG synthesis.

Originally, chloroplast PAP was believed to utilize prokaryotic PA as its substrate and to produce only prokaryotic DAG in chloroplasts. However, characterization of the mutants *trigalactosyldiacylglycerol 1* (*tgd1*), *tgd2* and *tgd3*, led to the idea that eukaryotic PA could be utilized for lipid metabolism in chloroplasts. In these mutants, incorporation of ER-derived lipid precursors into thylakoid lipids and accumulation of triacylglycerols and trigalactolipids were observed (Xu et al., 2003). TGD1 was identified as a permease-like protein, a possible component of a multipartite ABC transporter, and the protein was shown to be localized to the inner envelope membrane of chloroplasts (Xu et al., 2003). Because incorporation of PA into chloroplasts was reduced in *tgd1–1* plants, TGD1 was suggested to be a part of a multi-component ABC-type PA transporter (Xu et al., 2003, 2005; Benning et al., 2006). TGD2 was also shown to be localized in the inner envelope membrane and was identified to be a PA-binding protein, containing a predicted mycobacterial cell entry domain (Awai et al., 2006a). TGD3 exhibited basal ATPase activity and was shown to be localized inside the inner envelope membrane (Lu et al., 2007). Orthologs of TGD1, TGD2, and TGD3 are predicted to be present in Gram-negative bacteria, and the respective genes are organized in operons, suggesting a common biochemical role for the gene products. Based on the current analysis, it is hypothesized that TGD1, TGD2, and TGD3 are components of the PA transporter required for the biosynthesis of ER-derived thylakoid lipids in Arabidopsis. Further studies are needed to reveal the mechanisms by which the PA transporter delivers the substrate to chloroplast PAP.

IV Regulation of Lipid Biosynthesis

A Regulation of Monogalactosyldiacylglycerol Synthesis in Photosynthetic Organs

In Arabidopsis, there are two types of MGD, Type A and Type B (Awai et al., 2001). It is now well known that these two types are conserved among many seed plants and expression studies suggest they are functionally distinct. RT-PCR analysis revealed that Type A was expressed in all organs analyzed, whereas Type B genes were highly

expressed in non-photosynthetic organs (*AtMGD2*; flower, *AtMGD3*; root) (Awai et al., 2001).

In photosynthetic organs, the Type A MGDG synthase MGD1 predominantly regulates MGDG synthesis. However, a substantial amount of biochemical research had been performed regarding regulation of MGDG synthesis before the two types of MGD were identified in Arabidopsis. In seed plants, chloroplasts develop from proplastids during germination. The most significant morphological change in plastids is the formation of the thylakoid membrane, which is comprised primarily of galactolipids. Indeed, we reported that MGD activity increases immediately following illumination of dark-germinated cucumber seedlings with concomitant accumulation of galactolipids (Ohta et al., 1995b). Moreover, detailed analyses of CsMGD in cucumber revealed robust regulation of MGDG synthesis by light and phytohormones (Yamaryo et al., 2003). Upon illumination, induction of *MGD* expression was followed by a subsequent increase in enzyme levels and MGDG production. When detached cotyledons were subjected to illumination, induction levels of MGD expression and activity decreased but accumulation of MGDG was not affected, suggesting that light is not merely an inducer of MGD expression but is also distinctly involved in MGDG production. On the other hand, cytokinin treatment induced mRNA levels of MGD in dark-germinated seedlings (Yamaryo et al., 2003). Because cytokinin is known to be transported from the roots to the hypocotyl in seedlings, excision of cotyledons may interrupt cytokinin signaling and thus reduce gene expression. This suggests that gene expression is regulated by light via cytokinin. However, full recovery of MGDG production has not been demonstrated by cytokinin treatment of dark-grown seedlings. Simultaneous treatment of detached cotyledons with illumination and cytokinin substantially recovered MGDG production, suggesting that light and cytokinin cooperatively regulate MGDG synthesis (Yamaryo et al., 2003). Further analysis revealed that red light is required for MGDG production in seedlings although illumination with far-red light is sufficient to induce gene expression of CsMGD. Because disruption of Phy B in the cucumber *lh* mutant did not affect MGDG synthesis, Phy A is likely to be involved in photoreception for MGDG synthesis (Yamaryo et al., 2003).

Other factors involved in the regulation of MGDG synthesis have also been reported, particularly for SoMGD (Joyard and Douce, 1987; Joyard et al., 1998). In addition, isolation of the MGD gene allowed expression and purification of recombinant MGD from *E. coli* (Miège et al., 1999; Yamaryo et al., 2006) and the regulation of MGD activity by the chloroplast redox state was subsequently explored. One of the reductants, dithiothreitol, elevated the activity of SoMGD and CsMGD (Miège et al., 1999; Yamaryo et al., 2006) and elimination of dithiothreitol from the assay mixture significantly reduced the activity. This effect was confirmed by addition of the thiol-blocking reagents *N*-ethylmaleimide or *o*-phenanthroline to the assay mixture, which inhibited activity similar to other redox-controlled enzymes. These results suggested that the thiol groups on the cysteine residues in MGD are involved in activation/inactivation of the enzyme (Yamaryo et al., 2006). Because oxidization of the recombinant CsMGD with $CuCl_2$ caused loss of the activity, redox regulation is reversible at least in vitro. Therefore, it is also possible that MGD activity is tightly controlled by the redox state in vivo in chloroplasts. Moreover, recovery of the activity was also observed by the addition of thioredoxin, which may be an actual reductant in vivo. However, further experiments examining the interactions between MGD and thioredoxin will be required to support this hypothesis. Secondly, we and others have reported activation of MGD by lipid molecules. These studies originated from the observation that solubilization of the enzyme from the envelope membranes during purification resulted in loss of activity. The first report demonstrated that addition of PG to the solubilized enzyme partially recovered the activity (Covès et al., 1988). It was therefore suggested that PG may activate the enzyme in vivo, but the concentration of PG in chloroplasts is insufficient for the activation. Later, our group reported that PA is more efficient than PG for activation of MGD in cucumber (Ohta et al., 1995a). Indeed, MGD activity increased 30-fold in the presence of 60 μM PA, which was within the range of PA concentrations in chloroplasts.

Identification of Arabidopsis MGDs made it possible to analyze knock-out mutants and to demonstrate other new functions of MGDs not

previously revealed by biochemical analysis of the native and recombinant enzymes. However, the first MGD1 mutant identified was fortuitously discovered from a screen of mutants in which photosynthesis was disrupted (Jarvis et al., 2000). This mutant had a T-DNA insertion, but because the insertion was upstream of the MGD1 gene, MGD1 function was not completely disrupted. In the mutant, the chloroplasts were smaller and the thylakoid membranes were diminished compared to WT chloroplasts. The morphological changes were observed only in chloroplasts and not in etioplasts, which suggested an important role for MGD1 photosynthetic function. With respect to the lipid composition of the mutant, the concentration of MGDG was decreased to almost half of WT and MGD activity was decreased by 75%. From these results, it was suggested that MGD1 contributes to the bulk of MGDG synthesis in chloroplasts. Interestingly, the DGDG content of the mutant remained the same as WT. Because DGDG is synthesized from MGDG, the decrease in MGDG should have affected DGDG synthesis. These studies led to the following questions; (1) Is Type B MGD able to compensate for Type A function? (2) What kind of additional effects are observed if MGD1 is completely knocked out? and (3) Is MGDG essential for the function of chloroplasts? To address these questions, a null mutant of MGD1, designated as *mgd1–2*, was recently isolated and characterized (Kobayashi et al., 2007). In the siliques of *mgd1/MGD1* heterologous mutants, about 25% of seeds were wrinkled as if the embryos were immature, suggesting that these seeds may be *mgd1–2/mgd1–2* homozygous mutants with an embryonic lethal phenotype. However, when the seeds were sown on a plate containing 1% sucrose, 10% of them were able to germinate and the mutant plants were very small and transparent. Lipid analysis of *mgd1–2* plants indicated that the chloroplast lipids MGDG, DGDG and SQDG were barely detectable, suggesting abnormal formation of chloroplasts. Indeed, electron microscopy revealed that the thylakoid membranes of chloroplasts were disrupted in the mutants. Thus, analyses of *mgd1* and *mgd1–2* clearly indicated that Type B MGD cannot compensate for MGD1 and the latter is essential for photosynthetic ability and chloroplast formation.

B Regulation of Monogalactosyldiacylglycerol Synthesis in Non-Photosynthetic Organs

In non-photosynthetic organs of Arabidopsis, RT-PCR analysis indicated that the Type B MGD genes were highly expressed: AtMGD2 was expressed in flowers and AtMGD3 was expressed in roots (Awai et al., 2001). *AtMGD3* expression in seedlings was high, but the level gradually decreased with growth (Awai et al., 2001). More detailed expression studies were subsequently performed using a GUS reporter gene. The expression of MGD2::GUS or MGD3::GUS was detected only in restricted organs of Arabidopsis, whereas expression of MGD1::GUS was observed in all green tissues (Kobayashi et al., 2004). Robust expression of both MGD2::GUS and MGD3::GUS in pollen tubes suggested that MGDG synthesis by Type B MGD is involved in the growth of pollen tubes. In seedlings, the expression of MGD3::GUS was detected as well as that of MGD1::GUS, which corresponded to the RT-PCR analysis. Most notable in roots, the expression of Type B::GUS markedly increased when plants were transferred to Pi-starved growth medium (Awai et al., 2001; Kobayashi et al., 2004).

In contrast to the extensive studies of MGDG synthesis in photosynthetic organs, in which the primary membrane lipid is MGDG, MGDG synthesis in non-photosynthetic organs such as flowers and roots has not been carefully examined, although some early studies reported the presence of a large amount of galactolipid (Kleinig and Liedvogel, 1978; Camara et al., 1983). Because the two types of Arabidopsis MGD enzymes exhibited different expression profiles, it was suggested that they have distinct functions, giving rise to the possibility that two distinct mechanisms for galactolipid synthesis are utilized (Awai et al., 2001; Kobayashi et al., 2004). In Type B::GUS transformants, concentrated GUS staining in the anthers and the elongating pollen tubes was observed, suggesting that Type B MGDG synthesis is important for the reproductive process. In flowers of *Petunia hybrida*, DGDG was identified as the major galactolipid, although the ratio of MGDG to DGDG in photosynthetic organs was approximately 2:1, as expected for photosynthetic membranes (Murphy, 1982; Nakamura et al., 2003). In addition, the activity of MGD

increased during flower development, especially in mature pistils. These results demonstrated that the biosynthesis of MGDG by Type B MGD is substantial in flowers.

In Arabidopsis roots, the amount of galactolipid is usually very small. However, upon transfer of plants to Pi-starved medium, the amount of DGDG rapidly increases without accumulation of MGDG (Härtel et al., 2000). This increase occurs via two sequential reactions: MGDG synthesis by Type B MGD (Awai et al., 2001; Kobayashi et al., 2004) and DGDG synthesis by DGD1/DGD2 (Kelly and Dörmann, 2002; Kelly et al., 2003). It has also been reported that the increase in DGDG compensates for the decrease in phospholipids upon Pi starvation in the root plasma membranes of oat (Andersson et al., 2003) and in Arabidopsis mitochondria (Jouhet et al., 2004). Importantly, T-DNA insertion mutants of type B MGD (MGD2 and MGD3) were recently isolated and characterized (Kobayashi et al., 2009). Under Pi-starved growth conditions, *mgd3–1* displayed significantly reduced accumulation of DGDG, especially in roots, suggesting that MGD3 mainly contributes to the increase of DGDG. Moreover, in *mgd2mgd3* mutants, DGDG accumulation in roots was nearly abolished, clearly showing that Type B MGDs are indispensable for remodeling of the membrane lipid upon Pi starvation in roots.

C Regulation of Lipid Synthesis under Phosphate-Limited Conditions

Pi is an essential nutrient for development and reproduction not only in plants but in most living organisms; Pi is crucial for signaling cascades and is also an indispensable component of nucleic acids and phospholipids (Raghothama, 1999). In plants, Pi in the soil or the growth medium is assimilated from the root surface. However, the availability of soluble Pi is often limited because Pi primarily exists in the form of insoluble salts such as calcium phosphate or aluminum phosphate. Therefore, significant changes in morphology and biochemical processes are observed when plants are placed under a Pi-limited growth environment. A detailed analysis of Pi starvation-induced changes in gene expression was performed using an *A. thaliana* whole genome Affymetrix gene chip and the resulting data correlated well with studies examining the biochemical processes of Pi starvation (Misson et al., 2005). The increase in the number of root hairs for increased efficiency of Pi uptake represents one of the morphological changes critical for adaptation to Pi starvation (Lynch, 1995). In addition, two significant changes in the membrane lipid composition are adopted under Pi starvation, as shown in Fig. 5.

The first change in membrane lipid composition involves an increase in DGDG levels followed by replacement of phospholipids with DGDG outside of chloroplasts. The increased DGDG synthesized in chloroplasts is transferred to the plasma membranes, mitochondrial membranes and tonoplast membranes presumably by direct contact between the outer envelope membrane of chloroplasts and these other membranes (Andersson et al., 2003; Jouhet et al., 2004; Andersson et al., 2005). In Arabidopsis, several genes for galactolipid biosynthesis (*MGD2/3*, *DGD1/2*) are reported to be activated upon Pi starvation (Awai et al., 2001; Kelly and Dörmann, 2002; Kelly et al., 2003). Because little increase in MGDG has been observed during Pi starvation, the activation of type B MGD is thought to strictly supply MGDG as a substrate for subsequent DGDG synthesis.

The formation of lateral roots and root hairs is induced under Pi starvation. This adaptation is similar to that triggered by auxin. Moreover, as observed for Type B MGD::GUS, intense GUS staining was observed in the epithem cells of hydathodes, the base of trichomes, stipules and lateral root branches during Pi starvation, similar to that observed for auxin-derived GUS activity from an auxin-responsive element::GUS (DR5::GUS) construct (Avsian-Kretchmer et al., 2002; Aloni et al., 2003; Kobayashi et al., 2006). Indeed, it was suggested that architectural changes of the roots induced by Pi starvation are related to an increase in auxin sensitivity (López-Bucio et al., 2003). When Pi-starved roots were treated with the auxin inhibitors 2,3,5-triiodobenzole (an auxin transport inhibitor), *p*-chlorophenoxyisobutyric acid (an inhibitor for auxin effects) or *N*-(1-naphthyl) phthalamic acid, an auxin transport inhibitor), GUS staining of type B MGD was diminished. These results suggested that expression of type B MGD in Pi-starved roots requires polar transport of auxin from the shoot (Kobayashi et al., 2006). The plant hormone cytokinin is known to affect root elongation and lateral root formation, inhibiting

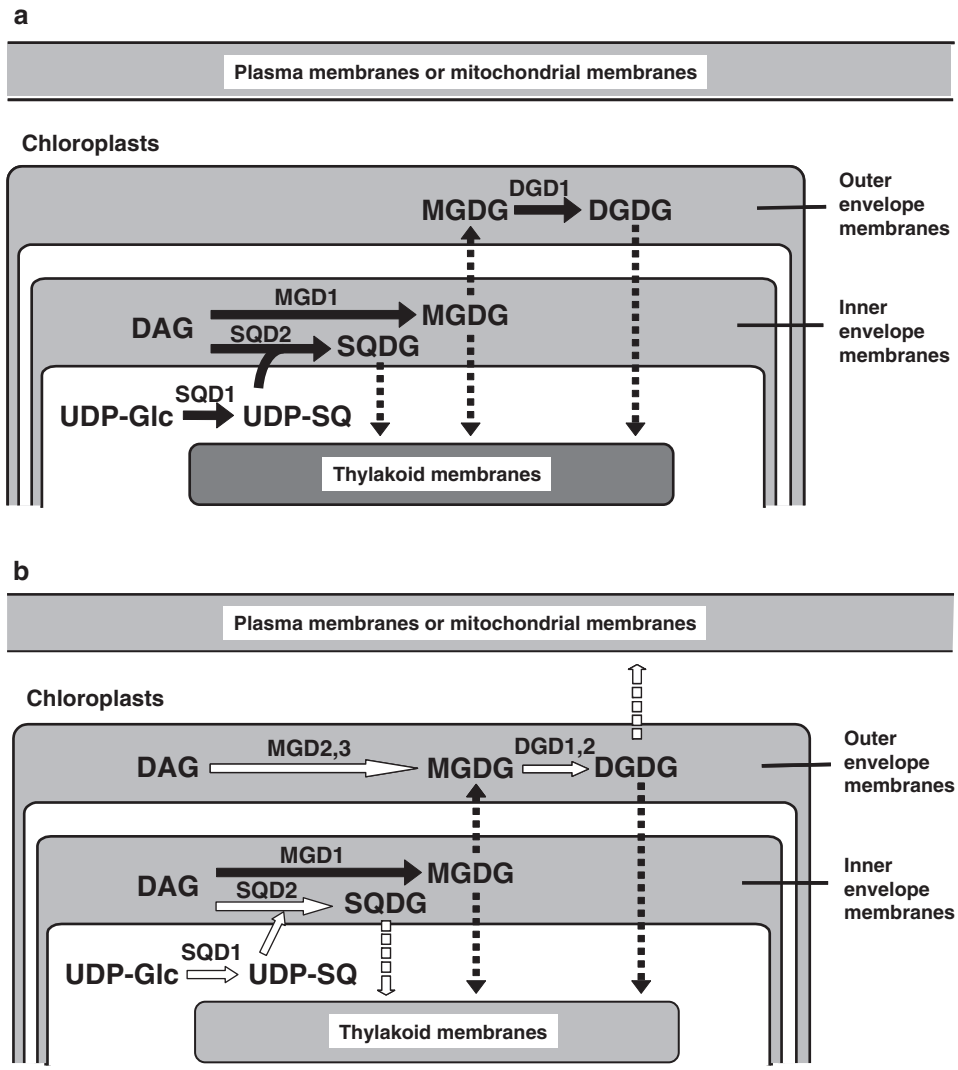

Fig. 5. Glycolipid biosynthesis in Arabidopsis leaves. (**a**) Plants grown under normal growth conditions. (**b**) Plants grown under phosphate-limited conditions. *Broken arrows* represent the movement of the lipids. *Black filled arrows* represent enzymatic reactions activated under normal growth conditions and *unfilled arrows* represent enzymatic reactions activated under phosphate-limited conditions. Abbreviations: MGD 1,2,3, monogalactosyldiacylglycerol synthase; DGD1,2, digalactosyldiacylglycerol synthase; SQD1, UDP-sulfoquinovose synthase; SQD2, sulfoquinovosyldiacylglycerol synthase; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; DAG, diacylglycerol; UDP-Glc, UDP-glucose; UDP-SQ, UDP-sulfoquinovose.

Pi-starved morphological changes. When Pi-starved plants were treated with 6-benzyladenine, a synthetic cytokinin, the expression of type B MGD was repressed in roots but not in shoots. Moreover, simultaneous treatment with 6-benzyladenine and indole-3-acetic acid, a representative auxin, recovered type B MGD expression, suggesting that effects of auxin and cytokinin are antagonistic with respect to expression of type B MGD during Pi starvation (Kobayashi et al., 2006). Such transcriptional regulation of type B MGD by auxin and cytokinin was also observed following membrane lipid alteration. The increase in DGDG upon Pi starvation was eliminated by treatment with 6-benzyladenine and 2,3,5-triiodobenzole in roots but not in shoots. Moreover, analyses of the

axr4–2aux1–7 double mutant, which is resistant to auxin (Hobbie and Estelle, 1995; Yamamoto and Yamamoto, 1999) revealed decreased expression of type B MGD and decreased accumulation of DGDG upon Pi starvation. From these data, it was suggested that auxin signaling may play an important role in Pi-starved roots in vivo. Although auxin signaling is essential for the activation of type B MGDs upon Pi starvation, auxin alone is not sufficient for inducing the expression of these genes (Kobayashi et al., 2006). Phosphite is an inactive analog of the Pi anion that mimics Pi in signaling pathways, thereby suppressing various responses induced by Pi-starvation (Ticconi et al., 2001; Varadarajan et al., 2002). Treatment of Pi-starved plants with phosphite abolished expression of type B MGD and accumulation of DGDG. However, indole-3-acetic acid treatment of these plants did not restore expression of type B MGD, indicating that suppression of Type B MGD by phosphite is independent of auxin (Kobayashi et al., 2006). These data suggested that the expression of Type B MGD is regulated by a Pi-sensing signaling system rather than by Pi availability in the cell. A recent report suggested that the expression of *MGD3* under Pi starvation is mainly controlled by the magnitude of cell division activity that determine the demand for Pi in Pi-starved plants (Lai et al., 2007).

The second change in bacteria and plant membrane lipid composition under Pi starvation involves a change in SQDG content (Gage et al., 1992; Benning et al., 1993; Güler et al., 1996; Essigmann et al., 1998; Härtel et al., 1998; Sato et al., 2000b; Yu et al., 2002; Yu and Benning, 2003; Sato, 2004). It has been suggested that SQDG can partially replace PG to maintain the anionic surface charge of thylakoid membranes in chloroplasts. Indeed, growth impairment of the SQDG-deficient *sqd2* mutant of Arabidopsis was only observed after severe Pi depletion (Yu et al., 2002). In the mutant plants, PG content remained high even under Pi-limited growth conditions. However, analysis of PG-deficient mutants revealed that PG and SQDG are not entirely interchangeable in Arabidopsis. Specifically, *pgp1* mutants defective for PGP synthase activity have severe growth and photosynthesis defects depending on the allele mutation (Hagio et al., 2002; Xu et al., 2002; Babiychuk et al., 2003). When a weak *pgp1–1* allele was combined with an insertion disruption allele of *sqd2* in Arabidopsis, the total anionic lipid content was reduced to one third of the WT levels, leading to a more severe impairment of growth and photosynthesis than in either mutant alone (Yu and Benning, 2003). These results suggest that anionic lipids such as SQDG or PG are essential for maintaining a functional photosynthetic membrane and therefore the total amount of these anionic lipids must be maintained under Pi-starved growth conditions.

V Concluding Remarks

Substantial advances in understanding the mechanisms of chloroplast lipid biosynthesis and function have been accomplished since their initial discovery. Both genetic and biochemical techniques, which have improved remarkably in this decade, allowed identification of most of the major genes involved in lipid biosynthesis. In addition, regulation of these genes in chloroplasts has been analyzed under a variety of growth conditions. The major genes identified to date are summarized in Table 2. However, the routes of substrate supply are not fully understood: the substrates UDP-Gal and DAG are required for MGDG and DGDG synthesis, whereas the substrates UDP-glucose, sulfite and DAG are required for SQDG synthesis. Analysis of substrate supply mechanisms, including identification of as yet unidentified enzymes/genes, will be an essential component of the research on chloroplast lipids in the next decade.

As genomic sequences of photosynthetic and nonphotosynthetic microorganisms have become available, searches for homologs of genes involved in chloroplast lipid biosynthesis among diverse organisms has been made possible, providing adequate tools for understanding the evolution of chloroplasts. Indeed, chloroplast lipid research is being expanded to encompass algae and cyanobacteria based on the hypothesis that the evolution of chloroplasts from cyanobacteria can be explained with respect to evolution of the membrane lipids. Both cyanobacteria and plant chloroplasts contain MGDG, but the biosynthetic pathways and enzymes involved in MGDG synthesis are different: cyanobacteria transfers glucose from UDP-glucose to DAG to

Table 2. Major genes involved in the biosyntheses of chloroplast lipids.

Pathway	Gene symbol	Locus
MGDG synthesis	*MGD1*	At4g31780
	MGD2	At5g20410
	MGD3	At2g11810
DGDG synthesis	*DGD1*	At3g11670
	DGD2	At4g00550
SQDG synthesis	*SQD1*	At4g31780
	SQD2	At5g01220
PG synthesis	*ATS1*	At1g32200
	ATS2	At4g30580
	CDS1	At1g62430
	PGP1	At2g39290
DAG synthesis	*NPC5*	At3g03540
	LPPγ	At5g03080
Lipid transport	*TGD1*	At1g19800
	TGD2	At3g20320
	TGD3	At1g65410

produce monoglucosyldiacylglycerol, followed by epimerization to generate MGDG, whereas chloroplasts transfer galactose from UDP-Gal to DAG to generate MGDG (Sato and Murata, 1982; Hölzl et al., 2005; Awai et al., 2006b). Determination of how and when these differences arose during the evolution of chloroplasts will provide important insights into the evolution of photosynthesis.

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Chapter 4

Lipids in Plant Mitochondria

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Summary

Mitochondria perform a variety of fundamental functions and are of pivotal importance in plant physiology and development. They possess a typical membrane lipid composition that is largely conserved in all eukaryotes. To establish and maintain their lipid pattern, they have to cooperate with other organelles, where a significant portion of their lipids are produced and subsequently assembled into the mitochondrial membranes. Certain lipids are, however, synthesized by the mitochondria themselves. Recent data provide new insight into the mitochondrial lipid biosynthetic pathways and the importance of their reaction products for mitochondrial structure and function. Mitochondrial de novo fatty acid synthesis, for instance, produces the precursor for the formation of lipoate, a sulfur-containing cofactor of several mitochondrial multi-enzyme complexes. This pathway has been shown to be indispensable for photoautotrophic growth of C_3 plants to meet the high demand of lipoylated glycine decarboxylase complexes catalyzing a key step in photorespiration. Polyprenyl diphosphates are channeled into mitochondrial ubiquinone synthesis by a *para*-hydroxybenzoate prenyltransferase. The central role of ubiquinone in oxidative phosphorylation is reflected in the embryo-lethal phenotype of plant mutants lacking mitochondrial prenyltransferase activity. In addition, de novo glycerolipid synthesis results in the formation of phosphatidylglycerol and cardiolipin, the typical mitochondrial membrane lipid with a unique tetraacyl structure. Mitochondria were found to require a defined level of these anionic lipids for proper structural integrity and function. Hereby phosphatidylglycerol can partly substitute cardiolipin. Cardiolipin is, however, essential for optimal mitochondrial functions and for maintaining mitochondrial activities under unfavorable conditions. Furthermore, cardiolipin has been shown to play a decisive role in controlling the dynamic equilibrium between mitochondrial fission and fusion and is indispensable for proper plant development.

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I Introduction

Mitochondria are highly dynamic, pleomorphic organelles found in nearly all eukaryotes. They appear to derive from one and the same α-proteobacterial ancestor and several basic features of their structure and function have been conserved in the various eukaryotes (Hackstein et al., 2006). Mitochondria are composed of a smooth outer membrane and an inner membrane of significantly larger surface area that surrounds the matrix and forms the inner boundary and the involuted cristae membrane interconnected by cristae junctions, small, relatively uniform tubular structures (Logan, 2006).

In higher plants, mitochondria usually have a spherical to sausage shape although more extreme morphologies are frequently seen. Within the various cell types, they are typically organized in a population of hundreds to thousands of physically discrete organelles rather than in tubular or extended reticular structures as observed in many other eukaryotes (Logan, 2006). But plant mitochondria like the organelles from other eukaryotes undergo frequent fission and fusion events not only in dividing but also in non-dividing cells (Arimura and Tsutsumi, 2005; Sheahan et al., 2005; Logan, 2006). The dynamic equilibrium between the opposing processes of fission and fusion controls mitochondrial shape, size and number and enables mitochondrial DNA recombination (Sheahan et al., 2005; Logan, 2006).

Biogenesis and maintenance of mitochondria require a close interaction with other organelles. Although mitochondria contain their own genome and protein-synthesizing machinery, the majority of the mitochondrial proteins is encoded by nuclear genes and is post-translationally imported into the organelles (Neupert and Herrmann, 2007). Similarly, some mitochondrial lipids are synthesized by the organelles themselves, but a significant portion is produced by extramitochondrial membranes and subsequently assembled into the mitochondrial membranes (Daum and Vance, 1997).

The role of mitochondria in providing the cell with ATP by oxidative phosphorylation in the cristae is well established. In addition, mitochondria are involved in a wide range of other metabolic processes, such as amino acid metabolism, biosynthesis of nucleic acids, iron–sulfur clusters, several cofactors and certain lipids (Nunes-Nesi and Fernie, 2008; Rébeillé et al., 2007). Moreover, they are implicated in cell signaling (Woodson and Chory, 2008) and programmed cell death essential for correct development and tissue homeostasis (Logan, 2006; Noctor et al., 2007).

It is also well known that plant mitochondria acting in concert with chloroplasts differ in various aspects from their animal counterparts. The unique demands placed on plant mitochondria are reflected, for instance, in their high light-dependent capacity for glycine oxidation, the presence of alternative oxidases, which bypass energy conservation, several homologues of type II NAD(P)H dehydrogenases at the inner and outer site of the inner membrane and a number of plant-specific metabolite carriers (Douce et al., 2001; Nunes-Nesi and Fernie, 2008; Rasmusson et al., 2008). Such features confer a high metabolic flexibility required for sustaining photosynthetic carbon assimilation and cell redox homeostasis under rapidly fluctuating conditions (Noctor et al., 2007; Nunes-Nesi and Fernie, 2008). In addition, the photosynthetic performance can be modulated by modifications in mitochondrial pathways like the tricarboxylic acid cycle or electron transport chain (Nunes-Nesi et al., 2008). Hence mitochondria are of pivotal importance in plant metabolism.

In this chapter, the typical lipid composition of mitochondrial membranes and the lipid biosynthetic pathways within the organelles are presented and recent data are highlighted, which provide strong evidence that lipids, especially those synthesized within the organelle, play a decisive role in sustaining structure and function of mitochondria.

Abbreviations: ACP – Acyl carrier protein; CDP-DAG – Cytidine 5′-diphosphate-diacylglycerol; CL – Cardiolipin; DGDG – Digalactosyldiacylglycerol; ER – Endoplasmic reticulum; GroP – Glycerol-3-phosphate; IPI – Isopentenyl diphosphate isomerase; KAS – β-Ketoacyl acyl carrier protein synthase; LPA – Lysophosphatidic acid, 1-acylglycerol-3-phosphate; mtKAS – Mitochondrial β-ketoacyl acyl carrier protein synthase; PHB – *Para*-hydroxybenzoate; PA – Phosphatidic acid; PC – Phosphatidylcholine; PE – Phosphatidylethanolamine; PG – Phosphatidylglycerol; PGP – Phosphatidylglycerophosphate; PI – Phosphatidylinositol; PS – Phosphatidylserine

II Lipid Composition of Mitochondrial Membranes

The various membrane systems of plant cells possess typical membrane lipid compositions, which remain relatively well conserved among tissues and plant species (Jouhet et al., 2007). Unlike plastidial membranes, where glycoglycerolipids predominate (see Chapter 3), mitochondrial membranes are largely constituted by phosphoglycerolipids, 1,2-diacyl-*sn*-glycero-3-phosphoryl (phosphatidyl) derivatives. They are characterized by 1,3-diphosphatidyl-*sn*-glycerol (cardiolipin, CL), the mitochondrial marker lipid in all eukaryotes that is largely confined to the inner membrane (Fig. 1a) and enriched in contact sites where the outer and inner membranes are closely opposed (Daum and Vance, 1997). Apart from mitochondria, CL is also found in the membrane of hydrogenosomes, mitochondria-derived organelles of protists (de Andrade Rosa et al., 2006), and the plasma membrane of bacteria (Dowhan, 1997), from which mitochondria have likely derived in the course of evolution.

Cardiolipin has a unique tetraacyl structure that consists of two phosphatidyl groups linked by a glycerol bridge (Fig. 1b). Although CL contains two phosphate groups, it carries a single negative charge under physiologically relevant pH values only, because one proton gets trapped in a bicyclic resonance structure formed by the two phosphates and the central hydroxyl group as reflected in the different pK values of the phosphate groups of CL (Haines and Dencher, 2002). The high pK_2 value and the ability of CL to form additional acid-anions between adjacent CL molecules makes CL singularly appropriate for buffering protons in its head group domain (Haines and Dencher, 2002). Due to its conical shape, CL can undergo phase transition from the lamellar to hexagonal stages, which are favored in the presence of divalent cations, for instance, and may play a role in the formation of membrane contact sites of inner and outer mitochondrial membranes. Finally, CL interacts strongly with many different proteins, which is perhaps the most important of its properties (Schlame et al., 2000; Schlame, 2008).

In mitochondria CL is a minor component besides other phosphoglycerolipids. Their total content reaches about 1 mol mg^{-1} protein and 0.5 µmol mg^{-1} protein in the outer and inner membrane, respectively (Douce, 1985). Sterols and sterol derivatives, which are enriched in the plasma membrane, are present in low but significant levels in mitochondrial membranes as well (Fig. 1a). They amount to 0.04–0.2 µmol mg^{-1} outer membrane protein and about 0.08 µmol mg^{-1} inner membrane protein in plant mitochondria (Manella and Bonner, 1975; Meance et al., 1976). These levels are, however, distinctly higher than the total content of ubiquinones being in the range of 3–5 nmol mg^{-1} protein only

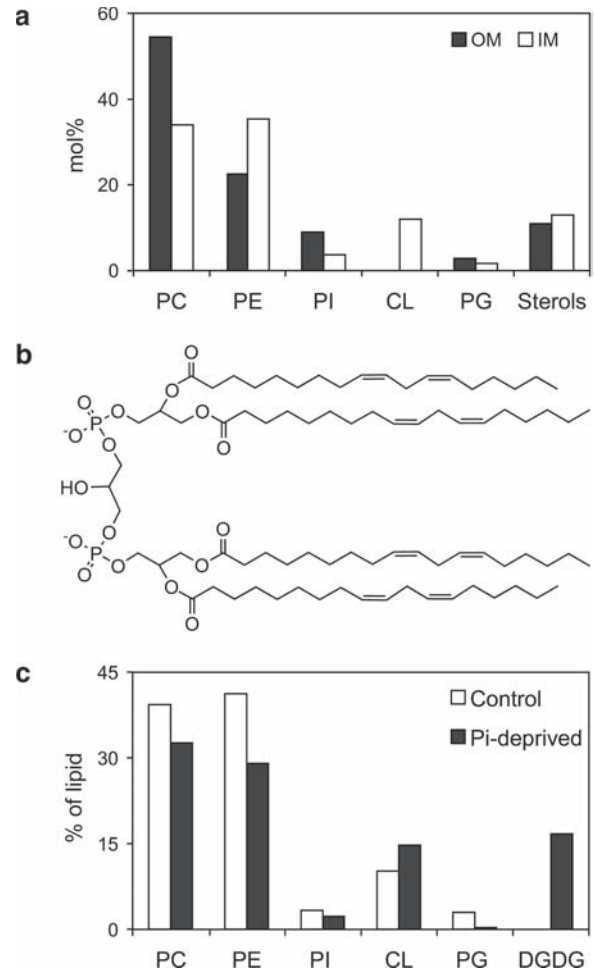

Fig. 1. Membrane lipids of plant mitochondria. (**a**) Membrane lipid composition of the outer (OM) and the inner membrane (IM) adapted with permission from Douce (1985). (**b**) Structure of a symmetric tetralinoleoyl species of cardiolipin. (**c**) Glycerolipid composition of mitochondria from *Arabidopsis* cell suspensions that were cultivated in the presence (control) or in the absence of phosphate (Pi-deprived). Adapted with permission from Jouhet et al. (2004).

(Schindler and Lichtenthaler 1984). Perhaps sphingolipids (see Chapter 5) are minor components of the outer mitochondrial membrane as well, as has been reported for the organelles from mammalians (Ardail et al., 2001), but this has not been explored in plants so far.

The phosphoglycerolipid composition of the mitochondrial membranes, which is largely conserved in all eukaryotes (Daum and Vance, 1997), consists of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as major components (Fig. 1a). Further components are the anionic phosphoglycerolipids phosphatidylinositol (PI) and phosphatidylglycerol (PG) besides CL, of which PI unlike CL is enriched in the outer membrane (Fig. 1a; Douce, 1985; Guillot-Salomon et al., 1997). Mitochondrial PC, PE and PI show fatty acid patterns similar to the respective lipids of the endomembrane system in line with their biosynthetic origin, but PC and PE species carrying polyunsaturated acyl groups in both positions are enriched in the inner membrane (Guillot-Salomon et al., 1997). Mitochondrial PG contains high levels (40–60%) of palmitic acid in its fatty acid mixture (Douce, 1985; Edman and Ericson, 1987; Dorne and Heinz, 1989; Caiveau et al., 2001) that is esterified in both glycerol positions unlike extramitochondrial PG and the lipid consists of at most 25% molecular species with two unsaturated acyl groups (Dorne and Heinz, 1989). On the other hand, CL, which is formed from PG, shows a fatty acid pattern strikingly different from that of PG. It almost exclusively carries unsaturated C_{18} acyl groups, especially linoleic and linolenic acids (Douce, 1985; Edman and Ericson, 1987; Dorne and Heinz, 1989; Caiveau et al., 2001). In addition, the available data suggest that CL from plants, similar to CL from other eukaryotes, is characterized by a high degree of molecular symmetry (Schlame et al., 2005).

Jouhet et al. (2004) reported that phosphate starvation, in response of which phosphoglycerolipids in the extraplastidial membranes are replaced by digalactosyldiacylglycerol (DGDG) synthesized in plastids, causes alterations in the mitochondrial glycerolipid composition as well. Interestingly, in the mitochondria from phosphate-deprived cells DGDG partly replaces PC, PE, PG and PI, but not CL, the level of which even increases in comparison with respective controls (Fig. 1c). The preservation of CL from degradation presumably explains why phosphate-limiting conditions hardly affect the respiratory activities of mitochondria and indicate an important role of CL in mitochondrial functions (Jouhet et al., 2004).

III Lipid Biosynthetic Pathways within Mitochondria

A Synthesis of Prenyl Diphosphate and Ubiquinones

Isoprenoids, a structurally and functionally diverse group of compounds, originate from the branched five-carbon-unit isopentenyl diphosphate and its isomer dimethylallyl diphosphate. Sequential condensation reactions of isopentenyl diphosphate with allylic diphosphates catalyzed by prenyl diphosphate synthases (Liang et al., 2002) result in the formation of linear polymers with increasing chain lengths. They serve as precursors for the synthesis of various isoprenoids, such as ubiquinones, the typical isoprenoids of mitochondria. Plants predominantly produce ubiquinone-9 and/or ubiquinone-10 with nine and ten isoprenoid units in their side chain, respectively (Lester and Crane, 1959; Ikeda and Kagei, 1979; Swiezewska et al., 1993; Hirooka et al., 2003). In addition, ubiquinone homologues with longer and shorter prenyl groups have been characterized as minor components in various plant species (Schindler and Lichtenthaler, 1984). Ubiquinones are important compounds of the respiratory electron transport chain. They also serve as radical scavengers (Ohara et al., 2004) and have been shown to be essential for embryo development in *Arabidopsis* (Okada et al., 2004).

In higher plants, isopentenyl diphosphate is synthesized via two pathways, the mevalonate pathway in the cytosol and the methylerythritol phosphate pathway in plastids (Bouvier et al., 2005). The different subcellular localization allows both pathways to operate independently. Nevertheless, several studies indicate that cooperation between the cytosolic and the plastidial pathway exists (Bouvier et al., 2005). Moreover, recent data suggest that the biosynthesis rate of the cytosolic pathway can be controlled by post-transcriptional regulation of mitochondrial RNA (Kobayashi et al., 2007).

Biochemical studies provided evidence that in addition to plastids and the endoplasmic reticulum (ER), mitochondria possess a prenylation system of their own (Lütke-Brinkhaus et al., 1984). Mitochondria, however, lack the respective enzymes for isopentenyl diphosphate formation and have to import precursors from the cytosol (Disch et al., 1998). Isopentenyl diphosphate is subsequently converted into dimethylallyl diphosphate by the action of isopentenyl diphosphate isomerases (IPIs; Fig. 2). In *Arabidopsis*, two IPIs have been identified (Campbell et al., 1998; Cunningham and Gantt, 2000). IPI1 is located in both plastids and cytosol whereas IPI2 is predominantly found in mitochondria (Okada et al., 2008; Phillips et al., 2008). Analyses of *Arabidopsis* mutants defective in one of the two IPI activities provided evidence that one enzyme can largely compensate for the lack of the other enzyme. The data indicate that mitochondria can import not only isopentenyl diphosphate but also dimethylallyl diphosphate or maybe isoprenic intermediates of longer chain lengths (Fig. 2) and, in addition, they can export these precursors (Okada et al., 2008; Phillips et al., 2008). Prenyl diphosphate synthases catalyzing the consecutive condensation of isopentenyl diphosphate with allylic substrates were found to be encoded by gene families in plants. The functional characterization of genes from *Arabidopsis* encoding prenyl diphosphate synthases resulted so far in the identification of two mitochondrial enzymes, namely, a farnesyl (C_{15}) diphosphate synthase (Cunillera et al., 1997; Manzano et al., 2006) and a geranylgeranyl (C_{20}) diphosphate synthase (Fig. 2; Okada et al., 2000). When the gene for the mitochondrial farnesyl diphosphate synthase was overexpressed in *Arabidopsis*, plants became more sensitive to oxidative stress under continuous light conditions (Manzano et al., 2006). The authors proposed that the overexpression leads to an increased flux of mevalonate-derived metabolites into mitochondria causing mitochondrial dysfunction.

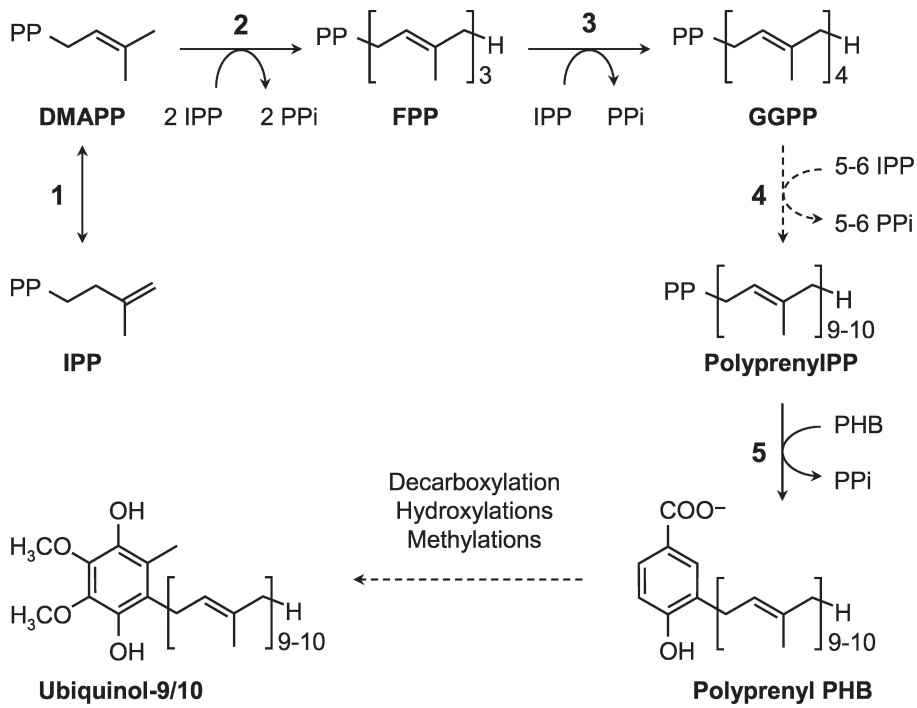

Fig. 2. Biosynthesis and channeling of polyprenyl diphosphates into the ubiquinone-biosynthetic pathway of higher plants. The reactions are catalyzed by isopentenyl diphosphate (IPP) isomerase (1), farnesyl diphosphate (FPP) synthase (2), geranylgeranyl diphosphate (GGPP) synthase (3), polyprenyl diphosphate (polyprenyl – PP) synthase (4) and PHB prenyltransferase (5). Decarboxylation, hydroxylations and methylations finally result in ubiquinol formation. DMAPP, dimethylallyl diphosphate.

Polyprenyl diphosphates are channeled into the mitochondrial ubiquinone-biosynthetic pathway by the action of a membrane-bound *para*-hydroxybenzoate (PHB) prenyltransferase (Fig. 2). Prenylation of PHB is followed by a decarboxylation step and several hydroxylations and methylations resulting in modifications at the aromatic head group. To date, the PHB prenyltransferases from *Arabidopsis* and rice (Okada et al., 2004; Ohara et al., 2006), as well as one methyltransferase from *Arabidopsis* (Avelange-Macherel and Joyard, 1998) have been identified as mitochondrial enzymes involved in ubiquinone synthesis. Homozygous mutation of *AtPPT1* was reported to be lethal (Okada et al., 2004), indicating that no other enzyme can compensate for the loss of this gene product. The mutation resulted in an arrest of embryo development at an early stage of zygotic embryogenesis, suggesting an essential role of ubiquinone (Okada et al., 2004). A common feature of PHB prenyltransferases from pro- and eukaryotes appears to be the broad substrate specificity with regard to prenyl diphosphates (Kawamukai, 2002; Bouvier et al., 2005; Ohara et al., 2006). This is even valid for the PHB prenyltransferase encoded by *slr0926* in the cyanobacterium *Synechocystis* sp. PCC 6803 (R. Sadre and M. Frentzen, unpublished). The enzyme is involved in the plastoquinone-biosynthetic pathway that shares similarities with ubiquinone biosynthesis in other prokaryotes.

The prenyl side chain lengths of the ubiquinones found in different organisms are determined by the prenyl diphosphate pool available to the prenyltransferases. This fact has allowed the development of transgenic rice plants with altered ubiquinone profile by the expression of a bacterial polyprenyl diphosphate synthase gene (Takahashi et al., 2006). Interestingly, overexpression of the yeast PHB prenyltransferase in either mitochondria or ER of tobacco plants led to increased levels of mitochondrial ubiquinones and conferred oxidative-stress resistance to the transgenic plants (Ohara et al., 2004). Based on these results, it seems likely that ubiquinone intermediates can be transported from the ER to mitochondria (Fig. 2). Moreover, the data indicate that ubiquinone biosynthesis is determined by PHB prenyltransferase activity and the available polyprenyl diphosphate pool.

B Synthesis of Fatty Acids and Lipoic Acid

Identification of mitochondrial acyl carrier proteins (ACPs; Chuman and Brody, 1989; Shintani and Ohlrogge, 1994; Meyer et al., 2007) and mitochondrial β-ketoacyl-ACP synthase (mtKAS; Yasuno et al., 2004) in conjunction with biochemical studies conducted with isolated organelles and suborganelle fractions (Wada et al., 1997; Gueguen et al., 2000) provide clear evidence that in plant cells not only plastids but also mitochondria possess a prokaryotically organized fatty acid synthase.

The mitochondrial pathway of fatty acid synthesis, which appears to be conserved in all eukaryotes (Hiltunen et al., 2005; Witkowski et al., 2007), differs in various aspects from the plastidial pathway. Plant mitochondria, except the organelles from Poaceae (Focke et al., 2003; Heazlewood et al., 2003), lack acetyl-CoA carboxylase and require malonate for fatty acid synthesis (Wada et al., 1997; Gueguen et al., 2000). Malonate imported into mitochondria, presumably via a dicarboxylate carrier (Rébeillé et al., 2007), can be converted into malonyl-ACP via malonyl-CoA by the combined action of malonyl-CoA synthetase and malonyl-CoA:ACP transacylase or by the action of a malonyl-ACP synthase, which is not detectable in plastids (Gueguen et al., 2000).

As in plastids, malonyl-ACP serves as a precursor to mitochondrial de novo synthesis of fatty acids. Unlike plastids, where three different KASs contribute to the construction of fatty acyl chains, the mtKAS catalyzes both initial condensation and subsequent elongation because it utilizes malonyl-ACP not only as donor but also as primer substrate (Fig. 3; Yasuno et al., 2004). Moreover, the mtKAS shows an unusual product profile caused by rearrangements in the active site upon primer binding (Olsen et al., 2004; Christensen et al., 2007) with two maxima, one for octanoyl-ACP and one near palmitoyl-ACP (Fig. 3; Yasuno et al., 2004). These results are in line with the data of labeling experiments showing that mitochondria effectively synthesize medium chain acyl-ACP thioesters, especially octanoyl-ACP from exogenously supplied malonate (Wada et al., 1997; Gueguen et al., 2000). These thioesters are slowly elongated so that longer acyl chains up to

C_{18} become detectable besides medium chain acyl groups after prolonged incubation times (Fig. 3; Gueguen et al., 2000). Hence the products of the mitochondrial fatty acid synthesis are strikingly different from those of the plastidial pathway and suggest that the pathways fulfill different functions.

In fact, unlike plastids, mitochondria predominantly channel their products into the pathway of lipoic acid biosynthesis (Fig. 3; Wada et al., 1997; Gueguen et al., 2000; Ewald et al., 2007). Lipoic acid covalently attached via an amide bond to the ε-amino group of a specific lysine residue, is an essential sulfur-containing cofactor of several mitochondrial multienzyme complexes, such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched chain α-ketoacid dehydrogenase and glycine decarboxylase while pyruvate dehydrogenase is located in plastids as well (Rébeillé et al., 2007). In mitochondria from photosynthetically active cells, the glycine decarboxylase complex is present at tremendously high concentrations within the matrix (Douce et al., 2001). Therefore, the H subunit of glycine decarboxylase represents the major sink for octanoyl-ACP in leaf mitochondria (Wada et al., 1997; Gueguen et al., 2000; Ewald et al., 2007). The conversion of octanoyl-ACP into an amide-bound lipoyl moiety is catalyzed by a lipoyl (octanoyl) transferase and a lipoic acid synthase (Fig. 3; Rébeillé et al., 2007). These enzymic activities have been demonstrated to occur in both mitochondria and plastids (Yasuno and Wada, 1998, 2002; Wada et al., 2001a, b).

Recent analyses of *Arabidopsis* mutants defective in the mtKAS clearly underline the importance of the mitochondrial pathway for lipoylation of the H subunit and thus for sustaining glycine metabolism (Ewald et al., 2007). They, however, also showed that mitochondria isolated from mutant leaves contain significant levels of lipoylated E2 subunits of pyruvate and α-ketoglutarate dehydrogenase. Moreover, the level of lipoylated E2 subunits of mitochondria from mutant roots was found to be very similar to that of wild-type mitochondria (Ewald et al., 2007). Provided that *Arabidopsis* does not possess a further mtKAS not readily identifiable by sequence similarity, these data suggest that a defect in the mitochondrial pathway of fatty acid synthesis can partially or even largely be compensated by an import of octanoyl or more likely lipoyl groups formed via the plastidial pathway of lipoate synthesis (Wada et al., 2001a; Yasuno and Wada, 2002) in conjunction with a lipoyl-protein ligase, recently identified in rice (Kang et al., 2007).

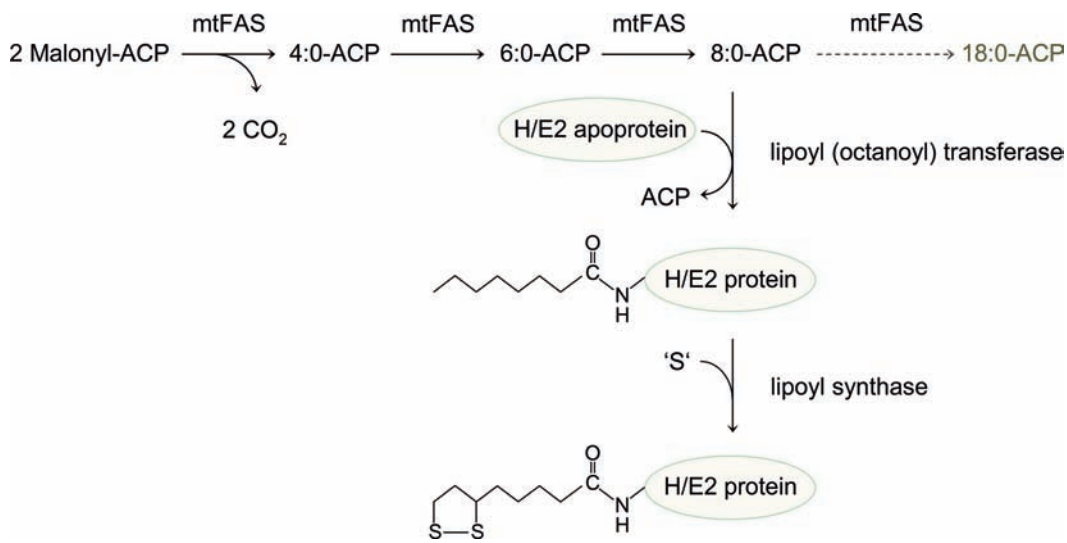

Fig. 3. Pathways of mitochondrial fatty acid and lipoic acid synthesis. The mitochondrial fatty acid synthase (mtFAS) composed of mtKAS, β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydratase and β-enoyl-ACP reductase catalyzes the initial condensation reaction yielding butyryl(4:0)-ACP and the subsequent malonyl-ACP dependent elongation reactions. Octanoyl(8:0)-ACP serves as the precursor to lipoylation of glycine decarboxylase H protein and to a certain extent of E subunits of ketoacid dehydrogenases.

C Synthesis of Glycerolipids

De novo biosynthesis of glycerolipids, the most abundant lipids in higher plants, occurs in plastids, mitochondria and the endomembrane system, mainly the ER. The three compartments have the ability to form PG in common (Frentzen, 2004). Unlike the microsomal pathway, which is essential for the biogenesis of the entire cellular membrane systems, mitochondria like plastids (see Chapter 3) synthesize a selected portion of their membrane lipids only. To establish, maintain and adapt the appropriate pattern of membrane lipids, the mitochondria have to cooperate with the other compartments, especially the ER where the various phosphoglycerolipids, such as PC, PE, PI and phosphatidylserine (PS), are synthesized and finally imported into the mitochondria via mitochondrion-associated membrane domains of the ER (see Chapter 16). Membrane contact between mitochondria and plastid envelope likely mediates DGDG transfer from the plastids to the mitochondria under certain conditions (Jouhet et al., 2004).

Within mitochondria PS can be converted to PE by the action of PS decarboxylase located at the outer surface of the inner mitochondrial membrane (Vance and Steenbergen, 2005). PS decarboxylases from yeast (Clancey et al., 1993; Trotter et al., 1993, 1995), mammalians (Kuge et al., 1991; Steenbergen et al., 2005) and plants (Rontein et al., 2003; Nerlich et al., 2007), which all belong to the group of pyruvoyl-dependent decarboxylases like bacterial PS decarboxylases (Vance and Steenbergen, 2005), have been characterized at the molecular level. These data revealed that the mitochondrial enzyme is conserved in all eukaryotes, but yeast cells possess one and *Arabidopsis* even two isozymes in the endomembrane system as well.

Analysis of yeast mutants showed that the mitochondrial PS decarboxylase is essential for the synthesis of mitochondrial PE, which cannot be completely substituted by the import of extramitochondrially formed PE (Trotter et al., 1993, 1995; Birner et al., 2001; Storey et al., 2001). In mammalians, the central role of the mitochondrial enzyme in cellular PE synthesis is reflected in the embryo-lethal phenotype of mice lacking PS decarboxylase activity (Steenbergen et al., 2005). On the other hand, analyses of *Arabidopsis* mutants defective in one, two or all three *PSD* genes encoding PS decarboxylase provide strong evidence that, in contrast to other eukaryotes, PS decarboxylation is not a major pathway for PE synthesis in plants (Nerlich et al, 2007). They are in line with those of Mizoi et al. (2006) showing that the Kennedy pathway largely contributes to PE synthesis in *Arabidopsis* and is essential for plant development. Mitochondria of the *Arabidopsis psd1 psd2 psd3* triple mutant, however, contain a slight but significant reduction in their PE content (Nerlich et al., 2007), suggesting that plant mitochondria, like the organelles from yeast, import PE less effectively than PS, although the difference is less distinct in the plant than in the yeast organelles.

1 De novo Biosynthesis of Cardiolipin

As depicted in Fig. 4, glycerolipid biosynthesis in plant mitochondria results in the formation of CL, the typical mitochondrial membrane lipid. After the stepwise acylation of glycerol-3-phosphate (GroP) catalyzed by a GroP acyltransferase and 1-acylglycerol-3-phosphate (lysophosphatidic acid, LPA) acyltransferase, a CDP-diacylglycerol (CDP-DAG) synthase activates phosphatidic acid (PA) in a CTP-dependent reaction. CDP-DAG then serves as substrate in the phosphatidylglycerophosphate (PGP) synthase reaction, the rate-limiting step of the pathway. PGP synthase transfers a phosphatidyl group from CDP-DAG to GroP yielding PGP, which is rapidly dephosphorylated to PG by a PGP phosphatase preventing the accumulation of the highly charged intermediate. Finally, a CL synthase transfers a phosphatidyl group from CDP-DAG to PG so that CL is formed (Fig. 4). The effectiveness of the mitochondrial CL synthase is reflected in the level of PG in the mitochondrial membranes being distinctly lower than that of CL (Fig. 1a). While the PG-biosynthetic pathway is conserved in nature (see Chapter 3), the mitochondrial CL synthesis differs from the bacterial one, where the CL synthase catalyzes a condensation reaction between two PG molecules that can only proceed in the presence of relatively high concentrations of substrates (Dowhan, 1997).

The first evidence that plant mitochondria contain all enzymic activities required for de novo synthesis of CL was provided by enzymic investigations with mitochondrial and submitochon-

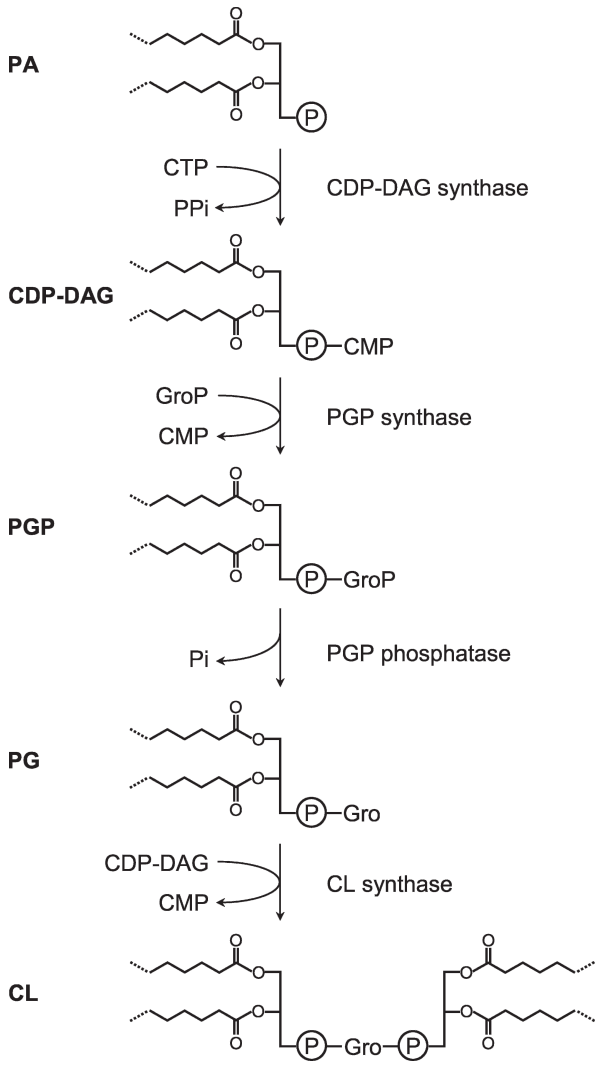

Fig. 4. Pathway of CL synthesis catalyzed by the integral membrane proteins of the inner mitochondrial membrane, namely CDP-DAG synthase, PGP synthase, PGP phosphatase and CL synthase (CMP, cytidyl monophosphate; P, phosphate group; Pi, inorganic phosphate; PPi, inorganic diphosphate).

drial fractions (Moore, 1982; Kleppinger-Sparace and Moore, 1985; Frentzen et al., 1990; Schlame et al., 1993; Frentzen and Griebau, 1994; Griebau and Frentzen, 1994). As outlined in the following, recent characterization of genes essential for CL synthesis and analyses of *Arabidopsis* mutants defective in the respective gene provided new insight into the pathway and the functions of the reaction products. These data will be presented in comparison with those of other eukaryotes.

All enzymes catalyzing the reaction sequence from PA to CL (Fig. 3) are integral proteins of the inner mitochondrial membrane as in the organelles of other eukaryotes (Schlame et al., 2000) while PA, depending on plant species, can be formed either in both mitochondrial membranes or in the outer membrane only (Moore, 1982; Frentzen et al., 1990). Mitochondria from other eukaryotes possess acyltransferases in their outer membranes, but appear to import PA from the ER for CL synthesis (Athenstaedt and Daum, 1999). In mammalian cells, two mitochondrial GroP acyltransferases have been identified, which preferentially channel their reaction products into storage rather than membrane-lipid synthesis (Gonzalez-Baró et al., 2007). On the other hand, yeast cells possess a dihydroxyacetone phosphate acyltransferase in their outer mitochondrial membrane that has been shown to contribute more pronouncedly to CL synthesis than to the synthesis of other glycerolipids (Athenstaedt and Daum, 1999). Hence these data indicate an efficient lipid-transfer mechanism between mitochondria and ER in the course of CL synthesis.

Progress has been achieved in the characterization of the two gene families of *Arabidopsis* that encode membrane-bound GroP and LPA acyltransferases (Zheng et al., 2003; Kim and Huang, 2004; Yu et al., 2004; Kim et al., 2005). But so far only one *Arabidopsis* gene, namely *AtGPAT1*, has been analyzed that likely encodes a mitochondrial GroP acyltransferase (Zheng et al., 2003). Disruption of *AtGPAT1* causes a massive arrest of pollen development, especially because of severe defects in tapetum differentiation and secretion. Because the differentiation of tapetum is known to be particularly sensitive to mitochondrial dysfunction, the observed phenotype of the *Atgpat1* mutant suggests that a lack of the acyltransferase causes mitochondrial dysfunction and failure of the organelles to instigate the programmed cell death of tapetum although other explanations cannot be ruled out at this time (Zheng et al., 2003).

Within the inner mitochondrial membrane, CDP-DAG synthase catalyzes the formation of CDP-DAG from CTP and PA that are synthesized in the mitochondria or imported from the ER (Fig. 4). In yeast cells, CDP-DAG synthase is encoded by a single gene that is targeted to both mitochondria and ER and is essential for cell viability and spore germination (Shen et al.,

1996). Two *CDS* genes encoding CDP-DAG synthase have been identified in mammalians, but the respective proteins were found to be associated with the ER only (Lykidis et al., 1997; Inglis-Broadgate et al., 2005), suggesting that mitochondrial CDP-DAG synthases of mammalians represent alternatively spliced forms or are encoded by further *CDS* genes. In plants, where CDP-DAG synthases are located not only in the inner mitochondrial membrane and the ER but also in the inner envelope membrane of plastids, the isofunctional enzymes are encoded by a small gene family that comprises five members in *Arabidopsis* (Beisson et al., 2003). The identity of *At1g62430* was demonstrated by Kopka et al. (1997) while functional expression studies in yeast revealed that the other four genes encode catalytically active enzymes as well (A. Haselier and M. Frentzen, unpublished). Proteome analysis showed that the CDP-DAG synthase encoded by *At3g60620* is located in the plastidial envelope membranes (Froehlich et al., 2003; Heazlewood et al., 2005). Experiments are currently in progress to determine the subcellular localization of the further *Arabidopsis* CDP-DAG synthase proteins, which resulted so far in the identification of another plastidial enzyme (A. Haselier and M. Frentzen, unpublished).

CDP-DAG serves as substrate for PG biosynthesis catalyzed by PGP synthase and PGP phosphatase in the inner mitochondrial membrane (Fig. 4). Unlike PGP phosphatase, of which the respective genes have not been identified from eukaryotes yet, genes encoding PGP synthase are known from yeast (Chang et al., 1998a; Dzugasová et al., 1998), mammalians (Kawasaki et al., 1999) and plants (Müller and Frentzen, 2001; Hagio et al., 2002; Xu et al., 2002; Babiychuk et al., 2003). In *Arabidopsis*, the PGP synthase isozymes of the three different compartments are encoded by two closely related genes only, termed *PGP1* and *PGP2*. While *PGP2* encodes the microsomal isozyme, *PGP1* encodes a preprotein that is targeted to both mitochondria and plastids (Müller and Frentzen, 2001; Babiychuk et al., 2003). The two *Arabidopsis* proteins exhibit substantial similarity to bacterial PGP synthases, but differ in mass, sequence and properties from the mitochondrial PGP synthases of yeast and mammalians (Chang et al., 1998a; Kawasaki et al., 1999; Müller and Frentzen, 2001). These data suggest that the mitochondrial PGP synthases of plants have an evolutionary origin different from the corresponding enzymes of other eukaryotes.

At the matrix site of the inner mitochondrial membrane, CL synthase finally catalyzes the formation of CL from PG and CDP-DAG (Fig. 4; Schlame et al., 2000). CL synthases from yeast (Jiang et al., 1997; Chang et al., 1998b; Tuller et al., 1998), plants (Katayama et al., 2004; Nowicki et al., 2005) and humans (Chen et al., 2006; Houtkooper et al., 2006; Lu et al., 2006) have been characterized at the molecular level and appear to be encoded by a single gene in the various eukaryotes. In contrast to PGP synthases, CL synthases from the various eukaryotes are highly homologous, display similar properties, and belong to the same protein family as bacterial and plant PGP synthases.

Synthesis of CL varies as a function of mitochondrial development. Expression of the yeast genes encoding both PGP and CL synthase is induced under conditions, which favor mitochondrial development (Schlame et al., 2000; Su and Dowhan, 2006a). In mammalian cells PGP synthase and CL synthase are also upregulated by factors stimulating mitochondrial biogenesis and increasing mitochondrial mass (Schlame, 2008). In plants, the CL synthase gene is ubiquitously expressed but upregulated in tissues and organs that are rich in mitochondria, such as root tips and developing embryos (Zimmermann et al., 2005; K. Katayama and H. Wada, unpublished), suggesting that CL biosynthesis in plants is under the control of factors that regulate mitochondrial biogenesis as well. Protein environment and proton motive force across the inner mitochondrial membrane can also affect CL synthase activity (Schlame et al., 2000; Gohil et al., 2004). In yeast cells, PGP synthase activity is posttranslationally regulated by reversible phosphorylations in response to inositol that causes a rapid decrease in PGP synthase activity (He and Greenberg, 2004). The activity of PGP synthase from mammalians appears to be under posttranslational control as well (Hatch et al., 2008). Whether the mitochondrial PGP synthase of plants is regulated by similar mechanisms remains to be elucidated. Recent data suggest that the CL biosynthetic pathway in yeast can be controlled by feed back mechanisms (Gu et al., 2004) and be fine-tuned by PG degradation catalyzed by a PG-specific phospholipase D (Simockova et al., 2008).

2 Remodeling of Cardiolipin

As outlined in section II, the fatty acid composition of mitochondrial PG differs appreciably from that of CL. These data in conjunction with the determined substrate specificities of CL synthase from plants (Frentzen and Griebau, 1994; Nowicki et al., 2005) suggest that the enzyme cannot establish the typical fatty acid pattern of CL. Therefore, it is likely that, in plants as in other eukaryotes, remodeling mechanisms of de novo synthesized CL exist (Schlame, 2008).

Studies with mitochondria isolated from rat liver suggested that phospholipid transacylation plays an important role in CL remodeling (Xu et al., 2003) and the respective transacylase was finally identified to be tafazzin (Xu et al., 2006a). Mutations in the gene G4.5 encoding tafazzin in humans are known to cause the Barth syndrome, a severe genetic disorder (Schlame and Ren, 2006). Meanwhile, mutants deficient or defective in tafazzin have been analyzed not only from humans (Schlame and Ren, 2006) but also from yeast (Vaz et al., 2003; Gu et al., 2004) and fruit flies (Xu et al., 2006b). These mutants are characterized by reduced levels of CL, severe alterations in CL molecular species composition and accumulation of monolysocardiolipin. Tafazzin, which belongs to the large family of acyltransferases using lysophospholipids as substrate, is associated with the mitochondrial membranes and exposed to the intermembrane space (Brandner et al., 2005; Claypool et al., 2006). To gain access to its substrate, CL must be translocated from the inner to the outer leaflet of the inner membrane and perhaps to a certain extent from the inner to the outer membrane as well. CL translocation is likely mediated by the phospholipid scramblase 3 (Liu et al., 2003a, b) in conjunction with proteins at contact sites between inner and outer membranes (Epand et al., 2007a, b; see Chapter 16).

Purified tafazzin from *Drosophila* was shown to catalyze the transfer of an acyl group from a phospholipid to a lysophospholipid, to display highest activities with PC and CL and the respective lysolipids and to possess specificity for linoleoyl groups (Xu et al., 2006a). It is equally active with *sn*-1- and *sn*-2-monolysocardiolipin, suggesting that it can essentially function as a positional isomerase (Schlame, 2008). Consequently, tafazzin can catalyze several transacylation reactions suitable for CL remodeling. Transacylations, which are near equilibrium reactions, cannot mediate the required unidirectional fatty acid transfer from phospholipids, such as PC and PE, to CL unless they are coupled to irreversible reactions. In this regard, deacylation and reacylation reactions catalyzed by phospholipase A_2 and acyl-CoA-dependent acyltransferase, respectively, might play a decisive role in converting the tafazzin products back to the substrates and thus completing the cycle (Schlame, 2008). In yeast, the CL-specific phospholipase encoded by *CLD1* (*YGR110W*) appears to be involved in the tafazzin cycle by producing monolysocardiolipins (Beranek et al., 2009).

Hence in recent years significant progress has been achieved in elucidating the CL remodeling pathway, but it is still unclear whether differences in CL composition of different cell types or organisms result from different fatty acid specificities of the respective tafazzin or from different environmental conditions, such as availability of acyl groups in the mitochondrial membrane lipids, or arises as a consequence of the chemical equilibrium between molecular species within the mitochondrial membranes as suggested by Schlame (2008).

3 Biological Functions of Phosphatidylglycerol and Cardiolipin

Biochemical studies provided evidence for the importance of CL in the structure and function of various mitochondrial enzymes, carriers and protein complexes involved in oxidative phosphorylation (Schlame et al., 2000; Schlame, 2008). The most prominent examples are ubiquinol:cytochrome *c* reductase (complex III), cytochrome *c* oxidase (complex IV) and ADP-ATP carrier where tightly bound CL has been identified in the protein crystals (Lange et al., 2001; Pebay-Peyroula et al., 2003; Shinzawa-Itoh et al., 2007). Furthermore, CL has been implicated in mitochondrial protein import, contact-site formation, cristae structure, maintenance of mitochondrial membrane permeability, and distribution of proton-motive force (Schlame et al., 2000; Schlame, 2008). Therefore, it was reasonable to predict that a deficiency in CL would result in alterations in cell respiration.

Surprisingly, mitochondrial function is not grossly perturbed in *crd1* yeast mutant strains

defective in CL synthase and thus lacking CL (Jiang et al., 1997; Chang et al., 1998b; Tuller et al., 1998). The *crd1* mutant cells are able to grow on non-fermentable carbon sources, presumably because they accumulate PG in the mitochondrial membranes in levels similar to those of CL in wild-type organelles. No significant changes in cell viability and mitochondrial functions were observed in HeLa cells either, in which the expression of CL synthase was markedly decreased (Choi et al., 2007; Huang et al., 2008). On the other hand, yeast mitochondria lacking CL show distinct defects in oxidative phosphorylation and protein import when their PG level is reduced (Jiang et al., 2000). Moreover, disruption of the *PGS1* gene encoding the PGP synthase in yeast results not only in a complete lack of PG and CL, but also in severe defects in both mitochondrial structure and function (Chang et al., 1998a). The *pgs1* mutant cells cannot grow on non-fermentable carbon sources because they fail to translate gene products essential for the electron transport chain (Chang et al., 1998a; Ostrander et al., 2001). A dramatic decrease in the levels of both PG and CL causes severe defects in mitochondrial structure and function of mammalians as well (Kawasaki et al., 1999; Han et al., 2005).

In summary, these data suggest that mitochondria require a defined level of PG and CL for their structure and function, but PG can largely substitute CL under optimal conditions. CL, however, is indispensable for optimal mitochondrial function and for maintaining mitochondrial activities under unfavorable conditions. It has been shown to be essential for optimizing the activity of the ADP/ATP carrier (Jiang et al., 2000), for maintaining mitochondrial DNA stability at elevated temperature (Zhong et al., 2004) and for sustaining inner membrane integrity and energetic coupling at maximal rates of respiratory electron transport or under stress (Koshkin and Greenberg, 2002; Zhong et al., 2004). Moreover, CL plays a decisive role in stabilizing higher ordered forms of chain complexes termed supercomplexes, which facilitate substrate channeling, prevent reactive oxygen species formation and improve biogenesis and stability of the electron transfer complexes (Zhang et al., 2002, 2005; Pfeiffer et al., 2003; Brandner et al., 2005; McKenzie et al., 2006; Chen et al., 2008a).

In addition, CL and PG have been implicated in other cellular processes (Joshi et al., 2009). For instance, translational regulation of the nuclear *COX4* gene has been shown to be mediated by a novel crosstalk pathway in which the lack of PG and CL in the mitochondrial membranes triggers the transmission of a signal back to the nucleus (Su and Dowhan, 2006b). One downstream effector of PG and CL is inositol phosphosphingolipid phospholipase C, which is activated by the anionic phospholipids and generates phytoceramide, a key regulator of respiratory growth (Vaena de Avalos et al., 2005). Recently evidence has provided that the anionic membrane lipids mediate an unexpected cross talk between mitochondria and the vacuole (Chen et al., 2008b). The analysis of yeast mutants thus provided exciting new insight into the in vivo functions of PG and CL.

Analysis of *Arabidopsis* mutants carrying a partially or completely inactivated *PGP1* gene that encodes both the plastidial and mitochondrial isozyme revealed that PGP1 is essential for plastidial PG biosynthesis and photoautotrophic growth (Hagio et al., 2002; Xu et al., 2002; Babiychuk et al., 2003; see Chapter 11). On the other hand, *Arabidopsis* mutants lacking PGP1 activity displayed no significant alterations in mitochondrial structure, protein composition and respiratory activities (Babiychuk et al., 2003). Although PGP synthase was hardly detectable in mitochondria isolated from the mutant plants, the organelle contained CL in levels very similar to wild-type mitochondria. These data suggest that mitochondria unlike plastids can compensate their defect in PG biosynthesis presumably by importing PG from the ER where the membrane lipid can still be formed by the PGP synthase encoded by the *PGP2* gene in the *Arabidopsis* mutant. In that way plant cells differ from yeast and mammalian cells that lacks the microsomal PG biosynthetic pathway and thus fail to synthesize PG and CL in mutants defective in mitochondrial PGP synthase (Chang et al., 1998a; Kawasaki et al., 1999).

Recent development and analysis of *cls-1* and *cls-2 Arabidopsis* mutants carrying a T-DNA insertion in exon 1 and exon 5 of the *CLS* gene, respectively, provided direct evidence that CL is essential for proper plant development (K. Katayama and H. Wada, unpublished). Seedlings of the homozygous mutant lines showed defects in root growth, presumably because of mitochondrial dysfunction. As shown in Fig. 5, these defects were more distinct with the *cls-2* than

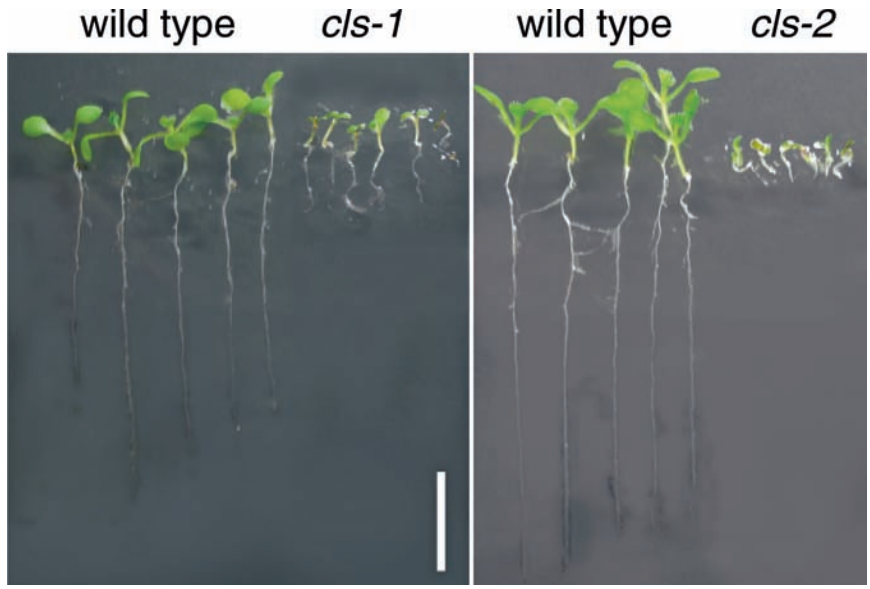

Fig. 5. Phenotype of homozygous *cls-1* and *cls-2* mutant seedlings from *Arabidopsis*, as well as wild-type controls cultivated for 7 days on sugar-containing agar plates. The mutant lines are affected in growth in comparison to the wild type, especially with regard to root growth. The *cls-2* mutant displays more severe defects than the *cls-1* mutant. The *white bar* corresponds to 1 cm (the figure was provided by K. Katayama and H. Wada (unpublished data) (See Color Fig. 4 on Color Plate 2).

with the *cls-1* mutant. While the radicula length of *cls-1* seedlings came to about 20% of that of wild-type controls, the radicula of *cls-2* seedlings was less than 10% in length and showed grossly altered columella cell organization. The defects in root growth caused retarded growth of the mutant plants and slightly affected leaf and flower development. Mutant plants produced fewer flowers than the wild type and after self-fertilization seed development was impeded, especially that of *cls-2* mutants, of which hardly any viable seeds could be harvested (K. Katayama and H. Wada, unpublished). Hence, the *Arabidopsis* mutants showed severe defects in tissues having highest levels of *CLS* gene expression (Zimmermann et al., 2005). The less distinct defects of the *cls-1* mutant in comparison to the *cls-2* mutant suggest that the CL synthase activity of *cls-1* is reduced but not abolished and this was confirmed by enzymic studies with isolated mitochondria (H. Akbari et al., unpublished).

Moreover, *cls* mutants, especially *cls-2*, exhibited aberrant mitochondrial morphology (K. Katayama et al., unpublished). In comparison to wild-type organelles, mitochondria of *cls-2* mutants were severalfold enlarged in both length and diameter. They developed cristae and few constrictions on the outer membrane. These data suggest that CL plays an important role in controlling the dynamic equilibrium between the opposing processes of plant mitochondrial fission and fusion by either inhibiting fusion or activating fission. In view of the data of Choi et al. (2006) the latter appears to be more likely, but this awaits clarification. To gain insight into the mechanisms controlling mitochondrial fusion–fission balance and the importance of CL in plant mitochondrial activities the developed *Arabidopsis* mutants are surely useful tools.

As mentioned before, mitochondria provide not only energy and metabolites for vital cellular functions but also the trigger for programmed cell death. Studies with mammalian cells suggest that CL plays an active role in mitochondrion-dependent steps of programmed cell death by facilitating the release of cytochrome *c* and further pro-apoptotic factors into the cytosol (Bayir et al., 2006; Gonzalvez and Gottlieb, 2007). In plants both plastids and mitochondria are involved in programmed cell death (Yao et al., 2004; Yao and Greenberg, 2006), but they appear to use similar types of regulation as mammalians (Noctor et al., 2007). The possible role of CL in the plant programmed cell death has, however, not been explored yet.

IV Concluding Remarks

To establish, maintain and adapt their typical membrane lipid composition, plant mitochondria have to import lipids from other compartments, but certain lipids are synthesized by the organelles themselves. Characterization of genes essential for the mitochondrial pathways of lipid biosynthesis and analysis of mutants defective in the respective genes provide new insight into the pathways, the involved lipid transport mechanisms and the in vivo functions of their reaction products. Considerable progress has especially been made with regard to the membrane lipids, PG and CL, which provide the lipid matrix with a negatively charged lipid–water interface and specifically interact with various proteins. A defined level of these anionic membrane lipids has been shown to be essential for proper structure and function of mitochondria. Hereby PG can partly substitute CL for sustaining basic mitochondrial activities, but CL is indispensable for optimal functions and proper morphology of mitochondria. These data in conjunction with those showing that thylakoid membranes require a defined level of anionic membrane lipids for biogenesis and maintenance of their photosynthetic machinery underline the importance of such membrane lipids in major cellular functions.

The elucidation of the molecular basis, by which CL affects plant–mitochondrial functions and the balance between fission and fusion, will be important goals for further experiments. This also applies to the mechanisms controlling the lipid-biosynthetic pathways, as well as the involved lipid transfer processes and the mechanisms regulating lipid homeostasis of mitochondria. Such investigations will extend our knowledge of the correlation between plant–mitochondria structure and function and the importance of the organelles in cell differentiation and cell death and thus in plant physiology and development.

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Chapter 5

Plant Sphingolipids: Structure, Synthesis and Function

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Summary

Sphingolipids are major structural components of endomembranes and dynamic regulators of basic cellular processes in plants. Advances during the past decade have revealed that sphingolipids are essential molecules in plants, and many of the genes for sphingolipid biosynthetic enzymes have been identified and characterized. In addition, improved methods for sphingolipid extraction and analysis have uncovered the immense structural complexity and quantitative importance of sphingolipids in plant cells. These advanced analytical methods have also been increasingly applied to the characterization of *Arabidopsis thaliana* mutants to provide unexpected insights into sphingolipid metabolism and function. Complementing these studies is a growing awareness that sphingolipids are one of the most abundant lipid components of the plasma membrane of plant cells and may play a role in the organization and function of membrane microdomains that are important for cell surface activities and for trafficking of proteins to the plasma membrane. Furthermore, sphingolipid metabolites including free and phosphorylated forms of long-chain bases and ceramides have been linked as bioactive regulators to a number of cellular processes (e.g., programmed cell death) that are important for abiotic stress resistance, plant development, and plant–pathogen interactions. This review provides a synopsis of the rapidly progressing field of plant sphingolipid biology and highlights gaps in our knowledge of the metabolism and function of these molecules in plants.

Abbreviations: ABA – Abscisic acid; acd – Accelerated cell death; ACP – Acyl carrier protein; AAL – *Alternaria alternata* f. sp. *lycopersici;* GBA – Bile acid β-glucosidase; DRM – Detergent-resistant membranes; ER – Endoplasmic reticulum; FATB – Fatty acid thioesterase B; GlcCer – Glucosylceramide; GLTP – Glycolipid transfer protein; GIPC – Glycosyl inositolphosphoceramide; HR – Hypersensitive response; IPC – Inositolphosphoceramide; L_d – Liquid-disordered phase; L_o – Liquid-ordered phase; LCB – Long-chain base; LCB-P – Long-chain base-1-phosphate; T_m – Melting temperature; PR – Pathogenesis-related; PI – Phosphatidylinositol; RNAi – RNA interference; SPT – Serine palmitoyltransferase; d18:0 – Sphinganine (dihydro sphingosine); SPHK – Sphingosine kinase; VLCFA – Very long-chain fatty acid; t18:0 – 4-Hydroxysphinganine (phytosphingosine); t18:1Δ8*trans* – 4-Hydroxy-Δ8*trans*-sphingenine; t18:1Δ8*cis* – 4-Hydroxy-Δ8*cis*-sphingenine; d18:1Δ4*trans* – Δ4*trans*-sphingenine (sphingosine); d18:1Δ8*trans* – Δ8*trans*-sphingenine; 18:1Δ8*cis* – Δ8*cis*-sphingenine; d18:2Δ4*trans*,8*cis* – Δ4*trans*,8*cis*-sphingadienine; d18:2Δ4*trans*,8*trans* – Δ4*trans*,8*trans*-sphingadienine

I Introduction

Sphingolipids are essential components of the endomembrane system in plants and other eukaryotes. Following their discovery in plants in the 1950s (Carter et al., 1958a, b), sphingolipids received only modest study due, in part, to the challenges associated with their analyses and the assay of sphingolipid metabolic activities in plant extracts. The quantitative significance of sphingolipids in plant cells was also not fully appreciated. However, sphingolipid biology has become an emerging area of plant lipid research during the past 5–10 years. The advances in sphingolipid research in plants have been guided by increased knowledge of sphingolipid metabolism in yeast and mammals (Lynch and Dunn, 2004). In addition, tools have been developed that make sphingolipid research in plants more tractable. These include functional genomic tools,

particularly collections of *Arabidopsis thaliana* insertion mutants that have enabled researchers to examine the consequences of altered gene expression on sphingolipid metabolism and function (e.g., Chen et al., 2006, 2008; Tsegaye et al., 2007; Dietrich et al., 2008). In addition, refined sphingolipid analytical protocols have been developed that allow for quantitative extraction of sphingolipids and comprehensive structural profiling of sphingolipids from plant tissues (Markham et al., 2006; Markham and Jaworski, 2007). Furthermore, an increasing number of forward genetic studies of mutants affected in plant development and pathogen resistance have uncovered new and unexpected roles of sphingolipids in plant biology (e.g., Koga et al., 1998; Stone et al., 2000; Brandwagt et al., 2002; Liang et al., 2003; Shi et al., 2007).

It is now recognized that sphingolipid function in plants is multi-faceted. Sphingolipids are primarily major structural components of endomembranes and have been estimated to compose ≥40% of the lipids of the plasma membrane and are also enriched in tonoplast (Verhoek et al., 1983; Yoshida and Uemura, 1986; Haschke et al., 1990; Sperling et al., 2005; Laloi et al., 2007). In addition to providing structural integrity to membranes, growing evidence supports a role of sphingolipids in detergent-resistant membrane (DRM) fractions or lipid rafts that are important for organization and function of proteins on the surface of plant cells and for trafficking of proteins through the Golgi to the plasma membrane (Mongrand et al., 2004; Borner et al., 2005; Laloi et al., 2007; Lefebvre et al., 2007). In addition, sphingolipids can function through biosynthetic intermediates and metabolites to mediate cellular processes, such as programmed cell death and ABA (abscisic acid)-dependent signal transduction (Ng et al., 2001; Coursol et al., 2003; Worrall et al., 2003).

Underlying the function of sphingolipids in plants is an intricate biosynthetic pathway that uses fatty acid and amino acid precursors to generate a wide array of sphingolipid structures and involves membrane-associated reactions in multiple subcellular compartments. The hundreds of different sphingolipid molecules that arise from this pathway have distinct physical and biochemical properties that can impact their functions in plant cells. Sphingolipid synthesis in plants is also balanced by catabolic pathways that not only provide a mechanism for the turnover of sphingolipids but can also generate bioactive molecules that regulate diverse cellular processes (Ng et al., 2001; Coursol et al., 2003; Liang et al., 2003).

In this review, we highlight current knowledge of sphingolipid structure, metabolism, and function in plants. We also describe gaps in our knowledge of sphingolipid biology that will likely be topics for future study in this rapidly evolving area of plant lipidology.

II Sphingolipid Structure

Like membrane glycerolipids, sphingolipids are amphiphilic molecules that contain a polar head group and two hydrophobic acyl chains (Fig. 1). The hydrophobic portion of sphingolipids is contained in their ceramide backbone, which is composed of a fatty acid bound through an amide linkage to a long-chain base (LCB). Fatty acids of plant sphingolipids typically range in chain length from 16 to 26 carbon atoms and are either saturated or monounsaturated with a *cis*-ω9 double bond (Imai et al., 2000). Small amounts of very long-chain fatty acids (VLCFAs) with odd numbers of carbon atoms (e.g., C21, C23, and C25) are also detectable in sphingolipids (Sastry and Kates, 1964; Carter and Koob, 1969). In addition, the fatty acid moiety frequently occurs with a α- (or C-2-) hydroxy group (Sastry and Kates, 1964; Carter and Koob, 1969).

The LCB is a unique component of sphingolipids and sphingolipid metabolites that is a combination of an amino acid (serine) and a fatty acid (typically palmitic acid, 16:0) (Fig. 2). In plants, LCBs contain 18 carbon atoms and are characterized by the presence of either two or three hydroxyl groups. Dihydroxy LCBs contain hydroxyl groups at the C-1 and C-3 positions, while trihydroxy LCBs contain an additional hydroxyl group at the C-4 position. Dihydroxy LCBs and trihydroxy LCBs occur in planta in the D-*erythro* and D-*ribo* configurations, respectively. The initial LCB produced in plants is sphinganine (or dihydrosphingosine), which is fully saturated and contains two hydroxyl groups.

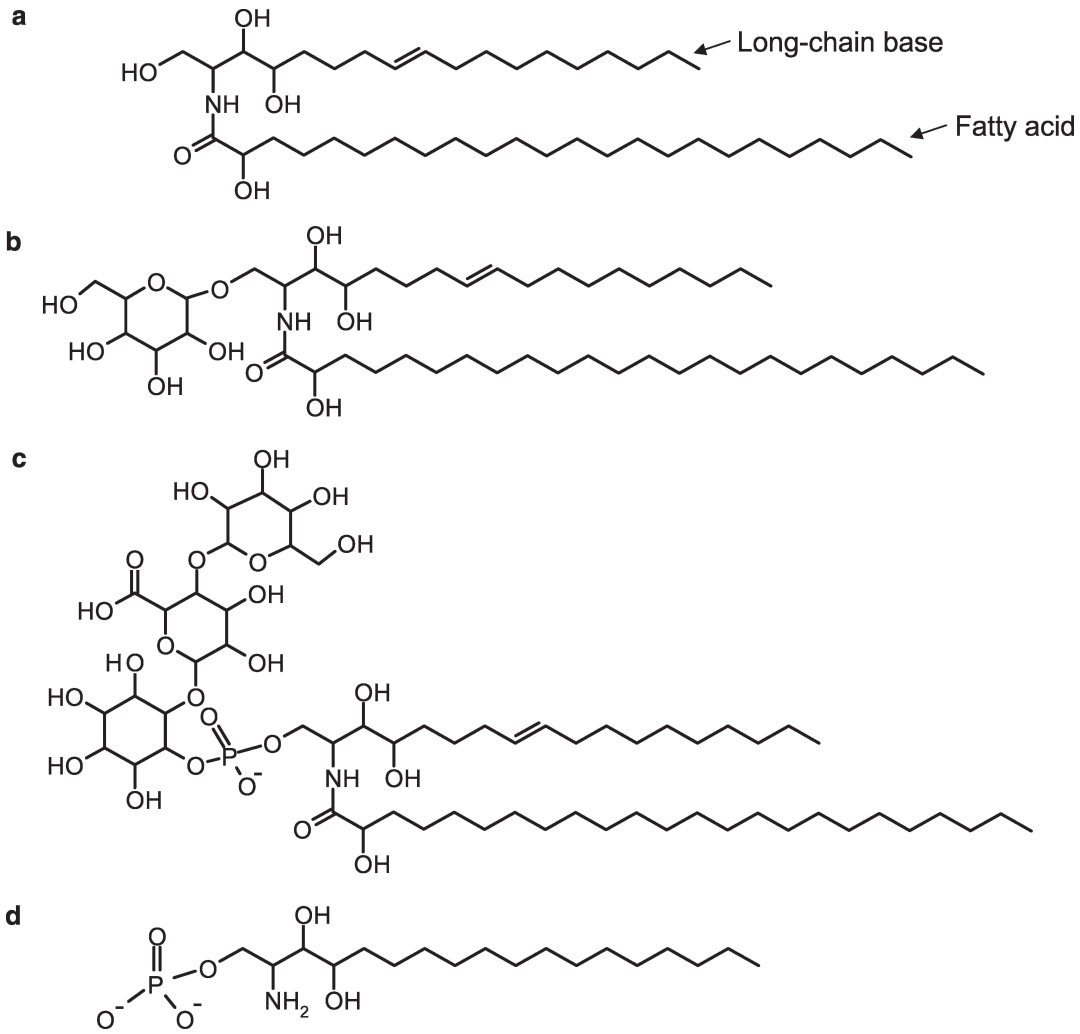

Fig. 1. Examples of complex sphingolipids and sphingolipid metabolites found in plants. Complex sphingolipids consist of a polar head group bound to a hydrophobic ceramide **(a)**. The ceramide backbone comprises a fatty acid bound to a C18 long-chain base through an amide linkage. The two major classes of complex sphingolipids in plants are glucosylceramides (GlcCers) and glycosyl inositolphosphoceramides (GIPCs), examples of which are shown in **(b)** and **(c)**. GlcCers contain a glucose bound through a 1,4 glycosidic linkage to a ceramide **(b)**. The GIPC shown in (**c**), a hexose-hexuronic acid-inositolphosphoceramide, is the major GIPC in *Arabidopsis thaliana*. Shown in **(d)** is a long-chain base-1-phosphate (LCB-P). Certain structural forms of LCB-Ps are believed to be involved in the regulation of cellular processes, such as ABA-dependent stomatal closure.

This LCB can be further modified by the addition of not only a C-4 hydroxyl group, but also by introduction of double bonds between the C-4 and C-5 atoms and the C-8 and C-9 atoms. The Δ4 double bond occurs exclusively in the *trans* configuration, whereas the Δ8 double bond can be either *cis* or *trans*. In contrast to mammals, Δ4-monounsaturated LCBs (Δ4*trans*-sphingenine or sphingosine) are typically of low abundance in plant sphingolipids. Instead, the Δ4 double bond is more frequently found in diunsaturated LCBs in combination with a Δ8 double bond (Lynch and Dunn, 2004). The most widely occurring LCBs in plant sphingolipids are sphinganine (or dihydrosphingosine; d18:0), 4-hydroxysphinganine (or phytosphingosine; t18:0), 4-hydroxy-Δ8*cis/trans*-sphingenine (Δ8*cis*- or *trans*-t18:1), and sphingadiene (Δ4*trans*-, Δ8*cis*- or *trans*-d18:2) (Lynch and Dunn, 2004) (Fig. 2). The relative amounts of these LCBs in ceramides of sphin-

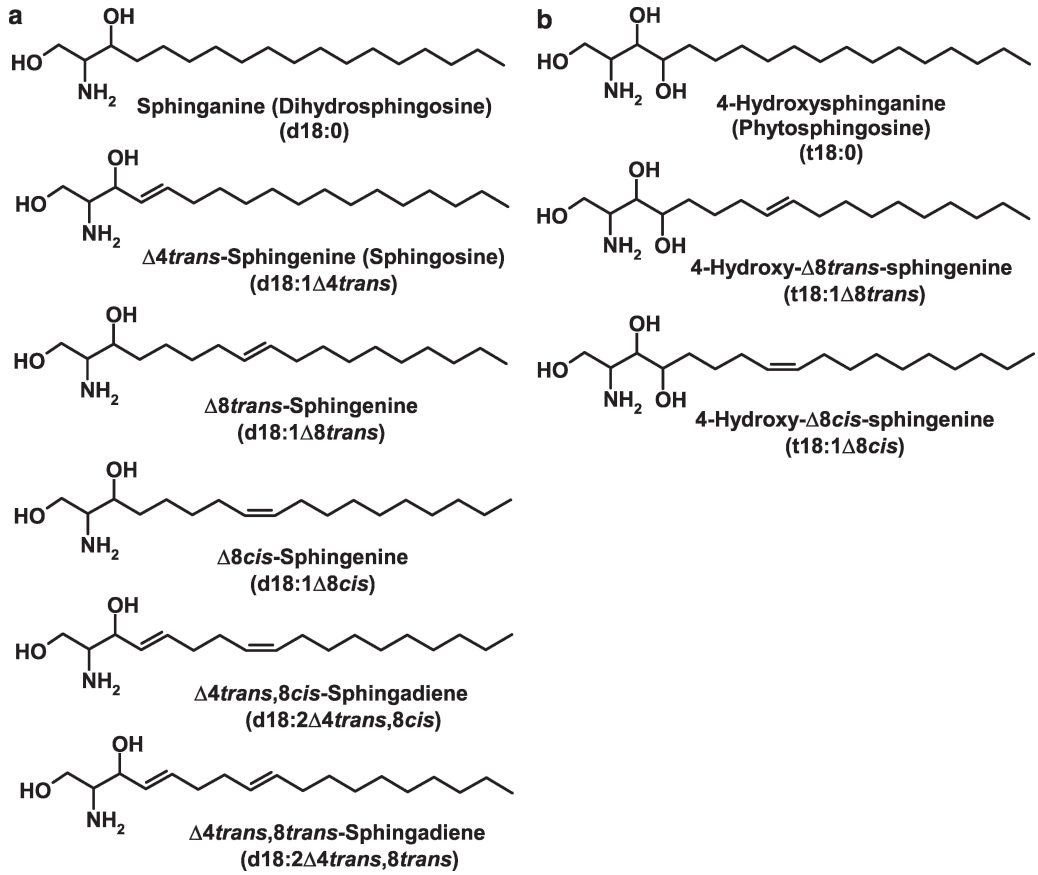

Fig. 2. Long-chain bases (LCBs) found in plant sphingolipids. Plant LCBs are derived from the condensation of serine and palmitoyl-CoA. **(a)** Dihydroxy LCBs contain hydroxyl groups at the C-1 and C-3 positions. **(b)** Trihydroxy LCBs contain an additional hydroxyl group at the C-4 position. Dihydroxy and trihydroxy LCBs can contain double bonds at the Δ8 position that are either in the *cis* or *trans* configurations. Dihydoxy LCBs can also contain a *trans* double bond at the Δ4 position. In the nomenclature used, d18:1Δ8*trans*, for example, indicates that the LCB is dihydroxy ("d") and contains 18 carbon atoms and one double bond at the Δ8 position that is in *trans* configuration. The "t" in the LCB names in **(b)** indicates that these molecules are trihydroxy LCBs.

golipids can vary widely among different organs of a single species and between different species. Although the vast majority of LCBs are found in ceramides, a small but detectable amount is present in plant cells in a free form or as a phosphate ester (Markham and Jaworski, 2007). The latter consist of a phosphate group bound to the C-1 hydroxyl of the LCB. LCB-1-phosphates (LCB-Ps) have been ascribed a number of bioactive properties in plants and other eukaryotes (e.g., Zhang et al., 1991; Dickson et al., 1997; Worrall et al., 2003; Taha et al., 2006) (Fig. 1).

The hydrophilic portion of sphingolipids consists of a polar head group that is bound to the C-1 atom of the LCB moiety of the ceramide. A large array of different head groups can be found in eukaryotes. These include carbohydrate residues that can be as simple as a glucose or galactose residue found in glucosyl- and galactosylceramides or as complicated as the extensive chains of sugar residues found in gangliosides. Other head groups include phosphocholine that is found in sphingomyelin and phosphoinositol-based moieties that are characteristic of yeast sphingolipids. To date, the two major classes of complex sphingolipids identified in plants are glucosylceramides (GlcCers) or glucocerebrosides and glycosyl inositolphosphoceramides (GIPCs) (Carter et al., 1958b, 1960; Kaul and Lester, 1975; Markham et al., 2006) (Fig. 1). The glucose residue in glucosylceramides

(GlcCers) is bound to the ceramide backbone in a 1,4 glycosidic linkage. GlcCers, together with free ceramides, are collectively referred to as neutral sphingolipids. The GIPCs, by contrast, are charged sphingolipids that contain a phosphoinositol group attached to the C-1 hydroxyl of the ceramide backbone through a phosphoester linkage (Carter et al., 1958b; Kaul and Lester, 1975). The inositol residue is substituted with additional sugar or sugar-derived residues. The major GIPC of *Arabidopsis thaliana*, for example, was recently identified as hexose-hexuronic acid-inositolphosphoceramide (Markham et al., 2006). The occurrence of even more complex GIPCs has been reported in tobacco. These include GIPCs with head groups containing two arabinose and two galactose moieties and two arabinose, two galactose, and one mannose moieties (Kaul and Lester, 1978).

Of the two major classes of sphingolipids, GlcCers have been more extensively characterized in plants. This is largely due to their ease of purification using traditional methods for extraction of plant lipids. GlcCers can also be enriched in lipid extracts based on the resistance of the amide bond between the fatty acid and LCB to mild alkaline hydrolysis. Similar treatment readily releases fatty acids from the glycerol backbone of the more abundant glycerolipids. GIPCs, however, are largely non-recoverable using the common chloroform/methanol-type extraction methods because of the high degree of polarity associated with their head groups. Recently, yeast lipid extraction protocols that use more polar solvents and heating have been adapted for the quantitative extraction of sphingolipids from plant leaves (Markham et al., 2006). Through the use of these protocols, it was recently determined that GIPCs are the most abundant class of sphingolipids in *Arabidopsis thaliana* leaves, accounting for approximately 60–65% of the total sphingolipids (Markham et al., 2006). GlcCers, by comparison, make up about 30% of the total sphingolipids in *Arabidopsis thaliana* leaves. In addition to GIPCs and GlcCers, ceramides, free LCBs, and LCB-Ps account for <10% of the total sphingolipid fraction in *Arabidopsis thaliana* leaves. It is also notable that phosphoceramides have been reported as anchors of arabinogalactan proteins in plasma membrane of pear and rose (Oxley and Bacic, 1999; Svetek et al., 1999). This is similar to the role that glycosylphosphatidylinositol (GPI) plays as a membrane anchor of proteins in plants and other eukaryotes (Paulick and Bertozzi, 2008). The quantitative significance of phosphoceramides as protein anchors in plants has yet to be determined (Oxley and Bacic, 1999; Svetek et al., 1999).

III Synthesis of Sphingolipids

A Ceramide Synthesis

1 Serine Palmitoyltransferase

The LCB component of ceramides results from the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine (Fig. 3). This reaction is catalyzed by serine palmitoyltransferase (SPT), a member of the pyridoxal phosphate-dependent α-oxamine synthase subfamily. The *Arabidopsis thaliana* SPT has been shown to consist of LCB1 and LCB2 subunits, which is similar to the subunit structure of other eukaryotic SPTs (Tamura et al., 2001; Chen et al., 2006; Dietrich et al., 2008). Although both LCB1 and LCB2 share structural similarity to α-oxamine synthases, the catalytic lysine residue that forms a Schiff base with pyridoxal phosphate resides in the LCB2 subunit (Tamura et al., 2001). Based on studies of the *Saccharomyces cerevisiae* enzyme, it is believed that the active site of SPT is at the interface of LCB1 and LCB2, and LCB1 functions to stabilize LCB2 (Gable et al., 2002; Han et al., 2004). SPT is generally regarded as the primary regulated step in sphingolipid biosynthesis in yeast and mammals, with regulation occurring at both the transcriptional and post-transcriptional levels (Hanada, 2003). Detailed studies on the biochemical properties and regulation of the plant SPT have yet to be reported. Studies of knock-out mutants for the LCB2 subunit of the *Arabidopsis thaliana* SPT have shown unequivocally that sphingolipid synthesis is essential for the viability of plant cells (Dietrich et al., 2008; Teng et al., 2008). The *Arabidopsis thaliana* LCB2 is encoded by two genes designated *LCB2a* (At5g23670; Table 1) and *LCB2b* (At3g48780) that are constitutively expressed and encode redundant polypeptides (Dietrich et al., 2008). Mutants containing a homozygous knock-out of one gene and a hetero-

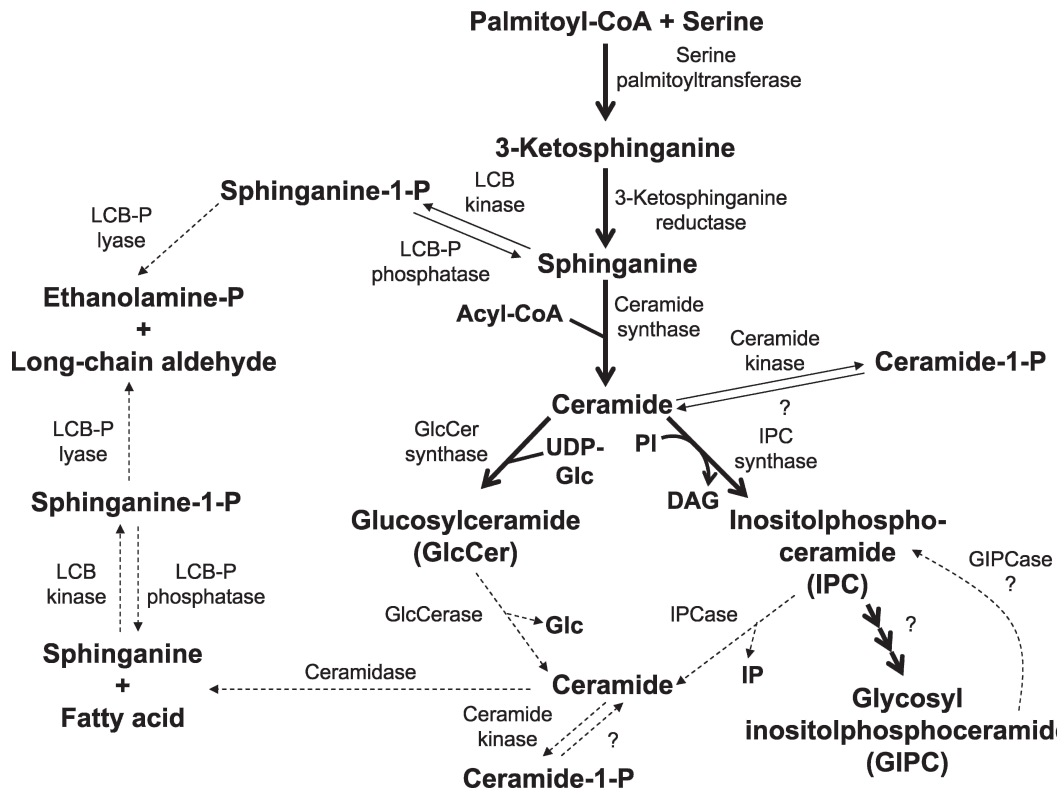

Fig. 3. Integrated pathways for the biosynthesis and turnover of sphingolipids in plants. *Solid arrows* indicate biosynthetic steps and dashed arrows indicate catabolic steps. *Arrows labeled with "?"* correspond to reactions that have yet to be identified in plants. Abbreviations: GlcCer, glucosylceramide; Glc, glucose; PI, phosphatidylinositol; DAG, diacylglycerol; IP, inositolphosphate; GlcCerase, glucosylceramidase; GIPC, glycosyl inositolphosphoceramide; GIPCase, glycosyl inositolphosphoceramidase; IPCase, inositolphosphoceramidase; LCB, long-chain base; LCB-P, long-chain base-1-phosphate; Ceramide-1-P, ceramide-1-phosphate.

zygous knockout for the second gene contain 50% aborted pollen, consistent with male gametophytic lethality resulting from loss of sphingolipid synthesis (Dietrich et al., 2008). In addition, partial RNAi suppression of the one *LCB1* gene (At4g36480; Table 1) of *Arabidopsis thaliana* was accompanied by reduced growth (Chen et al., 2006). However, the sphingolipid LCB content of these plants on a dry weight basis was unaffected, suggesting that plants compensate for down-regulation of sphingolipid synthesis by reduced growth (Chen et al., 2006).

2 3-Ketosphinganine Reductase

In the second step of LCB synthesis, 3-ketosphinganine, the product of SPT, is reduced by the enzyme 3-ketosphinganine reductase to form sphinganine (d18:0), the simplest LCB found in plants (Fig. 3). 3-Ketosphinganine reductase is encoded by two genes in *Arabidopsis thaliana* (At3g06060, *KSR-1* and At5g19200, *KSR-2*; Table 1). Although both genes contribute to the reductase activity and are essential, *KSR-1* is more highly expressed in *Arabidopsis thaliana* leaves and is the major contributor to in vivo sphinganine production (M. Chen and E.B. Cahoon, unpublished data). The saturated dihydroxy LCB d18:0 resulting from the combined activities of SPT and 3-ketosphinganine reductase is available for incorporation into ceramides. However, in plant sphingolipids, d18:0 is typically a minor LCB (Sperling et al., 2005; Markham et al., 2006). Instead, the majority of the d18:0 undergoes C-4 hydroxylation to form the trihydroxy LCB t18:0, a reaction that most likely occurs prior to incorporation of the LCB into ceramides. LCB C-4 hydroxylation is described in more detail below.

Table 1. Putative and characterized *Arabidopsis* genes involved in sphingolipid metabolism.

Arabidopsis gene (AGI code)	Designated gene symbol	Function (characterized or predicted)	*Saccharomyces cerevisiae* or mammalian homolog	References
At4g36480	*LCB1*	Subunit of serine palmitoyltransferase	*LCB1*	Chen et al., 2006
At5g23670	*LCB2a*	Subunit of serine palmitoyltransferase	*LCB2*	Tamura et al., 2001; Dietrich et al., 2008
At3g48780	*LCB2b*			
At3g06060	*KSR1*	3-Ketosphinganine reductase	*TSC10*	Beeler et al., 1998; Dunn et al., 2004
At5g19200	*KSR2*			
At1g69640	*SBH1*	LCB C-4 hydroxylase	*SUR2/SYR2*	Haak et al., 1997; Grilley et al., 1998; Chen et al., 2008
At1g14290	*SBH2*			
At3g61580	*SLD1*	LCB Δ8 desaturase	None	Sperling et al., 1998; Ryan et al., 2007
At2g46210	*SLD2*			
At2g34770	*FAH1*	Fatty acid α-hydroxylase	*FAH1/SCS7*	Haak et al., 1997; Mitchell and Martin, 1997
At4g20870	*FAH2*			
At4g04930	None	LCB Δ4 desaturase	*DES1*, *DES2*	Ternes et al., 2002; Michaelson et al., 2009
At3g25540	*LOH1*	Ceramide synthase	*LAC*, *LAG1*	Brandwagt et al., 2000; Spassieva et al., 2002
At3g19260	*LOH2*			
At1g13580	*LOH3*			
At2g37940	*IPCS1*	IPC synthase	*IPCS*[a], *SMS1*	Yang et al., 2005; Denny et al., 2006
At3g54020	*IPCS2*			
At2g29525	*IPCS3*			
At2g19880	*GCS*	Glucosylceramide synthase	*GCS*	Leipelt et al., 2001
At5g51290	*ACD5/AtCERK*	Ceramide kinase	*LCB4*, *LCB5*	Liang et al., 2003
At5g23450	*AtLCBK1*	LCB kinase		Imai and Nishiura, 2005
At4g21540	*SPHK1*	LCB kinase		Worrall et al., 2008
At2g46090	*SPHK2*	Putative LCB kinase		
At1g27980	*AtDPL1*	LCB-1-phosphate lyase	*DPL1*	Tsegaye et al., 2007; Worrall et al., 2008
At3g58490	*LCB-PP1*	LCB-1-phosphate phosphatase	*LCB3/YSR*, *YSR3*	Worrall et al., 2008
At5g03080	*LCB-PP2*			
At4g22330	*AtCES1*	Putative alkaline ceramidase	*YPC1*, *YDC1*	Mao et al., 2000a, b; Lynch and Dunn, 2004
At1g07380	None	Putative neutral ceramidase	*ASAH2*	Pata et al., 2008
At2g38010	None			
At5g58980	None			
At5g49900	None	Putative glucosylceramidase	*GBA2*	Boot et al., 2007

At1g33700	None			
At4g10060	None			
At3g24180	None			
At4g29680	None	Putative inositolphosphoceramidase	*ENPP7*	Duan et al., 2003a, b
At4g29690	None			
At4g29700	None			
At3g06460	*AtELOs*	Putative condensing enzyme for very long-chain fatty acid synthesis	*ELO1*, *ELO2*, *ELO3*	Lynch and Dunn, 2004
At3g06470				
At1g75000				
At4g36830				
At1g01120	*KCS1*	3-Ketoacyl-CoA synthase	None	Todd et al., 1999
At1g04220	*KCS2*			Paul et al., 2006
At1g07720	*KCS3*			Blacklock and Jaworski, 2006
At1g19440	*KCS4*			Blacklock and Jaworski, 2006
At1g25450	*KCS5/CER60*			Fiebig et al., 2000; Costaglioli et al., 2005
At1g68530	*KCS6/CER6/CUT1*			Fiebig et al., 2000; Costaglioli et al., 2005
At1g71160	*KCS7*			Blacklock and Jaworski, 2006
At2g15090	*KCS8*			Joubes et al., 2008
At2g16280	*KCS9*			Paul et al., 2006
At2g26250	*KCS10/FDH*			Yephremov et al., 1999
At2g26640	*KCS11*			Blacklock and Jaworski, 2006
At2g28630	*KCS12*			Joubes et al., 2008
At2g46720	*KCS13/HIC*			Gray et al., 2000; Costaglioli et al., 2005
At3g10280	*KCS14*			Joubes et al., 2008
At3g52160	*KCS15*			Joubes et al., 2008
At4g34250	*KCS16*			Blacklock and Jaworski, 2006
At4g34510	*KCS17*			Trenkamp et al., 2004
At4g34520	*KCS18*			Joubes et al., 2008
At5g04530	*KCS19/FAE1*			Paul et al., 2006
At5g43760	*KCS20*			Paul et al., 2006
At5g49070	*KCS21*			Joubes et al., 2008
At1g67730	*KCR*	3-Ketoacyl-CoA reductase	*YBR159*	Beaudoin et al., 2002
At1g24470	*KCR*?			
At5g10480	*HCD*, *PAS2*	3-Hydroxyacyl-CoA dehydratase	*PHS1*	Bach et al., 2008
At5g59770	*HCD*?			
At3g55360	*ECR*, *CER10*	Enoyl-CoA reductase	*TSC13*	Gable et al., 2004; Zheng et al., 2005

[a] Inositolphosphoceramide synthase (IPCS).

3 Very Long-Chain Fatty Acid Synthesis

The fatty acid component of plant ceramides consists of C16 fatty acids and very long-chain fatty acids (VLCFAs) with chain-lengths up to 26 carbon atoms. Interestingly, the fatty acid compositions of ceramide backbones of GlcCers and GIPCs have marked differences. In particular, GlcCers tend to have a higher content of C16 fatty acids compared to GIPCs, which are more enriched in VLCFAs (Sperling et al., 2005; Markham et al., 2006).

The VLCFA components of ceramides arise from acyl chain elongation in two carbon increments involving four reactions: (1) condensation of malonyl-CoA with an acyl-CoA chain, (2) reduction of the resulting 3-ketoacyl-CoA intermediate, (3) dehydration of the resulting 3-hydroxyacyl-CoA intermediate, and (4) reduction of the enoyl-CoA product of the dehydration reaction to form the two carbon elongated acyl-CoA chain (Blacklock and Jaworski, 2006).

Enzymes and corresponding genes for each of the four reactions have now been identified in plants (see Chapter 2). Genes for two classes of enzymes are capable of catalyzing the initial condensation reaction: 3-ketoacyl-CoA synthases (KCSs) and the structurally unrelated ELOs. The most characterized of these are the KCSs. Twenty-one KCS genes occur in the *Arabidopsis thaliana* genome, including *FAE1* (*FATTY ACID ELONGATION1*) or (*KCS19*) that is involved in the synthesis of VLCFAs found in seed oils and several *KCS*s that are associated with the synthesis of VLCFAs in surface waxes (e.g., *KCS1*, *KCS6* or *CER6*, and *KCS10* or *FDH*) (Joubes et al., 2008). A KCS that is specifically involved in sphingolipid VLCFA synthesis has yet to be identified. KCS-type enzymes do not occur in *Saccharomyces cerevisiae*. Instead, the initial condensation reaction for the synthesis of yeast VLCFAs, including those found in sphingolipids, involves ELO-type polypeptides (Toke and Martin, 1996). Four genes for ELO-related polypeptides occur in *Arabidopsis thaliana* (Table 1), but their in planta functions have yet to be reported. Although KCSs and ELOs share little sequence homology, plant KCSs can functionally replace ELOs in the elongation of fatty acids in *Saccharomyces cerevisiae*, suggesting that these enzymes have overlapping activities and can both interact with the other enzymes of the fatty acid elongation pathway for VLCFA synthesis (Paul et al., 2006).

Genes for the second reaction in fatty acid elongation, 3-ketoacyl-CoA reductase, have been identified as *GLOSSY8* alleles in maize (Dietrich et al., 2005), and an *Arabidopsis thaliana* homolog (At1g67730; Table 1) (Beaudoin et al., 2002) has been shown to complement the corresponding mutant (*ybr159*Δ) of *Saccharomyces cerevisiae*. Double mutants for the two maize *GLOSSY8* alleles develop kernels that contain normal endosperm, but the embryos of these seeds do not develop properly (Dietrich et al., 2005). In addition, kernels from the double mutant display large reductions in ceramide content (Dietrich et al., 2005).

Identification of genes for the 3-hydroxyacyl-CoA dehydratase, which catalyzes the dehydration step of fatty acid elongation, has long been elusive. However, a *Saccharomyces cerevisiae* enzyme Phs1p was recently shown to function as a dehydratase in fatty acid elongation (Denic and Weissman, 2007). The *Arabidopsis thaliana* homolog of the *PHS1* gene is the previously identified *PASTICCINO2* gene (At5g10480; Table 1) (Bach et al., 2008). Knockout mutants of this gene display embryo lethality, and the *pas2-1* partial mutant accumulates 3-hydroxyacyl-CoAs and has severe reductions in VLCFA content in sphingolipids, waxes, and seed oils (Bach et al., 2008).

The last step in fatty acid elongation, the reduction of the 2, 3-*trans* enoyl-CoA intermediate, is catalyzed by enoyl-CoA reductase. To date, only one enoyl-CoA reductase (At3g55360; Table 1) gene has been identified in *Arabidopsis thaliana* (Gable et al., 2004; Zheng et al., 2005), but it is probable that an additional enzyme(s) with enoyl-CoA reductase activity might be also present (Zheng et al., 2005). This possibility arises from the finding that knock-out of the enoyl-CoA reductase gene produces dwarfed mutants with an impairment in cell expansion that show a reduced content of VLCFAs in wax, seed oils and GlcCers, however, substantial amounts of VCLFAs are retained by the mutants (Zheng et al., 2005).

4 Ceramide Synthases

The final step in the assembly of ceramides is the condensation of a LCB with a fatty acid-CoA, a

reaction that is catalyzed by ceramide synthase (Fig. 3), sometimes referred to as sphinganine *N*-acyltransferase. Three genes for acyl-CoA-dependent ceramide synthases have been identified in *Arabidopsis thaliana* (At3g25540, *LOH1*; At3g19260, *LOH2*; and At1g13580, *LOH3*; Table 1) based on homology to yeast and mammalian ceramide synthases. The first ceramide synthase gene was identified in *Saccharomyces cerevisiae* as a locus whose deletion resulted in a 50% increase in cell life span (D'Mello et al., 1994). Based on this observation, the gene was designated *LONGEVITY ASSURANCE GENE1* or *LAG1*. *LAG1* and the related *LAC1* were subsequently shown to encode acyl-CoA-dependent ceramide synthases in *Saccharomyces cerevisiae* (Guillas et al., 2001; Schorling et al., 2001), and six homologs of *LAG1* and *LAC1* designated *LASS1-6* have been identified in human and mouse (Mizutani et al., 2005; Pewzner-Jung et al., 2006). Functional characterization of the *Arabidopsis thaliana* ceramide synthase-related genes has yet to be reported. Instead, the tomato *Asc-1* (*Alternaria stem canker-1*) gene that encodes a homolog of *LAG1* and *LAC1* has received attention because its presence results in resistance to the plant pathogen *Alternaria alternata* and to the mycotoxins fumonisin B_1 and *Alternaria alternata* f. sp. *lycopersici* (AAL) toxin (Brandwagt et al., 2000; Spassieva et al., 2002). It is now well established that these mycotoxins are potent inhibitors of acyl-CoA-dependent ceramide synthases, and that this inhibition results in accumulation of free LCBs (Abbas et al., 1994). As such, it appears that programmed cell death associated with *Alternaria alternata* infection of *asc-1* mutants is due to the buildup of cytotoxic free LCBs in response to the AAL toxin, and expression of the wild-type *Asc-1* gene is able to mitigate this effect (Brandwagt et al., 2002; Spassieva et al., 2002).

Recent analysis of intact sphingolipids from leaves of *Arabidopsis thaliana* points to patterns in the compositional makeup of ceramide backbones. For example, C16 fatty acids are more frequently paired with dihydroxy LCBs, and conversely, VLCFAs tend to be paired with trihydroxy LCBs (Markham and Jaworski, 2007; Chen et al., 2008). It is likely that these patterns reflect differing substrate specificities of the ceramide synthases found in plant cells. Although the substrate specificities of plant ceramide synthases have yet to be reported, the six mouse ceramide synthase genes, referred to as *Lass* (*Longevity assurance*) genes, have been shown to encode enzymes with distinct activities with fatty acyl-CoAs of differing chain lengths (Mizutani et al., 2005). For example, Lass6 is most active with C14 and C16 acyl-CoAs, while Lass2 displays the highest activity with C22 and C24 acyl-CoAs (Mizutani et al., 2005). Indirect evidence also points to the likelihood that ceramide synthases within a given plant species have different substrate specificities. In this regard, results from radiolabeling studies conducted with wild-type tomato and the *asc-1* mutant suggest that different molecular species of sphingolipids are produced in these lines (Spassieva et al., 2002). In addition, a recently described *Arabidopsis thaliana* mutant that produces only the dihydroxy form of LCBs accumulates primarily sphingolipids with ceramides that contain C16 fatty acids rather than VLCFAs (Chen et al., 2008). This observation is consistent with two functional classes of ceramide synthases in *Arabidopsis thaliana*: one class that primarily combines dihydroxy LCBs with C16 fatty acids and a second class that generates a preponderance of ceramides with trihydroxy LCBs and VLCFAs.

A second type of ceramide synthase activity was originally described in *Saccharomyces cerevisiae* that involves the condensation of a fatty acid and LCB through an acyl-CoA-independent mechanism (Mao et al., 2000a, b). This activity is catalyzed by the alkaline ceramidases YPC1 and YDC1. These enzymes function primarily in the breakdown of ceramides into free fatty acids and LCBs, but are also capable of catalyzing the reverse reaction to generate ceramides (Mao et al., 2000a, b). The so-called "reverse ceramidase" activity is not inhibited by fumonisin B_1, and expression of *YPC1* and *YDC1* rescues *Saccharomyces cerevisiae* cells from fumonisin B_1-induced growth inhibition (Mao et al., 2000a, b). Similar reverse ceramidase activity has been demonstrated in vitro with recombinant mammalian alkaline ceramidases (El Bawab et al., 2000, 2001). Although *Arabidopsis thaliana* contains a gene for a homolog of *Saccharomyces cerevisiae* alkaline ceramidases (At4g22330; Table 1), the quantitative significance, if any, of this enzyme for ceramide synthesis in vivo has yet to be determined.

B Synthesis of Complex Sphingolipids

Most of the ceramides produced in plant cells subsequently serve as substrates for the attachment of polar head groups to form complex sphingolipids. These reactions consist predominately of the incorporation of a glucose residue to form GlcCer or the incorporation of an inositolphosphoryl moiety to form an inositolphosphoryl ceramide (IPC), the precursor of GIPCs.

1 Glucosylceramide Synthesis

The attachment of the glucose head group to a ceramide is catalyzed by GlcCer synthase (Fig. 3). Plant GlcCer synthase has received only limited study to date. A cotton GlcCer synthase cDNA was identified based on partial homology to the human GlcCer synthase and was shown to restore GlcCer production to a *Pichia pastoris* GlcCer synthase knockout mutant (Leipelt et al., 2001; Hillig et al., 2003). Interestingly, recombinant expression of the cotton GlcCer synthase not only generated GlcCers, but also produced small amounts of sterol glucosides in a *Pichia pastoris* GlcCer synthase/sterol glucosytransferase double mutant (Hillig et al., 2003). Whether this mixed activity occurs in planta remains to be determined. Based on enzyme assays conducted with the recombinant cotton enzyme, the glucose donor for the plant GlcCer synthase-mediated reaction is UDP-glucose (Hillig et al., 2003). Determination of whether GlcCers are essential in plants has been hindered by the lack of a T-DNA insertion mutant for the one putative GlcCer synthase gene (At2g19880; Table 1) in *Arabidopsis thaliana*.

2 Inositolphosphoceramide Synthesis

This synthesis of IPCs occurs via the transfer of the head group of phosphatidylinositol (PI) onto ceramide (Fig. 3). In addition to IPC, the second product of this reaction is diacylglycerol formed from the PI substrate. This activity is catalyzed by IPC synthase. Though IPC synthase activity has been assayed in plant extracts, the identification of plant genes for this enzyme has yet to be reported. In *Saccharomyces cerevisiae*, IPC synthase is encoded by the *AUR1* gene (Nagiec et al., 1997). This gene takes its name from the fact that *Saccharomyces cerevisiae* IPC synthase is strongly inhibited by aureobasidin A, as well as other fungal toxins including rustimicin and khafrefungin (Mandala et al., 1997, 1998b). IPC synthase activity in microsomes of wax bean (*Phaeolus vulgaris* L.) is also inhibited by aureobasidin A and rustimicin (Bromley et al., 2003). Despite this similarity in sensitivity to inhibitors, no homologs of the *Saccharomyces cerevisiae* IPC synthase have been identified to date in plants. Another possibility is that the plant gene is more closely related to the recently identified IPC synthases from protozoans. Indeed, homologs of these genes do occur in *Arabidopsis thaliana* (Table 1), and one of these genes has recently been shown to function as an IPC synthase (Wang et al., 2008). Following synthesis of IPCs, additional as yet uncharacterized glycosylation reactions presumably give rise to the more structurally complex GIPCs that are found in plants (Fig. 3).

C Subcellular Location of Sphingolipid Synthesis

Our knowledge of the spatial layout of sphingolipid synthesis in plant cells is based on enzyme assays conducted with enriched membrane fractions and more recently on confocal microscopy of sphingolipid biosynthetic enzymes fused to fluorescent proteins. Studies with confocal microscopy have shown that the *Arabidopsis thaliana* LCB2 and LCB1 subunits of serine palmitoyltransferase reside in the ER (Tamura et al., 2001; Chen et al., 2006), as does the 3-ketosphinganine reductase (M. Chen and E.B. Cahoon, unpublished). These results, therefore, indicate that LCB synthesis occurs in the ER. Moreover, ceramide synthase activity has been identified in the ER in *Phaseolus vulgaris* seeds (Lynch and Dunn, 2004), which is consistent with the localization of the *Saccharomyces cerevisiae LAG1*- and *LAC1*-encoded ceramide synthases (Barz and Walter, 1999). In addition, glucosylceramide synthase activity in cotton was assigned to the ER (Hillig et al., 2003), while IPC synthase activity in *Phaseolus vulgaris* was detected in Golgi (Bromley et al., 2003), which is also the subcellular location of the *Saccharomyces cerevisiae* enzyme (Levine et al., 2000). Furthermore, a fluorescent protein tagged version of the recently identified IPC synthase from *Arabidopsis thaliana* was also localized in Golgi by confocal microscopy

(Wang et al., 2008). The picture that emerges is that ceramide synthesis occurs in the ER, but the sites of GlcCer and GIPC synthesis are physically separated between the ER and Golgi apparatus (Fig. 4).

The apparent spatial separation of GlcCer and GIPC synthesis may, in part, explain the structural differences found in the ceramide backbones of these complex sphingolipids. As noted earlier, the ceramides of GlcCers contain higher amounts of C16 fatty acids and dihydroxy LCBs. GIPCs, instead, are more enriched in ceramides with VLCFAs and trihydroxy LCBs. In the current model of sphingolipid synthesis in plants, two spatially separated and structurally divergent pools of ceramides would be required to support GlcCer and GIPC synthesis, and selective transport of specific ceramides to the Golgi for GIPC synthesis would be necessitated. This transport could be achieved by vesicular and/or non-vesicular mechanisms. Sphingomyelin synthesis in mammals is a relevant example of how non-vesicular transport of ceramides contributes to the biosynthetic processes of complex sphingolipids. In this example, ceramide is transported from its site of synthesis in the ER to the Golgi apparatus, where the phosphocholine head group of sphingomyelin is attached (Hanada et al., 2007). In mammals, ceramide trafficking from the ER to Golgi is mediated by ceramide transfer proteins (CERTs), which display specificity for different ceramide structures (Kudo et al., 2008). The demonstration of CERT-like proteins in plants has yet to be reported. However, the in vitro sphingolipid transfer properties of two polypeptides from *Arabidopsis thaliana* with homology to human and bovine glycolipid transfer proteins have been partially characterized. These polypeptides, designated ACD11 and AtGLTP1 (GLTP1, glycolipid transfer protein 1),

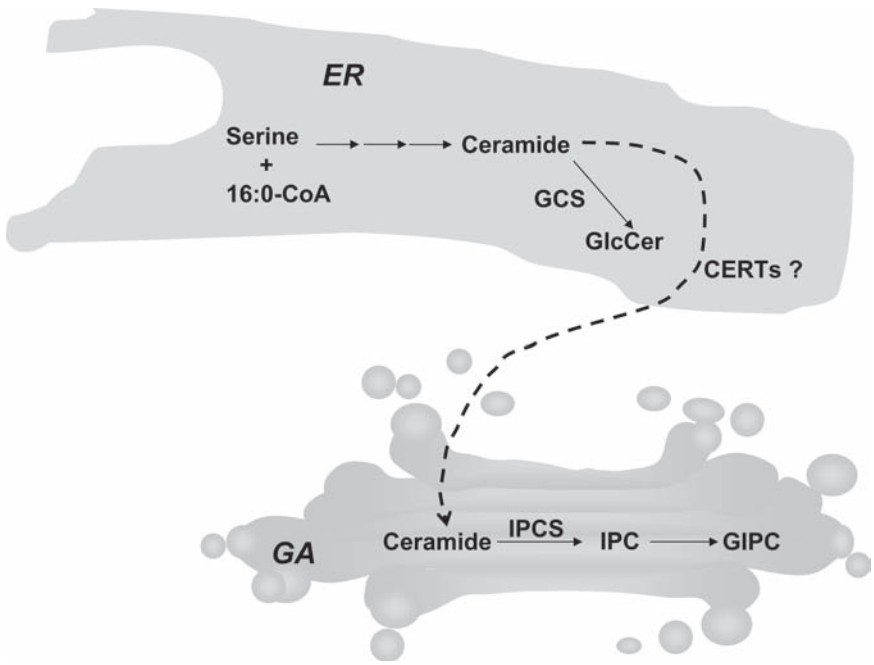

Fig. 4. Possible spatial separation of glucosylceramide (GlcCer) and glycosyl inositolphosphoceramide (GIPC) biosynthesis in plant cells. Evidence from assay of sphingolipid biosynthetic enzymes in enriched membrane fractions and from confocal microscopy of fluorescent protein-tags of these enzymes indicates that long-chain bases, ceramides, and GlcCers are synthesized primarily in the ER and inositolphosphoceramides (IPC) and likely GIPCs are synthesized in the Golgi apparatus (GA). Given that GlcCers and GIPCs have distinct ceramide composition, this model evokes the possibility that specific types of ceramides are transported by vesicular and/or non-vesicular routes (such as that mediated by CERTs in mammals) to the Golgi apparatus for GIPC synthesis. Abbreviations: GCS, glucosylceramide synthase; IPCS, inositolphosphoceramide synthase.

are encoded by At2g34690 and At2g33470, respectively. In the case of ACD11, the purified *E. coli*-expressed polypeptide was assayed for its ability to transfer [^{3}H] sphingolipids from a negatively charged donor phospholipid vesicles to a neutral acceptor vesicles. Of the three sphingolipids tested, the highest activity was measured with the sphingosine (d18:1Δ4). The protein displayed > ten-fold lower activity with galactosylceramide and ceramide. It should be noted that sphingosine is typically of very low abundance relative to other LCBs in *Arabidopsis thaliana*, and galactosylceramides are not known to occur in *Arabidopsis thaliana*. For AtGLTP1, the purified recombinant protein was assayed for its ability to transfer fluorescent sphingolipids between phospholipid or galactolipid vesicles (West et al., 2008). These studies revealed that AtGLTP1 is about eight times more active with glucosylceramide than with galactosylceramide and about 30 times more active with glucosylceramide than with lactosylceramide (West et al., 2008). These findings provide compelling evidence for a role of ACD11 and AtGLTP1 in sphingolipid transfer in *Arabidopsis thaliana* cells. Still, the in vivo substrates and the in planta contributions of these proteins to the transport of sphingolipids among different membranes (e.g., ER to Golgi) remain to be determined.

D Long-Chain Base Modification Reactions

The eight LCBs that derive from sphinganine (d18:0) are formed by at least one of three enzymes: (1) LCB C-4 hydroxylase, (2) LCB Δ8 desaturase, and (3) LCB Δ4 desaturase (Fig. 5). These enzymes are non-heme diiron oxo proteins that contain the three histidine "boxes" that are characteristic of fatty acid desaturases and hydroxylases, and other members of this enzyme family (Shanklin and Cahoon, 1998). The LCB Δ8 desaturase is particularly intriguing because it typically can introduce the Δ8 double bond of LCBs in either the *cis* or *trans* orientations (Sperling et al., 1998; Beckmann et al., 2002). By contrast, the LCB Δ4 desaturase lacks this bifunctional activity and instead introduces a double bond at the Δ4 position exclusively in the *trans* orientation (Ternes et al., 2002). Although each of these enzymes can function alone to generate either saturated trihydroxy LCBs (i.e., t18:0) or monounsaturated dihydroxy LCBs (i.e., d18:1Δ4*trans*, d18:1Δ8*trans*, and d18:1Δ8*cis*), four of the nine LCBs found in plants are the products of the combined activities of two of these enzymes. In this regard, the activities of the LCB C-4 hydroxylase and the LCB Δ8 desaturase give rise to the two most abundant LCBs in *Arabidopsis thaliana* leaves: t18:1Δ8*trans* and t18:1Δ8*cis*. In addition, the concerted activities of the LCB Δ4 desaturase and the LCB Δ8 desaturase produce the diunsaturated dihydroxy LCBs d18:2Δ4*trans*,8*trans* and d18:2Δ4*trans*,8*cis*, which are typically enriched in GlcCers of plants, such as tomato. To date, the plant LCB C-4 hydroxylase and LCB Δ8 desaturases have been the most extensively characterized.

1 Long-Chain Base C-4 Hydroxylation

The LCB C-4 hydroxylase catalyzes the introduction of a hydroxyl group at the C-4 position of a dihydroxy LCB to form a trihydroxy LCB (Fig. 5a). Homologs of the *Saccharomyces cerevisiae* LCB C-4 hydroxylase gene *SUR2* occur in plants (Sperling et al., 2001; Imamura et al., 2007; Chen et al., 2008). Two LCB C-4 hydroxylase genes have been identified in *Arabidopsis thaliana* (At1g69640, *SBH1*; At1g14290, *SBH2*; Table1) and both have been shown to restore the synthesis of trihydroxy LCBs when expressed in *Saccharomyces cerevisiae sur2* mutants (Sperling et al., 2001). Although definitive evidence has yet to be reported regarding the nature of the substrate for plant LCB C-4 hydroxylases, it is likely that C-4 hydroxylation occurs primarily on free dihydroxy LCBs prior to incorporation into ceramide (Wright et al., 2003) and the trihydroxy LCB t18:0 is the predominate free LCB in *Arabidopsis thaliana* leaves (Markham and Jaworski, 2007; Chen et al., 2008). The functional significance of LCB C-4 hydroxylation in *Arabidopsis thaliana* was recently examined by the generation of double mutants and RNAi suppression lines for the two hydroxylase genes (Chen et al., 2008). Based on the lack of a growth phenotype in the *Saccharomyces cerevisiae sur2* mutant (Haak et al., 1997; Grilley et al., 1998), it was anticipated that the *Arabidopsis thaliana* mutants would have no obvious phenotypes. Instead, double mutants were found to be severely dwarfed and did not progress from vegetative to reproductive growth

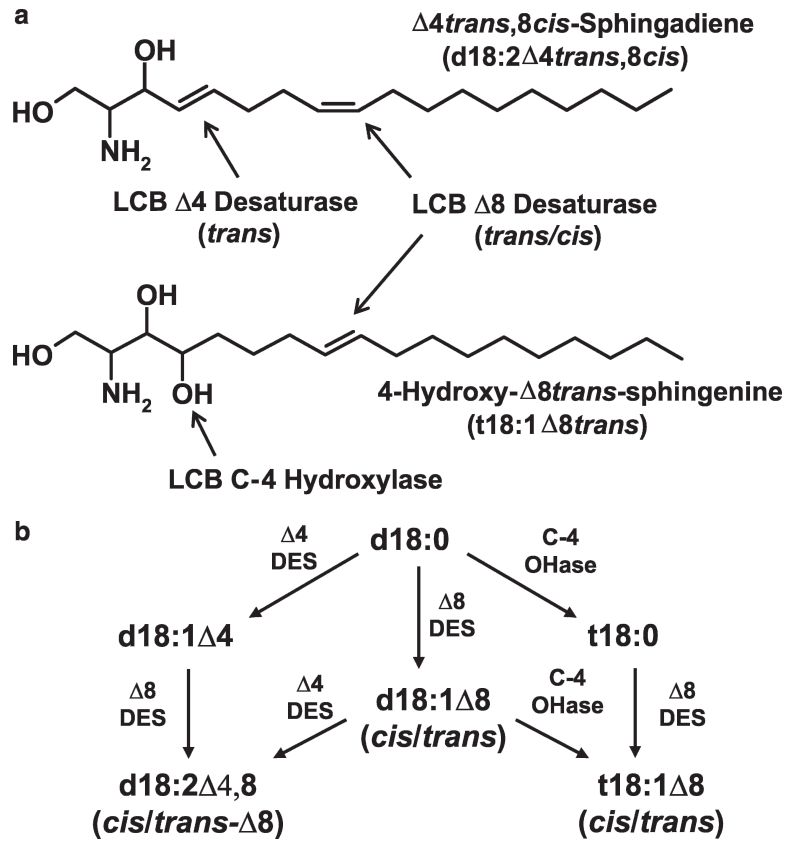

Fig. 5. Long-chain base modification reactions in plants. **(a)** The C-4 hydroxyl group and Δ4 and Δ8 double bonds that are found in plant long-chain bases (LCBs) arise from the concerted activities of the LCB C-4 hydroxylase and LCB Δ4 and Δ8 desaturases, respectively. **(b)** These enzymes can work separately or in combination to generate the complete complement of LCBs found in plant sphingolipids. The LCB Δ8 desaturase can function to introduce a double bond in either the *cis* or *trans* configuration, and the LCB Δ4 desaturase functions exclusively as a *trans* desaturase. Of note, the sequence of these reactions and the exact nature of the substrates (e.g., free LCB, ceramide) have yet to be definitively established. C-4 OHase, LCB C-4 hydroxylase. Δ4 DES, LCB Δ4 desaturase. Δ8 DES, LCB Δ8 desaturase.

(Chen et al., 2008). In addition, the degree of growth reduction in RNAi lines was found to be more severe as the relative content of trihydroxy LCBs decreased. Unexpectedly, the sphingolipid content in double mutants was 2.5- to three-fold higher than in wild type plants, and the accumulation of sphingolipids was primarily the result of increased amounts of molecular species with C16 fatty acids, rather than the more typical VLCFAs, in all sphingolipid classes (Chen et al., 2008). As described above, the increased levels of ceramide backbones with C16 fatty acids and dihydroxy LCBs is likely reflective of the substrate specificities of ceramide synthases in *Arabidopsis thaliana*. Overall, these results indicate that LCB C-4 hydroxylation is a critical sphingolipid structural modification for growth and for the regulation of sphingolipid content and composition in *Arabidopsis thaliana*. These findings also suggest that the synthesis of trihydroxy LCBs is important for mediating flux into the sphingolipid biosynthetic pathway, perhaps through regulation of SPT activity, to meet the demands for growth.

2 Long-Chain Base Δ8 Desaturation

The LCB Δ8 desaturase is absent in *Saccharomyces cerevisiae* and mammalian cells, but does occur in plants and many fungi. The LCB Δ8 desaturase was first identified in plants as a novel peptide consisting of an *N*-terminal cytochrome b_5 domain fused to a desaturase-like polypeptide

(Sperling et al., 1998). Heterologous expression of the *Arabidopsis thaliana* and *Brassica napus* cDNAs in *Saccharomyces cerevisiae* allows the biosynthesis of *cis* and *trans* isomers of t18:1Δ8 (Sperling et al., 1998). These results demonstrate that the plant LCB Δ8 desaturase is bifunctional with regard to the stereospecificity of double bond insertion. In most plants, the *trans* isomers of Δ8 unsaturated LCBs predominate in the total sphingolipid extract. However, the *cis* isomer of t18:1Δ8 is much more enriched in GlcCers relative to GIPCs in plants, such as *Arabidopsis thaliana* (Sperling et al., 2005; Markham et al., 2006; Markham and Jaworski, 2007). It is not yet clear if this difference in stereoisomer composition results from distinct LCB Δ8 desaturases that may be associated with GlcCers and GIPCs. Recently, a plant LCB Δ8 desaturase from the legume *Stylosanthes hamata* was shown to produce more of the *cis* isomer of t18:1Δ8 when expressed in *Saccharomyces cerevisiae* (Ryan et al., 2007). This is in contrast to findings with the *Arabidopsis thaliana* and *Brassica napus* enzymes, which generated mostly the *trans* isomer upon expression in *Saccharomyces cerevisiae* (Sperling et al., 1998). These studies demonstrate that LCB Δ8 desaturase with different stereoselective properties have evolved in plants. Mechanistic studies with the sunflower LCB Δ8 desaturase indicate that the bifunctionality of this enzyme arises from two different conformations that the LCB substrate can assume in the active site (Beckmann et al., 2002). Interestingly, expression of the *Stylosanthes hamata* desaturase in *Arabidopsis thaliana* was shown to not only increase the content of t18:1Δ8cis but also confer increased tolerance to aluminum toxicity (Ryan et al., 2007). This finding strongly indicates that the relative amounts of *cis–trans* isomers of unsaturated LCBs in sphingolipids impact the plant's ability to adapt to at least some abiotic stresses. However, it has yet to be established if LCB Δ8 unsaturation is essential in plants. A number of metabolic questions regarding the LCB Δ8 desaturase also remain unanswered. For example, it is not known if this enzyme uses a free LCB, ceramide, or complex sphingolipid as a substrate. In addition, it has not been established if distinct LCB Δ8 desaturases are involved in the synthesis of monounsaturated and diunsaturated LCBs. This is particularly relevant given the occurrence of two LCB Δ8 desaturase genes in *Arabidopsis thaliana* (At3g61580, *SLD1*; At2g46210, *SLD2*; Table 1).

3 Long-Chain Base Δ4 Desaturation

The LCB Δ4 desaturase introduces the *trans*-Δ4 unsaturation that is found in GlcCers of many plant species, but is typically absent in GIPCs (Fig. 5) (Markham et al., 2006). In contrast to mammals, Δ4 monounsaturated LCBs are present in low abundance in plant sphingolipids (Sperling et al., 2005; Markham et al., 2006). Instead, Δ4 double bonds occur together with *cis*- or *trans*- Δ8 double bonds in the diunsaturated LCB d18:2Δ4,8 (sphingadiene). The plant LCB Δ4 desaturase is most related to animal LCB Δ4 desaturases, including the *Drosophila melanogaster* Δ4 desaturase encoded by the *DEGENERATIVE SPERMATOCYTE-1* gene (*DES-1*) (Ternes et al., 2002). In addition, the mouse LCB Δ4 desaturase DES2 has been shown to act as a bifunctional enzyme that can also catalyze C-4 hydroxylation of dihydroxy LCBs (Omae et al., 2004). This gene family also includes LCB Δ4 desaturases from a number of fungal species (Ternes et al., 2002). Functional identification of plant LCB Δ4 desaturases has only recently been reported. In this study, the *Arabidopsis thaliana* LCB Δ4 desaturase (At4g04930, Table 1) was shown to restore the synthesis of d18:2 to a *Pichia pastoris* Δ4 desaturase null mutant (Michaelson et al., 2009). It is notable that functional expression of plant LCB Δ4 desaturases has not been achievable in *Saccharomyces cerevisiae* (Michaelson et al., 2009). This may be reflective of the substrate specificity of the plant Δ4 LCB desaturase. Because Δ4 unsaturation is found almost entirely in GlcCers, one possibility is that the LCB Δ4 desaturase uses GlcCers as substrates. However, GlcCers do not occur in *Saccharomyces cerevisiae*. Another possibility is that the Δ4 desaturase activity requires the presence of a Δ8 double bond in LCB substrates, given that Δ4 monounsaturated LCBs are of low abundance in plants. Like the previous metabolic scenario, *Saccharomyces cerevisiae* does not have a Δ8 desaturase, and to our knowledge, the co-expression of plant LCB Δ4 and Δ8 desaturases in *Saccharomyces cerevisiae* has not been reported.

As with the LCB C-4 hydroxylase and Δ8 desaturase, the substrate for the plant LCB Δ4

desaturase has yet to be defined. In addition to the possibility that this enzyme uses a GlcCer substrate (see above), it cannot be ruled out that the Δ4 unsaturation is introduced into ceramides, which are then selectively used for GlcCer synthesis. The latter hypothesis is supported by the observation that in *Pichia pastoris* knock-out of the LCB Δ4 desaturase results in a complete loss of GlcCers (Michaelson et al., 2009). This observation supports the idea that Δ4 unsaturation is introduced prior to the attachment of the glucose head group and as such, is necessary for channeling of ceramides into GlcCers in fungi.

The functional significance of the LCB Δ4 desaturase in plants is also not clear. Some plants are enriched in Δ4 unsaturated LCBs in GlcCers (e.g., tomato), whereas other species (e.g., *Arabidopsis thaliana*) contain very low levels of these LCBs in GlcCers (Markham et al., 2006). The near absence of Δ4 unsaturated sphingolipids in *Arabidopsis thaliana* suggests that the LCB Δ4 desaturase may be of little importance in this plant. Indeed, a T-DNA knock-out of the corresponding gene (At4g04930) did not affect the growth and development of *Arabidopsis thaliana* (Michaelson et al., 2009). In addition, changes in stomatal aperture in response to ABA treatment was also not affected in the *Arabidopsis thaliana* LCB Δ4 knockout mutant relative to the wild-type control (Michaelson et al., 2009). This finding brings into question the purported role of Δ4 unsaturated LCB-1-phosphates in ABA-dependent stomatal closure. Still, it cannot be ruled out that the Δ4 desaturase has some important physiological functions in plants, such as tomato, that contain high levels of these LCBs in GlcCers.

E Fatty Acid α-Hydroxylation

A distinctive feature of the ceramide component of sphingolipids of most eukaryotes, including plants, is the presence of a α-hydroxyl group on the fatty acid moiety. In plant species characterized to date, most of the fatty acids of sphingolipids contain this structural feature, which results from the activity of the sphingolipid fatty acid α-hydroxylase. The α-hydroxylase gene (*FAH1* or *SCS7*) was first discovered in *Saccharomyces cerevisiae* (Haak et al., 1997; Mitchell and Martin, 1997). By homology, one of the two *Arabidopsis thaliana* α-hydroxylase genes (At2g34770; Table 1) was identified and subsequently shown to restore α-hydroxylation of 26:0 upon expression in the corresponding *Saccharomyces cerevisiae* mutant (Mitchell and Martin, 1997). Like the LCB Δ8 desaturase, the *Saccharomyces cerevisiae* α-hydroxylase contains a cytochrome b_5 domain at its *N*-terminus (Mitchell and Martin, 1997). This domain, however, is absent in the plant sphingolipid fatty acid α-hydroxylases identified to date (Mitchell and Martin, 1997). As with the LCB modification reactions described above, the substrate for fatty acid α-hydroxylation has not been established in plants, nor has it been determined in yeast or mammals. Results from studies conducted in *Tetrahymena pyriformis* point to α-hydroxylation occurring on the intact complex sphingolipid and/or ceramides rather than on free fatty acids or acyl-CoAs prior to incorporation into ceramides (Kaya et al., 1984). Notably, pools of non-hydroxy ceramides and hydroxy ceramides are detectable in *Arabidopsis thaliana* leaves (Markham and Jaworski, 2007; Chen et al., 2008). In contrast to the high content of α-hydroxy fatty acids in *Arabidopsis thaliana* complex sphingolipids (i.e., GlcCers and GIPCs), ceramides lacking hydroxylated fatty acids are approximately three times more abundant than ceramides with hydroxylated fatty acids (Markham and Jaworski, 2007; Chen et al., 2008). This observation is consistent with α-hydroxylation occurring after incorporation of the fatty acid into ceramides in plants. The functional significance of sphingolipid fatty acid α-hydroxylation is not known. *Saccharomyces cerevisiae* mutants devoid of sphingolipid α-hydroxy fatty acids are viable and lack noticeable growth defects (Haak et al., 1997; Mitchell and Martin, 1997). As with the LCB Δ8 and Δ4 desaturases, determination of the function of sphingolipid α-hydroxylation in plants awaits the identification of null mutants for this structural modification.

F Long-Chain Base-1-Phosphates: Synthesis and Turnover

Long-chain base-1-phosphates (LCB-Ps; Fig. 1) are bioactive metabolites that are generated from the phosphorylation of LCBs arising either from de novo synthesis or from the catabolism of sphingolipids (Fig. 3). LCB-Ps in mammals and *Saccharomyces cerevisiae* have been shown to

function as regulators of diverse activities including cell proliferation, differentiation, and apoptosis (Zhang et al., 1991; Mao et al., 1999; Mandala et al., 2000; Le Stunff et al., 2002a; Johnson et al., 2003; Taha et al., 2006). Plant LCB-Ps have received attention because of evidence linking these molecules to signal transduction in ABA-dependent stomatal closure (Ng et al., 2001; Coursol et al., 2003; Worrall et al., 2003). The levels of LCB-Ps in plant cells are controlled primarily by the biosynthetic activity of LCB kinases and the catabolic activities of LCB-P phosphatases and lyases. Based on the reported role of LCB-Ps in guard cell function (Ng et al., 2001; Coursol et al., 2003; Worrall et al., 2003) and plant cell death (Shi et al., 2007), the interplay of these activities may be important for triggering or attenuating key physiological processes in plants.

1 Long-Chain Base Phosphorylation

LCB-Ps are synthesized by phosphorylation of the C-1 position of free LCBs through the activity of LCB kinases (Fig. 3). In *Saccharomyces cerevisiae*, two LCB kinases, LCB4 and LCB5, have been identified. Of these, LCB4 accounts for approximately 97% of the LCB kinase activity in *Saccharomyces cerevisiae* cells (Nagiec et al., 1998). Similarly, two sphingosine kinases (SphK1 and SphK2) occur in mammals (Liu et al., 2000, 2002). *Arabidopsis thaliana* contains four genes with homology to the mammalian *SphK1*: At5g23450 (*AtLCBK1*), At4g21540 (*SPHK1*), At2g46090 (*SPHK2*), and At5g51290 (*ACD5* or *AtCERK*) (Table 1). Of these genes, *AtLCBK1* was the first to be shown to encode a functional LCB kinase (Imai and Nishiura, 2005). The *Escherichia coli*-expressed AtLCBK1 phosphorylated a range of naturally occurring LCBs, including d18:0, d18:1Δ4, d18:2Δ4,8, and t18:0 (Imai and Nishiura, 2005). At4g21540 was also recently shown to encode a functional LCB kinase (Worrall et al., 2008). Triton X-100-solubilized extracts from human embryonic kidney 293 cells expressing this gene were able to phosphorylate d18:0, t18:0, t18:1Δ8, and d18:2Δ4,8 (Worrall et al., 2008). This gene was designated *SPHK1* because of its in vitro activity with sphingosine (or Δ4*trans*-sphingenine, d18:Δ4*trans*) and its likely relation to previous studies of the role of sphingosine-1-P in mediation of stomatal closure (Worrall et al., 2008). Despite this, sphingosine is a very minor LCB in most organs of *Arabidopsis thaliana* and is likely not the primary in vivo substrate of this enzyme. In contrast to the findings with SPHK1, recombinant expression of At2g46090, designated *SPHK2*, in human embryonic kidney 293 cells failed to yield an active protein (Worrall et al., 2008). As such, it is unclear if SPHK2 is a true LCB kinase in *Arabidopsis thaliana*. The final LCB kinase-like gene in *Arabidopsis thaliana* At5g51290 was demonstrated to encode a ceramide kinase rather than an LCB kinase (see below) (Liang et al., 2003).

The two demonstrated LCB kinases in *Arabidopsis thaliana* AtLCBK1 and SPHK1 contain a putative ATP-binding domain (GDGXXXEXXNGXXXR), which is also conserved in other SPHK kinases from fungi and animals (Liu et al., 2002). Interestingly, the in vitro substrate profiles of AtLCBK1 and SPHK1 do not perfectly overlap (Imai and Nishiura, 2005; Worrall et al., 2008). Of most significance, the activity of recombinant AtLCBK1 with t18:0 is about 20% of that with d18:1Δ4 (Imai and Nishiura, 2005). By comparison, SPHK1 is nearly equally active with d18:0 and t18:0 (Worrall et al., 2008). In addition, a T-DNA knockout of *SPHK1* resulted in the loss of nearly all sphingosine phosphorylation activity in membrane extracts from *Arabidopsis thaliana* leaves (Worrall et al., 2008). This suggests that SPHK1, rather than AtLCBK1, contributes primarily to the LCB kinase activity in *Arabidopsis thaliana* leaves, although AtLCBK1 was shown to be constitutively expressed (Imai and Nishiura, 2005).

The subcellular localization of the plant LCB kinase has not been fully established. In *Saccharomyces cerevisiae*, there are conflicting reports regarding the localization of these enzymes. For example, LCB4 was localized to ER, Golgi, and late endosomes using an epitope-tagging strategy (Hait et al., 2002; Funato et al., 2003), but this enzyme was found on the cell perimeter using immunofluorescence microscopy with antibodies against LCB4 (Kihara et al., 2005). LCB kinase activity in plants is primarily membrane associated, and this activity has been identified in ER and Golgi-enriched fractions from maize microsomes (Crowther and Lynch, 1997; Coursol et al., 2005; Worrall et al., 2008). Specific subcellular

localization of AtLCBK1 and SPHK1 polypeptides awaits further study.

The in planta functions of LCB kinases have been explored by the characterization of *Arabidopsis thaliana SPHK1* overexpression lines and lines containing a T-DNA disruption in *SPHK1*. In these studies, stomatal aperture was greater in the T-DNA disruption line and smaller in the overexpression line relative to a wild-type control in response to exogenous ABA treatment (Worrall et al., 2008). These results are consistent with a role of LCB-Ps in ABA-dependent stomatal closure. In addition, seeds from the *SPHK1*-overexpression line displayed slower germination than seeds from wild-type plants (Worrall et al., 2008). Conversely, seeds from the *SPHK1*-disruption line displayed faster germination than the control. The delayed germination of seeds from the overexpression line was enhanced by exogenous ABA (Worrall et al., 2008). These results point to a role of LCB-Ps in mediating germination, and this activity may be a component of ABA signaling processes.

2 Long-Chain Base-1-Phosphate Catabolism

The turnover of LCB-Ps can proceed either by dephosphorylation to form free LCBs via the activity of LCB-P phosphatases (Mao et al., 1997) or by hydrolysis of the LCBs to generate long-chain aldehydes and phosphoethanolamine via the activity of LCB-P lyases (Saba et al., 1997) (Fig. 3). Two LCB-P phosphatase genes *YSR2* (or *LCB3*) and *YSR3* and one LCB-P lyase gene *DPL1* occur in *Saccharomyces cerevisiae* (Qie et al., 1997; Mandala et al., 1998a; Mao et al., 1999; Bach et al., 2008). Deletion of either of the two LCB-P phosphatase genes results in the accumulation of primarily d18:0-1-P, but does not result in any detectable alterations in growth (Mandala et al., 1998a; Mao et al., 1999). Conversely, overexpression of *YSR2* suppresses growth of *Saccharomyces cerevisiae* cells (Mao et al., 1999). A notable phenotype associated with disruption of *YSR2* is enhanced thermotolerance, which suggests a role of LCB-Ps in stress adaptation in *Saccharomyces cerevisiae* (Mao et al., 1999). The *YSR2* mutant is also incapable of synthesizing sphingolipids from exogenous LCBs (Qie et al., 1997). The *Saccharomyces cerevisiae* LCB-P lyase gene *DPL1* (or *BST1*) was initially identified as a gene whose overexpression could rescue cells from growth inhibition by exogenous sphingosine (Saba et al., 1997). As observed with *YSR2* and *YSR3*, disruption of *DPL1* results in the accumulation of LCB-Ps (Saba et al., 1997). Interestingly, double mutants of *DPL1* and *YSR2* are non-viable, but can be rescued by knockout of the LCB kinase gene *LCB4* (Kim et al., 2000). These observations indicate that high levels LCB-Ps can result in lethality in *Saccharomyces cerevisiae*.

Based on homology to the *Saccharomyces cerevisiae YSR2*, *YSR3*, and *DPL1* genes, *Arabidopsis thaliana* contains at least one LCB-P phosphatase (*AtLCB-PP1*, At3g58490; Table 1) gene and one LCB-P lyase gene (*AtDPL1*, At1g27980; Table 1). Although the *AtLCB-PP1*-encoded polypeptide shares only moderate homology with *YSR2* and *YSR3*, it contains a phosphohydrolase domain that is found in LCB-P phosphatases from other eukaryotes (Le Stunff et al., 2002b). Biochemical characterization of the polypeptide encoded *AtLCB-PP1* or homologs from other plant species has yet to be reported. However, the *Arabidopsis thaliana DPL1* homolog *AtDPL1* has been studied in detail (Niu et al., 2007; Tsegaye et al., 2007). Expression of the *AtDPL1* gene is able to rescue the growth inhibition of the *Saccharomyces cerevisiae dpl1* mutant in response to exogenous LCBs (Niu et al., 2007; Tsegaye et al., 2007). The AtDPL1 polypeptide also restores LCB-P lyase activity to the *Saccharomyces cerevisiae dpl1* mutant (Tsegaye et al., 2007). This enzyme, like LCB kinases, has membrane-associated activity, and a fluorescent protein fusion of this polypeptide localizes to the ER (Tsegaye et al., 2007). In addition, T-DNA mutants for the *AtDPL1* gene lack LCB-P lyase activity and show increased accumulation of LCB-Ps (Tsegaye et al., 2007). These plants do not display growth phenotypes but are hypersensitive to fumonisin B_1, which is likely due to higher levels of LCBs and LCB-Ps accumulation in the mutant in response to the mycotoxin relative to wild-type plants (Tsegaye et al., 2007). It is also notable that expression of *AtDPL1* is strongly upregulated in response to senescence (Niu et al., 2007; Tsegaye et al., 2007). This implies that sphingolipid turnover is an active process during senescence or alternatively that modulation of LCB-P levels plays some physiological role during senescence. Although *AtDPL1* mutants have now been characterized,

the overall importance of LCB-P turnover in *Arabidopsis thaliana* awaits the generation of LCB-P phosphatase and lyase mutants that completely lack the ability to degrade LCB-Ps.

G Ceramide Phosphorylation

Phosphorylation of ceramides has become a topic of interest because of the link between this reaction and programmed cell death observed in the *acd5* mutant of *Arabidopsis thaliana*. As described in more detail below, mutants of the *ACD5* gene (*AtCERK*, At5g51290; Table 1) display early senescence and a spontaneous increase in salicylic acid and enhanced expression of pathogenesis-related genes, presumably due to the buildup of free ceramides. The *ACD5* gene encodes a polypeptide that is most closely related to mammalian ceramide kinases and more distantly related to LCB kinases (Liang et al., 2003). Consistent with this identification, the *ACD5*-encoded enzyme displays ceramide kinase activity that introduces a phosphate group at the C-1 position of a free ceramide to form ceramide-1-phosphate (Liang et al., 2003) (Fig. 3). Partially purified enzyme from expression of *ACD5* in *Escherichia coli* was most active with ceramides containing C6 or C8 fatty acids coupled to a dihydroxy LCB (Liang et al., 2003). The enzyme showed little or no activity with sphingosine or diacylglycerol, indicating that it is neither an LCB kinase nor a diacylglycerol kinase (Liang et al., 2003). The most obvious function of the ceramide kinase is to mediate programmed cell death by regulating the relative amounts of free and phosphorylated ceramides. No other function has yet been ascribed to the plant ceramide kinase, and it is not known if a ceramide phosphatase functions in combination with the plant ceramide kinase, in a manner analogous to LCB kinases and phosphatases.

IV Sphingolipid Turnover

A Ceramide Turnover

Ceramidases catalyze the catabolism of ceramides by cleaving amide bonds to yield LCBs and fatty acids (Fig. 3). Three classes of ceramidases have been reported in eukaryotes whose activities differ based on pH. These enzymes are classified as acid, neutral and alkaline ceramidases. The human acid ceramidase is the first purified and characterized enzyme of the acid ceramidase class (Sugita et al., 1972). Mutations in the corresponding gene are the basis for a lysosomal disorder known as Farber disease (Koch et al., 1996; Ferlinz et al., 2001). There is no apparent homolog of human acid ceramidase in *Arabidopsis thaliana* based on homology searches using the human acid ceramidase as query.

Neutral ceramidases catalyze the hydrolysis of ceramide in the neutral pH range; however, they also have the abilities to catalyze the reverse reaction of ceramide synthesis by using a LCB and free palmitic acid as substrates. As described above, this activity is not inhibited by fumonisin B_1, in contrast to the acyl-CoA-dependent ceramide synthase (El Bawab et al., 2001). From database searches for homologs of the human neutral ceramidase *ASAH2*, three candidate neutral ceramidase genes occur in *Arabidopsis thaliana* (At1g07380, At2g38010, and At5g58980; Table 1), and only one candidate neutral ceramidase occurs in rice (*OsCDase*, Os01g0624000). All of the putative *Arabidopsis thaliana* and rice neutral ceramidase polypeptides contain the conserved peptide sequence GDVSPN in their amidase catalytic domain NXGDVSPNXXC (Galadari et al., 2006; Pata et al., 2008). To date, the rice *OsCDase* gene is the only plant neutral ceramidase-like gene that has been characterized. The recombinant OsCDase enzyme generated in *Saccharomyces cerevisiae* ceramidase mutants was more active with a fluorescent-labeled C12-d18:1Δ4 substrate than with a C12-t18:0 substrate and was active over a broad pH range, with maximal activity at pH 5.7 to 6.0. In addition, expression of the rice enzyme in *Saccharomyces cerevisiae* was accompanied by an increase in ceramides containing very-long chain fatty acids with t18:0 LCBs. The increase in these ceramides, rather than a decrease that would be expected with ceramidase activity, suggests that OsCDase may also have ceramide synthase (or reverse ceramidase) activity. In addition, the fluorescent protein-tagged OsCDase was shown to localize in ER and Golgi apparatus (Pata et al., 2008). This finding is somewhat unexpected because the bulk of sphingolipids in plant cells is believed to be associated with the plasma

membrane and tonoplast. As such, it is unclear at this point if neutral ceramidases are involved in sphingolipid turnover in planta or have other functions in plant cells.

The final type of eukaryotic ceramidase is the alkaline ceramidase. In *Saccharomyces cerevisiae*, two alkaline ceramidase genes *YPC* and *YDC* have been identified (Mao et al., 2000a, b). *YPC* was isolated from a screen designed to identify genes that would confer fumonisin B_1 resistance to *Saccharomyces cerevisiae*. The YPC polypeptide was subsequently shown to function as both an alkaline ceramidase and as an acyl-CoA-independent ceramide synthase (Mao et al., 2000a). YPC and YDC have distinct in vitro substrate preferences. YPC is more active with ceramide substrates containing t18:0 (Mao et al., 2000a), whereas YDC is more active with ceramide substrates d18:0 (Mao et al., 2000b). *Arabidopsis thaliana* contains one uncharacterized homolog of *YPC* and *YDC* (At4g22330; Table 1).

B Complex Sphingolipid Turnover

GlcCers and GIPCs are the two most abundant sphingolipid classes in plants, yet little is known about plant catabolic enzymes associated with the removal of their head groups. These activities are likely to be critical for the maintenance of the optimal content and composition of sphingolipids in membranes. The degradation of GlcCers in mammals is catalyzed by glucosylceramidase (or cerebrosidase), which converts glucosylceramide to ceramide and glucose by cleavage of the glycosidic linkage between these moieties (Brady et al., 1965) (Fig. 3). Two forms of glucosylceramidase occur in mammals: lysosomal glucosylceramidase and non-lysosomal glucosylceramidase. Mutation in the human lysosomal glucosylceramidase is the genetic basis for Gaucher's disease, which is characterized by hyperaccumulation of GlcCers, particularly in spleen (Ho and O'Brien, 1971). No homolog of the human lysosomal glucosylceramidase is detectable in the *Arabidopsis thaliana* genome database. A non-lysosomal glucosylceramidase was recently identified as the earlier described bile acid β-glucosidase (GBA2) in mice (Yildiz et al., 2006; Boot et al., 2007). At least four homologs of GBA2 occur in *Arabidopsis thaliana* (Table 1) that share 40–50% amino acid sequence similarity with the mammalian GBA2. The activities of the corresponding *Arabidopsis thaliana* enzymes have not been reported.

Turnover of GIPCs, the most abundant sphingolipid class in *Arabidopsis thaliana*, has not been determined (Fig. 3). In the *Saccharomyces cerevisiae*, removal of the phosphoinositol head group of inositol phosphate-containing sphingolipids is catalyzed by the ISC1 polypeptide that was identified based on limited homology with bacterial neutral sphingomyelinases (Sawai et al., 2000; Matmati and Hannun, 2008). The bacterial enzymes catalyze the release of the phosphocholine head group of sphingomyelin to generate free ceramide. However, no homolog of the *Saccharomyces cerevisiae ISC* is detectable in database searches of *Arabidopsis thaliana*. In mammals, acid, alkaline, and neutral classes of sphingomyelinases have been identified (Marchesini and Hannun, 2004; Duan, 2006; Ohlsson et al., 2007). While *Arabidopsis thaliana* lacks homologs of mammalian acid and neutral sphingomyelinases, three tandemly arranged genes that encode as yet uncharacterized polypeptides with 30% identity to the human intestinal alkaline sphingomyelinase occur in *Arabidopsis thaliana* (At4g29680, At4g29690, At4g29700; Table 1).

Overall, catabolic reactions are undoubtedly of importance for maintenance of sphingolipid homeostasis and for the generation of bioactive metabolites in plant cells. However, the turnover of sphingolipids in plants has received only limited characterization to date. As such, catabolic enzymes, including those involved in ceramide degradation and removal of head groups of complex sphingolipids will likely be key targets for future research of sphingolipid metabolism in plants.

V Sphingolipid Function

A Sphingolipids as Membrane Structural Components

1 Distribution and Functions of Sphingolipids in Membranes

Based on research conducted in the 1950s and early 1960s (Carter et al., 1958a, b, 1960, 1961), it is now recognized that GIPCs and GlcCers are

the principal complex sphingolipids in plants (Fig. 1). More recently, GIPCs and GlcCers were determined to compose approximately 60% and 30% of the sphingolipids of *Arabidopsis thaliana* leaves, respectively (Markham et al., 2006). The remainder of the sphingolipids in *Arabidopsis thaliana* leaves consists of ceramides, free LCBs, and LCB-1-Ps. Measurement of the complete complement of sphingolipids in specific membranes in plants, however, has yet to be reported. Through the use of indirect measurements, sphingolipids have been estimated to compose >40% of the total lipids in plasma membrane in various plant species (Sperling et al., 2005). In addition, numerous reports have identified GlcCers as major lipid constituents of plasma membrane, tonoplast, Golgi apparatus, and detergent resistant membrane fractions isolated from plasma membrane and Golgi (e.g., Verhoek et al., 1983; Yoshida and Uemura, 1986; Lynch and Steponkus, 1987; Haschke et al., 1990; Cahoon and Lynch, 1991; Mongrand et al., 2004; Sperling et al., 2005; Laloi et al., 2007). For example, GlcCers reportedly compose 30% of the total lipids in plasma membrane from spring oat (Uemura and Steponkus, 1994) and nearly 20% of the total lipids in tonoplast from oat mesophyll (Verhoek et al., 1983). These studies have undoubtedly underestimated the total sphingolipid content of plasma membrane and tonoplast because the abundant GIPC class was not included in measurements of total lipids due to difficulties in the extraction and isolation of these lipids (Markham et al., 2006). Because many of the sphingolipid biosynthetic enzymes have been localized to the ER, it is also presumed that this organelle is enriched in sphingolipids (Tamura et al., 2001; Hillig et al., 2003; Chen et al., 2006, 2008). With the recent reports of improved methods for sphingolipid extraction and mass spectral analysis, the comprehensive characterization of sphingolipid compositions of specific membranes should be forthcoming.

In addition, two recent studies have highlighted the importance of sphingolipids for the integrity of the endomembrane system. In these reports, *Arabidopsis thaliana* pollen was examined by electron microscopy to determine membrane defects associated with the loss of sphingolipids in plants deficient in sphingolipid synthesis due to knockout of the two *LCB2* genes of serine palmitoyltransferase (Dietrich et al., 2008; Teng et al., 2008). These studies revealed aberrant vesiculation of the ER, the absence of Golgi bodies, and the lack of the Golgi-derived intine layer on the periphery of the mutant pollen. Sphingolipids also play critical roles in endomembrane-associated processes in plant cells. For example, defects in endocytic membrane trafficking were observed in an *Arabidopsis thaliana* mutant with reduced content of very-long chain fatty acids in sphingolipids (Zheng et al., 2005). The results collectively show that sphingolipids are not only quantitatively important components of the endomembrane system in plants, but also are essential for the ontogeny and function (e.g., membrane trafficking) of endomembranes.

It is likely that alterations in sphingolipid content and composition can significantly impact the physical properties of plasma membrane and tonoplast, given the abundance of sphingolipids in these membranes. Because the plasma membrane and tonoplast are of primary importance for osmotic adaptation and ion fluxes, changes in sphingolipid content and composition almost certainly impact the ability of plants to respond to stresses, such as drought, freezing, salt, and heavy metals. Indeed, GlcCer content of plasma membrane has been shown to decrease in a number of plant species during the events of cold acclimation that lead to enhanced freezing tolerance (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994; Uemura et al., 1995). In addition, enrichment of the *cis*-Δ8 isomer of t18:1 in LCBs of *Arabidopsis thaliana* sphingolipids was recently shown to increase aluminum tolerance (Ryan et al., 2007). With the availability of mutants and the identification of genes for key sphingolipid metabolic enzymes, researchers are now in a position to dissect the roles of specific structural features of sphingolipids in environmental stress resistance in order to rationally engineer desirable compositions of plasma membrane and tonoplast for improved crop productivity.

2 Physicochemical Behavior of Sphingolipids in Aqueous Solutions and Microdomain Formation

The biological properties of sphingolipids are derived from their amphiphilic nature and their behavior in aqueous solutions. Since biological membranes are highly organized complex mixtures

of hundreds of lipid and protein species in which the performance of individual lipids is difficult to evaluate, most of the knowledge in this field has been obtained from studies of model membranes. LCBs, such as d18:1Δ4*trans*, tend to form micelles in aqueous environments, showing similar critical micellar concentration values (6–112 μM) to other single chain amphiphiles, such as lysophospholipids and aliphatic-type detergents (Le Stunff et al., 2002c; Garmy et al., 2005). The ability to form micelles is important for the signaling role of these compounds, although there is low probability that LCBs physically leave the membrane environment where they are generated. Complex sphingolipids in aqueous solutions have the tendency to associate in complex, self-organized structures, such as liposomes and bilayers that can serve as model systems for biological membranes. Their polar and non-polar structural moieties contribute to this formation. The aliphatic carbon chains of the fatty acid and the LCB moieties contribute to the high melting temperatures (T_m) of sphingolipids, and promote the hydrophobic effect that spontaneously and cooperatively maintains them together with other membrane lipids, giving them a relatively rigid conformation in the membrane (Pascher, 1976; Pascher and Sundell, 1977; Ramstedt and Slotte, 2006). The hydrophilic head groups of sphingolipids permit lipid–lipid or lipid–protein interactions that either reinforce or decrease the non-polar cohesion of the hydrophobic tails, since phosphate and carbohydrate groups may introduce hydrogen bonding or steric and coulombic repulsions with neighboring molecules (Brown, 1998).

Sphingolipids tend to segregate with sterols in very highly packed and ordered membrane regions, in a phase characterized as L_o (liquid-ordered phase) (Mayor and Rao, 2004; Simons and Vaz, 2004). L_o is an ordered, solid-like gel state that is different to the L_d (liquid-disordered phase) or less ordered fluid liquid crystalline state that most of the membrane glycerolipids adopt (Ohvo-Rekilä et al., 2002). The spontaneous and favored association between sterols and sphingolipids seems to be promoted by three factors: (1) the effective length of the rigid sterol ring system that is equivalent to a 17-carbon all *trans* hydrocarbon chain, matching the LCB extension (Wu and Chi, 1991; Brown, 1998); (2) the establishment of a hydrogen bond between the 3-β–OH group of the sterol and the amide group of the ceramide (Curatolo, 1987; Massey, 2001); and (3) the limited contribution of the sterol polar head group to steric hindrance with head groups of adjacent lipids (Xu et al., 2001; London, 2002).

Most of the membrane lipids are in the L_d phase, which is fluid and laterally homogenous in terms of lipid and protein composition. Sphingolipids and sterols become transiently associated to form an L_o phase, a physical region that moves together as a patch or "lipid raft" with free lateral mobility in the L_d phase lipids (Xu et al., 2001; Ohvo-Rekilä et al., 2002; Binder et al., 2003) (Fig. 6). The size, half-life, and formation dynamics of these membrane domains are not well understood. Data available from animal systems suggest that microdomains may constitute membrane structured regions ranging from 10 to 100 nm, perhaps containing from 100 to 100,000 lipid molecules (Simons and Tomre, 2000). The microdomains can fuse to form wider structures resembling membrane platforms for which cellular residence time varies from milliseconds to hours (Simons and Tomre, 2000; Binder et al., 2003; Ramstedt and Slotte, 2006). It is not clear whether some proteins facilitate lipid association or if the lipids promote the specific association of proteins in order to form functional microdomains. The existence of lipid rafts or microdomains does not contradict the essence of the fluid mosaic model for membrane structure, but rather expands upon it (Edidin, 2003; Vereb et al., 2003). In this regard, the lipids in the L_d phase not only possess individual motion consistent with the Singer and Nicolson model (Singer and Nicolson, 1972), but also the lipids in the L_o phase that form the microdomain undergo free lateral diffusion as a patch in the L_d phase behave as a raft (Fig. 6).

Lipid rafts or microdomains possess structural characteristics that accomplish specific cellular functions. In yeast and animal cells, lipid rafts may act as supramolecular platforms to recruit proteins by favoring efficient physiological interactions. The establishment of favorable molecular interactions is critical to membrane processes such as signaling, polarity, and trafficking, responses to biotic and abiotic stresses, and cell specialization and cell maintenance (Hearn et al., 2003; Hering et al., 2003; Schuck and Simons, 2004). Lipid microdomains have also received extensive

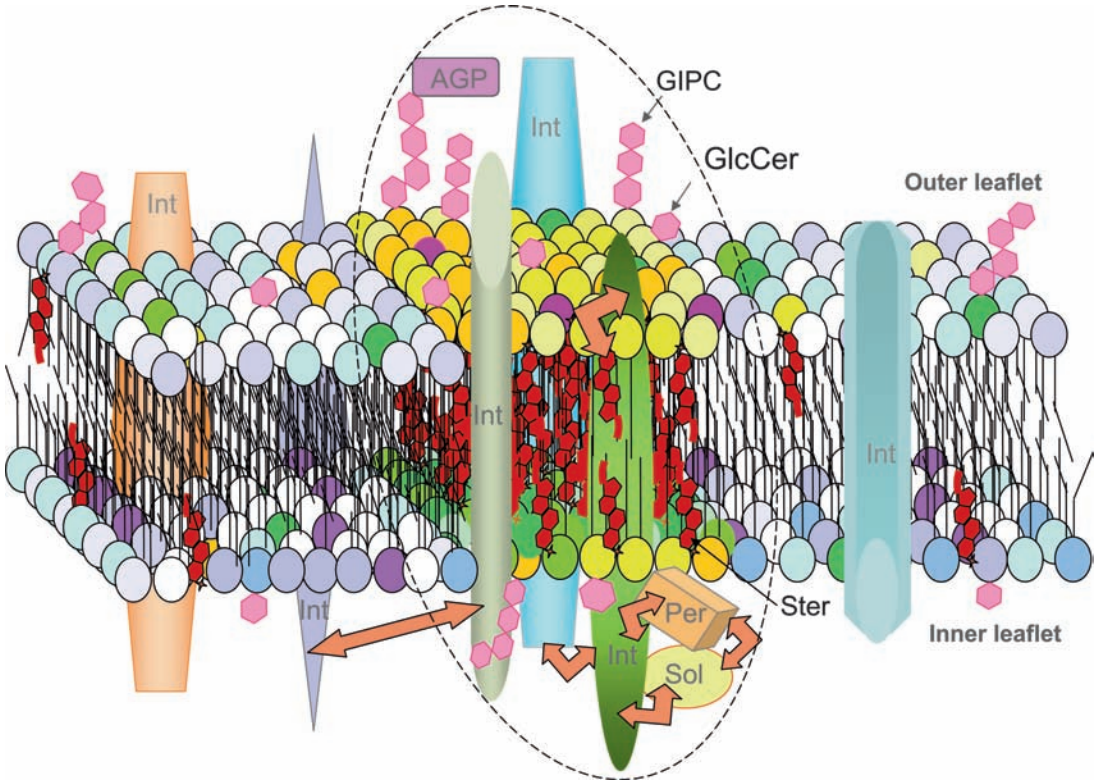

Fig. 6. Sphingolipids as structural building blocks of membrane microdomains or lipid rafts. Sphingolipids, including glucosylceramides (GlcCers) and glycosyl inositolphosphoceramides (GIPC), can spontaneously associate with high affinity to sterols (Ster) in cell membranes, forming transient domains that provide a site where specific interactions between lipid and/or proteins are favored (the microdomain is indicated by circled area of membrane). However, these assembly sites may function to exclude proteins in order to avoid undesirable functional collisions as well. *Arrows* indicate favored or unfavored interactions of proteins. Integral membrane proteins (Int), arabinogalactan proteins (AGP), peripheral (Per) and soluble (Sol) proteins are representative proteins associated to these regions (illustration by Laura Carmona-Salazar) (See Color Fig. 5 on Color Plate 3).

attention with regard to the role of rafts as portals for infection by viral, bacterial, and protozoan pathogens (van der Goot and Harder, 2001; Mañes et al., 2003; Lafont et al., 2004; Riethmuller et al., 2006).

3 Sphingolipid-Rich Microdomains in Plant Membranes

Evidence for microdomain formation in plants and in other eukaryotes comes from the isolation of detergent resistant membranes (DRMs) or detergent insoluble membranes, which are obtained from membranes treated with detergents at 4°C. Given the highly cohesive hydrophobic and hydrophilic interactions among sterols and sphingolipids, these regions are difficult to dissolve and can be isolated for biochemical studies (Schroeder et al., 1994). Whether these preparations actually constitute the microdomains present at the membrane or are just collected components of the original membrane structures is a controversial matter (Schroeder et al., 1994; Simons and Ikonen, 1997; Munro, 2003). However, to date, DRMs are the best biochemical method for studying the composition of lipid rafts and their cell function (Lingwood and Simons, 2007).

Membrane microdomains were recognized and intensively studied in animal cells at least 12 years earlier (Simons and Ikonen, 1997; Brown and London, 1998). Peskan et al. (2000) obtained the first preparation that suggested the existence of membrane microdomains in plants. Since then, DRM fractions have been isolated from plasma membranes, Golgi apparatus, or microsomes from a number of different plant sources

(Mongrand et al., 2004; Shahollari et al., 2004; Borner et al., 2005; Laloi et al., 2007; Lefebvre et al., 2007). This distribution is the result of the synthesis and assembly of the raft components in the endoplasmic reticulum and Golgi apparatus, and the delivery to the plasma membrane or other target membranes by vesicle trafficking routes (Futerman, 2006).

Free sterols and sphingolipids are enriched in DRMs and respectively constitute approximately 30% and 20–40% of the total lipid content of DRMs (Mongrand et al., 2004; Laloi et al., 2007; Lefebvre et al., 2007). Most studies to date have reported only GlcCers in plant-derived DRMs and have overlooked the more abundant GIPCs, as these molecules possess a high degree of polarity and are not extracted with solvent systems that have been traditionally used in plant lipidology. As a result, reports describing the lipid content of DRMs from plants have almost certainly underestimated levels of sphingolipids. Nevertheless, the enrichment of sphingolipids in DRMs obtained from different membrane sources ranges from 1.3- to 5-fold relative to the sphingolipid content of the bulk membranes, and sphingolipids with trihydroxy and dihydroxy LCBs are found in plasma membranes and isolated DRM (Mongrand et al., 2004; Borner et al., 2005; Laloi et al., 2007; Lefebvre et al., 2007).

Proteins are also major components of plant lipid rafts. The number of identified proteins in DRM varies from 15 to 270, depending on the sensitivity and resolution of the methods employed (Mongrand et al., 2004; Borner et al., 2005; Lefebvre et al., 2007). DRM proteins fall into several groups related to their possible function: (1) signaling proteins, such as leucine-rich repeat receptor-like kinases, serine/threonine kinases, calcium-dependent kinases, small GTP binding proteins, glycosylphosphatidylinositol (GPI) anchored proteins and proteins responsive to pathogen elicitors and those involved in defense responses to biotic and abiotic stress; (2) transmembrane transport proteins, such as proton pumps, water channels, anion and cation carriers, and putative plant hormone transporters; (3) intracellular trafficking proteins, such as cytoskeletal proteins and proteins characteristic of secretory vesicles or trafficking assembly complexes; and (4) metabolic enzymes, especially those involved in the synthesis of cell wall polymers (Peskan et al., 2000; Mongrand et al., 2004; Shahollari et al., 2004; Borner et al., 2005; Morel et al., 2006; Lefebvre et al., 2007). Recently, an E3 ligase, a protein possibly involved in fumonisin B_1 induction of programmed cell death has been reported in DRMs from *Arabidopsis thaliana* (Lin et al., 2008).

The interaction of sphingolipids with membrane proteins has been studied. Biochemical experiments, supported by several crystallographic structures of membrane proteins, have shown that the surrounding lipids influence the activity of membrane proteins (Camara-Artigas et al., 2002; Long et al., 2007). Like most membrane lipids, sphingolipids may affect protein activity by modifying protein conformations. As essential components of membrane microdomains, sphingolipids may contribute to protein function by facilitating protein–protein interactions required for biological activity. Given the types of proteins found in microdomains (Borner et al., 2005), alterations in the content and composition of sphingolipids likely impacts important cellular activities, such as synthesis and degradation of cell walls and intercellular movement of plant hormones.

Membrane thickness in microdomains is greater than in non-microdomains, due to the presence of longer and more extended hydrophobic chains. This suggests that integral proteins must match their hydrophobic residues to reside in either of these regions in such a way that migration to another region brings about conformational changes necessary for protein secretion (Allende et al., 2004). It has been hypothesized that the carbohydrate moiety of sphingolipid head groups may interact with an aromatic residue of a sphingolipid-binding domain consisting of a hairpin structure that stabilizes the lipid–protein interaction (Fantini, 2003).

What is the role of membrane microdomains in plant cells? Experiments in plant and animal cells have revealed microdomains as sites for pathogen entry, and have identified molecular components that may give some clues to function (Mañes et al., 2003; Bhat et al., 2005; Grennan, 2007). Many of the proteins identified in microdomains suggest a role as metabolic or secretion centers for housekeeping activities. They could also be sites where the recognition of external signals and their initial transduction takes place.

Special attention must be given to the recruitment of signaling proteins involved in defense responses to pathogens. It is thought that specific and optimized lipid microenvironments for protein–protein interactions mediate these events in plants, resulting in an efficient and rapid response to a metabolic demand or to a stress situation, as demonstrated for yeast and animal systems (Bagnat et al., 2000; Hering et al., 2003). The same phenomena could be occurring in cases where polarization of membrane components is required, such as pollen tube formation. In addition to proteins, microdomain lipids may participate in these functions. Based on recent reports that LCBs, in their free or phosphorylated form, act as mediators in signal transduction pathways (Ng et al., 2001; Coursol et al., 2003), a role for membrane microdomains as a sphingolipid signaling reservoir can also be envisioned.

B Sphingolipids as Signaling Molecules

In contrast to the large amount of information available from animals and yeast, relatively little is known about the signaling properties of sphingolipids in plants (Worrall et al., 2003). Recently, several reports have documented bioactive functions of sphingolipids in drought stress, programmed cell death, and pathogenesis (Fig. 7).

1 Role of Sphingolipids in Drought Stress Signaling and Regulation of Stomatal Closure

LCBs play a role in signaling in plant cells by mediating stomatal closure. Stomata are pores in the epidermis of leaves and stems that are important for carbon dioxide uptake during photosynthesis and for the reduction of water loss by transpiration. Guard cells regulate stomatal

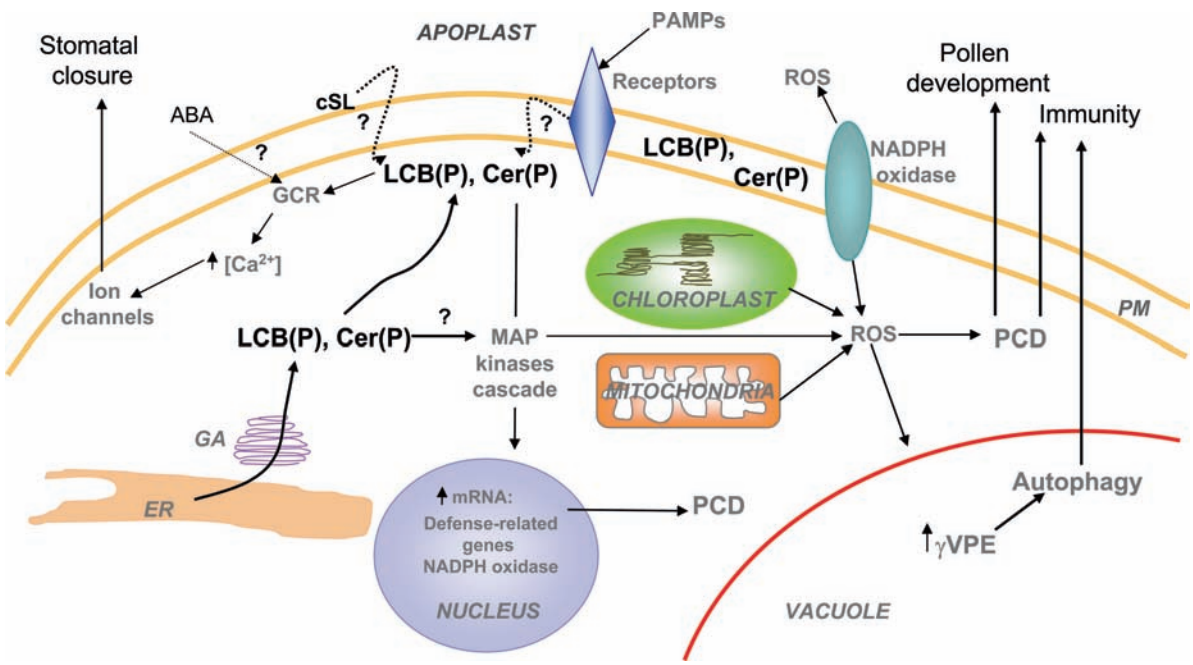

Fig. 7. Bioactive roles of sphingolipid metabolites. Free or phosphorylated long chain bases (LCB(P)) and ceramides (Cer(P)) are intracellular mediators in transduction pathways that lead to control of stomata closure and programmed cell death. Given their demonstrated role in the generation of reactive oxygen species (ROS) and the initiation of programmed cell death (PCD), it is possible that these sphingolipid metabolites also contribute to pollen development and immunity to pathogens. With regard to plant–pathogen interactions, sphingolipid metabolites may be associated with pathogen-induced triggering of MAP kinases cascades and may serve as downstream components of signal transduction pathways mediated by pathogen-associated molecular patterns. It is unknown what the relative contributions of de novo sphingolipid synthesis and sphingolipid degradation are to the production of bioactive LCBs and ceramides. Abbreviatons: cSL, complex sphingolipids; ER, endoplasmic reticulum; GA, Golgi apparatus; PM, plasma membrane; GCR, G-protein coupled receptor; PAMPs, pathogen-associated molecular patterns; γVPE, vacuolar processing enzyme (See Color Fig. 6 on Color Plate 4).

opening and closure by responding to diverse environmental and chemical cues, such as light intensity, temperature, water status, CO_2 levels and hormonal signals. The plant hormone ABA regulates stomatal opening and closure by elevating cytosolic Ca^{2+} levels, which, in turn, activates plasma membrane anion channels in guard cells. The resulting K^+ efflux causes loss of turgor and stomatal closing (Schroeder et al., 2001). The role of LCBs in this ABA-mediated signaling cascade was first shown as a 2.4-fold increase in sphingosine (d18:1Δ4*trans*)-1-phosphate (S1P) levels in drought-treated *Commelina communis* plants compared to control plants. Exogenously applied S1P induces stomatal closure at low concentrations (4–6 μM), while the 1-phosphate ester of d18:0 does not show this effect. The transient increase in cytosolic Ca^{2+} concentration appears to be the link between S1P and the ABA-mediated signal transduction pathway leading to ion channel activation (Ng et al., 2001). Further evidence for the involvement of S1P and phytosphingosine-1P was obtained by direct assay of LCB kinase activity in mesophyll and guard cell protoplasts in *Arabidopsis thaliana* leaves (Coursol et al., 2003, 2005). Treatment of *Arabidopsis thaliana* plants with ABA was found to activate the LCB kinase in vivo, and this activity was sensitive to *N*,*N*-dimethylsphingosine, a known inhibitor of mammalian LCB kinases (Coursol et al., 2003). The target of S1P in animal cells is a G-protein coupled receptor, which in turn interacts with a transmembrane G-protein. G-proteins are a family of signal-transducing proteins consisting of multiple subunits. The involvement of G-proteins in the signal-transduction pathway for S1P in plants was also suggested by Coursol and co-workers (Coursol et al., 2003), who showed that *Arabidopsis thaliana* mutants lacking the G-protein α-subunit were unable to respond to exogenously applied S1P (Fig. 7).

2 Sphingolipid-Associated Programmed Cell Death and Autophagy

Programmed cell death is an essential event in plant development and stress responses, sharing some features with apoptosis in animal cells (van Door and Woltering, 2005). The pharmacological approach of adding exogenous LCBs and ceramides to plant cell cultures and tissues has supported the role of these molecules as potential mediators of programmed cell death. Synthetic C2 ceramide (50 μM) induces hydrogen peroxide production and cell death in *Arabidopsis thaliana* cell culture in a calcium-dependent manner. Cytosolic Ca^{2+} increases about twofold a few seconds after ceramide addition and its role in promoting cell death downstream is shown by the effect of La^{3+}, a calcium channel inhibitor (Townley et al., 2005). Free LCBs have similar effects and appear to be more active. The application of d18:1Δ4*trans*, d18:0, or t18:0 at a concentration of 2 μM to *Arabidopsis thaliana* leaves causes an accumulation of reactive oxygen intermediates and cell death (Shi et al., 2007). In contrast, the phosphorylated intermediates of the free LCBs do not have this activity and even prevent cell death when they are applied simultaneously with the non-phosphorylated form. Interestingly, this protective effect shows a structural specificity, as each phosphorylated LCB was only able to block programmed cell death initiated by its corresponding free LCB (Shi et al., 2007), suggesting that the ratio of specific phosphorylated and non-phosphorylated LCBs might act as cell death regulators.

A possible control mechanism could be achieved through autophagy, an event necessary for protein and organelle degradation. Macroautophagy, usually referred to as simply autophagy, is a conserved mechanism in eukaryotic cells. This process requires the formation of double membrane vesicles that engulf cytoplasmic contents and sometimes organelles. Once these vesicles are acidified, their external membranes fuse with lysosomes (in animals) or the vacuole (in plants), and the vesicles with only inner membranes are introduced into these structures to be degraded through proteolysis (Klionsky, 2005).

Recently, it has been shown that autophagy is a crucial process in initiating the hypersensitive response of programmed cell death, and also in preventing programmed cell death during innate immunity. In *Nicotiana benthamiana*, disruption of the *BECLIN1* gene, the ortholog of conserved mammalian and yeast tumor suppressor genes, causes unrestricted local and systemic cell death upon pathogen challenge. *BECLIN1*-silenced plants show a reduction of double-membrane vesicles in the vacuole (Liu et al., 2005). The exact role of the BECLIN1 protein in regulat-

ing autophagy is not known. However, in mammalian cells, enhanced levels of BECLIN1 are associated with ceramide-mediated autophagy (Scarlatti et al., 2004). S1P induces autophagy in animal cells, but in contrast to ceramide-induced autophagy, BECLIN1 protein levels are not modified (Lavieu et al., 2006).

3 Sphingolipids in Plant–Pathogen Interactions

Plants recognize a wide array of microbial-derived molecules known as pathogen-associated molecular patterns through specific molecular interactions with the products of plant resistance genes (Jones and Dangl, 2006). This interaction initiates a response by the plant to arrest pathogen colonization. When this response is particularly accelerated and amplified, the reaction is termed a hypersensitive response (HR). Lipid-derived molecules, such as jasmonic acid, have been implicated in plant defense signaling (Shah, 2005). Recent evidence also implicates sphingolipids as potential signal molecules in plant defense pathways. This hypothesis is based on the biological activity of AAL toxin and fumonisin B_1, which are produced by necrotrophic plant pathogenic fungi. Both toxins are sphingosine-analog mycotoxins that disrupt sphingolipid synthesis as they target the acyl-CoA-dependent ceramide synthase (Fig. 3). AAL toxin is a known pathogenicity factor required for *Alternaria alternata* f. sp. *lycopersici* infection of tomato (Gilchrist and Grogan, 1976). Fumonisins are required for the development of foliar disease symptoms produced by *Fusarium verticillioides* infection (Glenn et al., 2008). As inhibitors of the acyl-CoA-dependent ceramide synthase, these toxins cause a marked increase of free LCBs. In *Lemna pausicostata* leaf discs, 1 μM fumonisin B_1 and 1 μM AAL toxin produce a 75- and a 129-fold increase in sphinganine levels, respectively (Abbas et al., 1994). In addition to augmenting LCB levels, both toxins produce cell death in various plant species (van Asch et al., 1992; Abbas et al., 1994; Stone et al., 2000). Fumonisin B_1-induced cell death in *Arabidopsis thaliana* has a number of features in common with HR, including the presence of localized lesions, reactive oxygen species production, and defense gene expression that depend on functional salicylic acid, ethylene and jasmonic acid-mediated pathways (Asai et al., 2000) (Fig. 7).

Resistance to these toxins has shed some light on the potential mechanisms leading to programmed cell death by sphingolipids. In tomato, the *Asc-1* gene confers resistance to AAL toxin and to the fungus *Alternaria alternate* f. sp. *lycopersici*. When homozygous tomato genotypes harboring the resistance gene are treated with AAL toxin, they do not accumulate the LCBs d18:0 and t18:0 to the same high levels as the susceptible *asc/asc* mutant (Abbas et al., 1994). In addition, the AAL toxin-resistant plants are able to synthesize complex sphingolipids, and cell death is not observed (Spassieva et al., 2002). Overexpression of *Asc-1* confers resistance to AAL toxin and *Alternaria alternata* in susceptible tomato genotypes and in *Nicotiana umbratica* (Brandwagt et al., 2002). The Asc-1 polypeptide is homologous to the *Saccharomyces cerevisiae LAG1*-encoded ceramide synthase (Spassieva et al., 2002), and its overexpression likely reduces the accumulation of cytotoxic LCBs.

Characterization of the *Arabidopsis thaliana* fumonisin B_1-resistant mutant *fbr11-1* provided direct evidence that the accumulation of free LCBs mediates cell death (Shi et al., 2007). In this mutant, the gene for the LCB1 subunit of serine palmitoyltransferase harbors a T-DNA insertion in its 3′ untranslated region leading to a reduced transcript level compared to wild type plants. Basal levels of LCBs are not altered in these plants, but the mutants are unable to achieve high levels of LCBs after treatment with fumonisin B_1 and are, therefore, unable to initiate the cell death response (Shi et al., 2007). The *fbr11-1* mutants do not accumulate superoxide anion and hydrogen peroxide when treated with fumonisin B_1, suggesting that reactive oxygen species mediate LCB-induced cell death. The fact that both AAL toxin-resistant tomato genotypes and fumonisin B_1-resistant *Arabidopsis thaliana* mutants accumulate LCBs upon treatment with mycotoxin, but to a lesser degree than the susceptible genotypes, suggests that a threshold level of free LCBs must be reached to trigger programmed cell death.

Other fumonisin B_1-resistant *Arabidopsis thaliana* mutants are available to study the transduction pathway, which, triggered by fumonisin B_1 and accompanied by increases in levels of LCBs, leads to programmed cell death. The *fbr6* mutant

survives and develops in the presence of fumonisin B_1 but shows an elongated petiole and exaggerated leaf margin serration in the absence of the toxin (Stone et al., 2005). The FBR6 polypeptide was identified as a putative transcriptional regulator (AtSPL14) with a highly conserved DNA binding domain (GTAC), nuclear localization signal and an ankyrin protein interaction motif. FBR6 belongs to the SQUAMOSA promoter binding protein family that have been linked to plant growth and development (Liang et al., 2008). Indeed, the *fbr6* mutant shows elongated petioles and enhanced leaf margin serration (Stone et al., 2005). Although the role of FBR6 in fumonisin B_1 resistance is not yet clear, identification of its target genes might help to explain this function. Other fumonisin B_1 resistant mutants identified to date include *fbr1* and *fbr2* (Stone et al., 2000). In the presence of 1 μM fumonisin B_1, *fbr1* and *fbr2* plants develop normally and show reduced levels of the plant defensin gene transcript *PDF1.2* when compared to wild type plants. Although levels of other resistance gene transcripts are not modified, these mutants show increased resistance to a virulent strain of *Pseudomonas syringae* pv. *maculicola* (Stone et al., 2000). Identification of the *FBR1* and *FBR2* gene products should provide further clarification of their potential role in sphingolipid metabolism.

Two *Arabidopsis thaliana* mutants with an accelerated cell death (acd) phenotype provide further evidence linking sphingolipid metabolism to the hypersensitive response. Mutants *acd-11*constitutively express cell death and HR-related genes and accumulate phytoalexins and callose. The *ACD11*-encoded polypeptide is related to a mammalian glycolipid transfer protein, and in vitro experiments have shown that the recombinant protein can transport sphingosine (d18:1) between membranes with high binding affinity (Brodersen et al., 2002). A second mutant, *acd5*, has a similar phenotype and develops normally but shows unrestricted cell death when infected by *Pseudomonas syringae*, in a salicylic acid-dependent pathway (Greenberg et al., 2000). The ACD5 protein has ceramide kinase activity and a balance between its product, ceramide-1-phosphate and ceramide might trigger the cell death program (Liang et al., 2003). As it is the case with LCB-induced programmed cell death, phosphorylation of ceramides appears to be a mechanism for reducing the potential cytotoxic effects of accumulated ceramides.

A critical step in the fumonisin-induced cell death pathway is protease activation of the vacuolar processing enzymes (VPE), a family of cysteine proteases responsible for the maturation of some proteins, which are found in both developing seed and vegetative tissues (Kuroyanagi et al., 2005). The vegetative forms are induced by wounding and senescence, and are thought to be involved in programmed cell death. Genetic evidence supporting the role of these enzymes in plant cell death comes from an *Arabidopsis thaliana* null mutant, which lacks all four VPE genes. In the VPE mutant, vacuoles do not collapse in fumonisin B_1-treated leaves, whereas wild type leaves show vacuole disruption and subsequent cell death (Kuroyanagi et al., 2005).

In addition, a sphingolipid-mediated signaling cascade involving sphingolipid-specific receptors, protein kinases, and GTPases has been identified in interactions between rice and a pathogenic fungus. In this regard, two classes of GlcCers ("Cerebrosides A and C") isolated from the rice pathogen *Magnaporthe grisea* (rice blast) have been shown to elicit HR-type cell death and the accumulation of the phytoalexin momilactone when applied to rice leaves (Koga et al., 1998). These responses ultimately promote resistance to rice blast and other pathogens. The elicitation could also be conferred by ceramides generated from Cerebrosides A and C, but fatty acid and LCB degradation products of these GlcCers or mammalian GlcCers were unable to elicit this response in rice, suggesting the presence of a receptor in rice that is able to specifically recognize fungal sphingolipids (Koga et al., 1998; Umemura et al., 2000). In addition, rice containing a mutation in the G protein α subunit displayed reduced response to the *Magnaporthe grisea* Cerebroside A and C elicitors, as measured by H_2O_2 accumulation and pathogenesis-related (PR) gene expression, and lacked resistance to rice blast (Suharsono et al., 2002). Resistance to rice blast could be restored in rice with the mutant heteromeric G protein α-subunit by overexpression of OsRac1, a homolog of RacGTPase and an important regulator of cell death and disease resistance in rice (Suharsono et al., 2002). This also restored H_2O_2 production and expression of PR genes in the host plants. Moreover, silenc-

ing of *OsRac1* resulted in reduced levels of the OsMAPK6 protein, a mitogen-activated protein kinase, and also lead to reduced activation of OsMAPK6 by the GlcCer elicitor (Lieberherr et al., 2005). These results are consistent with a sphingolipid-induced cascade that involves transmission of a signal from a sphingolipid receptor in rice to the heteromeric G protein α-subunit and then to the downstream OsRac1 and OsMAPK6 that triggers the production of reactive oxygen species and PR gene expression and ultimately disease resistance in the host plant (Suharsono et al., 2002; Lieberherr et al., 2005).

C Relevance of Sphingolipids to Chloroplasts and Photosynthesis

Although sphingolipids have not been previously identified in chloroplast membranes, the biosynthesis of sphingolipid long-chain bases is directly linked to metabolic reactions that occur in chloroplasts. In this regard, the palmitoyl-CoA precursor of LCBs is formed by de novo fatty acid synthesis in chloroplasts. This substrate of serine palmitoyltransferase (SPT) arises from the release of palmitic acid from acyl carrier protein (ACP) by palmitoyl-ACP thioesterase or fatty acid thioesterase (FATB) for export to the cytosol. It has been shown that null mutants for *FATB* display reduced growth (Bonaventure et al., 2003). The basis for this phenotype is not clear given that no differences were detected in the content of waxes and sphingolipids on a dry weight basis between the wild type and mutant (Bonaventure et al., 2003). One possibility is that the reduced growth results from restricted synthesis of sphingolipids due to the reduced availability of the palmitoyl-CoA substrate. In fact, the phenotype of these mutants is similar to that of *Arabidopsis thaliana* lines with partial RNAi suppression of the LCB1 subunit of serine palmitoyltransferase (Chen et al., 2006). Like the *FATB* mutants, the *LCB1* RNAi lines do not have reduced sphingolipid content on a dry weight basis (Chen et al., 2006). An interpretation of this result is that plants adjust their growth based on the availability of sphingolipids, which can be determined by the pool sizes of the palmitoyl-CoA precursor or by the activity of serine palmitoyltransferase. As such, fatty acid biosynthetic reactions, including the release of palmitic acid from ACP, in chloroplasts can play a key role in regulating sphingolipid synthesis in the ER. This, in turn, impacts the growth and physiology of the plant.

In addition, it has recently been shown that complete or partial loss of LCB C-4 hydroxylation results in up to a three-fold increase in total sphingolipid content in *Arabidopsis thaliana* leaves (Chen et al., 2008). Lipidomic analyses conducted on these plants revealed decreases in levels of principally chloroplast-specific lipids, monogalactosyldiacylglycerol and phosphatidylglycerol, relative to the wild-type controls (Chen et al., 2008). These changes were largely accounted for by reductions in molecular species that contain C16 fatty acids. One explanation for the altered content of monogalactosyldiacylglycerol and phosphatidylglycerol in the C-4 hydroxylase deficient plants is that the increased demand for palmitoyl-CoA to support enhanced sphingolipid synthesis is compensated for by reductions in the flux of palmitic acid into chloroplast lipids. The examples of the FATB and LCB C-4 hydroxylase mutants illustrate the interdependency of lipid metabolic pathways in the chloroplast and ER.

Furthermore, several lines of evidence point to a relationship between sphingolipid function and chloroplasts. It has been shown that disruption of sphingolipid synthesis by application of fumonisin B_1 results in light-dependent cell death in leaves of jimsonweed (*Datura stratonium* L.) (Abbas et al., 1992) and *Arabidopsis thaliana* (Stone et al., 2000). In the case of jimsonweed, chloroplast membrane integrity is lost in response to fumonisin B_1 treatment, as evidenced by the destruction of the chloroplast outer envelope, disorganization of thylakoid membranes, and large reductions in chlorophyll content (Abbas et al., 1992). Fumonisin B_1-associated programmed cell death appears to be closely linked to the generation of reactive oxygen species (Asai et al., 2000; Shi et al., 2007). HR-related programmed cell death, such as that observed in *ACD5* and *ACD11* mutants, also appears to be mediated through reactive oxygen species (Greenberg et al., 2000; Brodersen et al., 2002; Liang et al., 2003). In the latter case, reactive oxygen species appear to be the product of light-dependent reactions in chloroplasts (Liu et al., 2007).

VI Concluding Remarks

The study of plant sphingolipids has advanced remarkably during the past 10 years. Methods for the quantitative extraction and comprehensive analysis of plant sphingolipids are now in place, and many of the genes that are involved in sphingolipid synthesis have been identified and functionally characterized. In addition, it is now established that sphingolipids are essential molecules in plants and are major structural components of plasma membrane, tonoplast, Golgi apparatus, and likely other endomembranes. As membrane constituents, sphingolipids have been implicated in biotic and abiotic stress resistance, membrane transport, and the organization of membrane microdomains. It has also become evident that sphingolipid-related molecules are important regulators of cellular processes. The best characterized of these functions is the role of LCBs and likely ceramides as initiators of programmed cell death. Despite this progress, much remains to be learned about sphingolipid metabolism and function in plants. The important areas for future study include: (1) understanding how sphingolipid synthesis is regulated, (2) unraveling the enzymes of sphingolipid catabolism and how they function to mediate sphingolipid levels and the production of signaling molecules, (3) determining how sphingolipids are transported within the cells and function in endomembrane trafficking, (4) understanding the precise roles of sphingolipids in signaling pathways, and (5) obtaining a more complete understanding of the contributions of sphingolipids to membrane microdomain formation and function. In addition, with the availability of mutants for enzymes associated with the synthesis and modification of sphingolipids, researchers can now begin to address the functional basis for the immense structural diversity that is found in plant sphingolipids. A further challenge that remains is the development of a single, unified analytical platform for profiling of the content and composition of sphingolipids, glycerolipids, and sterols in plant organs and isolated membranes. With this capability, it will be possible to understand how alterations in one component of lipid metabolism impact the complete lipid metabolic network. With the momentum that has been gained in the study of plant sphingolipids, it is likely that many of these questions will be addressed in the coming decade.

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Chapter 6

Lipids in Algae, Lichens and Mosses

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Summary

Lipids from algae, lichens and mosses are highly diverse and differ from prokaryotic cyanobacteria and vascular plants in many aspects. Although in lower eukaryotes most of the lipids have functions similar to those in vascular plants, the chain length and the desaturation degree can be significantly higher than that observed in vascular plants. This is due primarily to the fact that these organisms are exposed to extreme environments with respect to drought and temperature. Algae, lichens and mosses may contain

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exotic lipids like betaine lipids, *n*-alkanes or halogenated lipids, which will be of industrial interest in the near future. In the present chapter, the lipid composition of algae, lichens and mosses will be presented with a special focus on lipids that are found exclusively in these groups. New data on the function of membrane lipids in algae, lichens and mosses will be discussed, and the effect of environmental changes on the lipid composition in the lower eukaryotes will be outlined briefly.

I Introduction

Algae, lichens and mosses share the characteristic that many species can tolerate relatively extreme conditions with respect to water, salt, pH, temperature and radiation. All of these environments induce specific alterations in the membrane structure in order to maintain membrane fluidity, ion selectivity and permeability. Therefore, it is not surprising that these species have developed special pathways, enzymes and lipid products to cope with their biotic and abiotic environments.

II Lipids in Algae

Eukaryotic algae inhabit a wide range of ecosystems in both freshwater and marine environments. They represent a group of diverse organisms and are classically divided into groups based on differences in ultrastructure and phylogeny. The primary research on algal lipids has been performed on green algae (Chlorophyta), brown algae and diatoms (both belonging to Heterokontophyta) and red algae (Rhodophyta). In the present review, data on green algal lipids are presented in special chapters due to the amount of available information, while the published results for the other groups are summarized. Early work on the lipid metabolism of algae concentrated on model organisms, such as *Chlamydomonas reinhardtii* (see also Chapter 7), but during the last years, increasing amounts of information about the other algal groups have become available. Work on algal lipids has included the elucidation of the pathways of lipid synthesis, as well as the genes and enzymes involved. Lipid research has also focused on changes in the algal lipid composition in response to environmental changes and on the role of lipids in stress response, innate immunity and protection against algal pathogens. The latter research topics in particular have led to the discovery, isolation and characterization of novel and unusual lipids that are not found in vascular plants. In the present chapter, we will briefly summarize the classical data on the lipid composition of various algal groups, taking into account recent data that have been generated using new techniques, such as mass-spectrometry (for more detailed information the reader is referred to the excellent reviews of Thompson, 1996; Harwood, 1998; Guschina and Harwood, 2006). We will then focus on the novel lipid compounds that have been discovered in recent years (also reviewed by Gerwick, 1994; Guschina and Harwood, 2006; see also the review on halogenated algal lipids by Dembitsky and Srebnik, 2002), and conclude with a chapter on the influence of abiotic effects on the lipid composition of algae, as well as a short description of different functions of lipids in the algal physiology. The chapter covering algal lipids is a follow-up to the excellent review "Membrane lipids in algae" by Harwood (1998) presented in the previous version of the book titled "Lipids in Photosynthesis: Structure, Function and Genetics". In the present chapter, the basics of algal lipids, that is, the lipid and fatty acid composition of different algal classes, are therefore not covered in such great detail as in the previous review written by Harwood (1998), and the reader is referred to his work. In the present review we have concentrated on the addition of novel information about algal lipids that has been gathered since, resulting in the new chapters addressing algal oxylipins and

Abbreviations: AA – Arachidonic acid; DDE – Diadinoxantin-de-epoxidase; DGTA – Diacylglycerolhydroxymethylalanine; DGTS – Diacylglyceryltrimethylhomoserine; DGDG – Digalactosyldiacylglycerol; DHA – Docosahexaenoic acid; EPA – Eicosapentaenoic acid; FCP – Fucoxanthin-chlorophyll-binding protein; LHC – Light-harvesting Chl a/b binding complex; MGDG – Monogalactosyldiacylglycerol; PC – Phosphatidylcholine; PE – Phosphatidylethanolamine; PG – Phosphatidylglycerol; PS – Photosystem; PUFA – Polyunsaturated fatty acids; SQDG – Sulfoquinovosyldiacylglycerol; TAG – Triacylglycerol; VDE – Violaxanthin de-epoxidase

the function of algal lipids. Other aspects that were briefly addressed in the previous review by Harwood, for example, environmental effects on the lipid composition, are now covered in closer detail.

A Structures

1 Lipid Classes

Chlorophyta

The lipid composition of green algae is dominated by membrane lipids, and under certain circumstances, such as stress conditions, larger accumulations of storage lipids can be found. The glycerolipids of green algae can be divided into phospholipids, galactolipids and sulfolipids (Fig. 1). Typical phospholipids of green algae are phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine and phosphatidylglycerol (PG). Galactolipids comprise monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), and the major sulfolipid is sulfoquinovosyldiacylglycerol (SQDG). While phospholipids contribute to different functional membranes, MGDG and SQDG are restricted to the thylakoid membranes of the chloroplast. DGDG can also be found in extraplastidial membranes, where it is able to replace bilayer-forming phospholipids, including PG, PC and PE (Jouhet et al., 2004; Andersson et al., 2005). Accumulation of DGDG is usually observed under phosphate starvation of vascular plants and algae (Härtel and Benning, 2000; Li et al., 2006). The concentration of the sulfolipid SQDG is generally higher in Chlorophyta than in vascular plants. Another important lipid of green algae is the betaine lipid diacylglyceryltrimethylhomoserine (DGTS), which has been found, for example, in *Volvox carteri* (Moseley and Thompson, 1980), *Dunaliella salina* (Norman and Thompson, 1985), and *C. reinhardtii* (Sato and Furuya, 1983). DGTS replaces the phospholipid PC to a large extent in marine green algae, whereas in freshwater green algae, higher concentrations of PC can be found (Eichenberger, 1982). It has been suggested that the high concentrations of DGTS in the marine species reflect the low phosphate concentration in the oceanic envi-

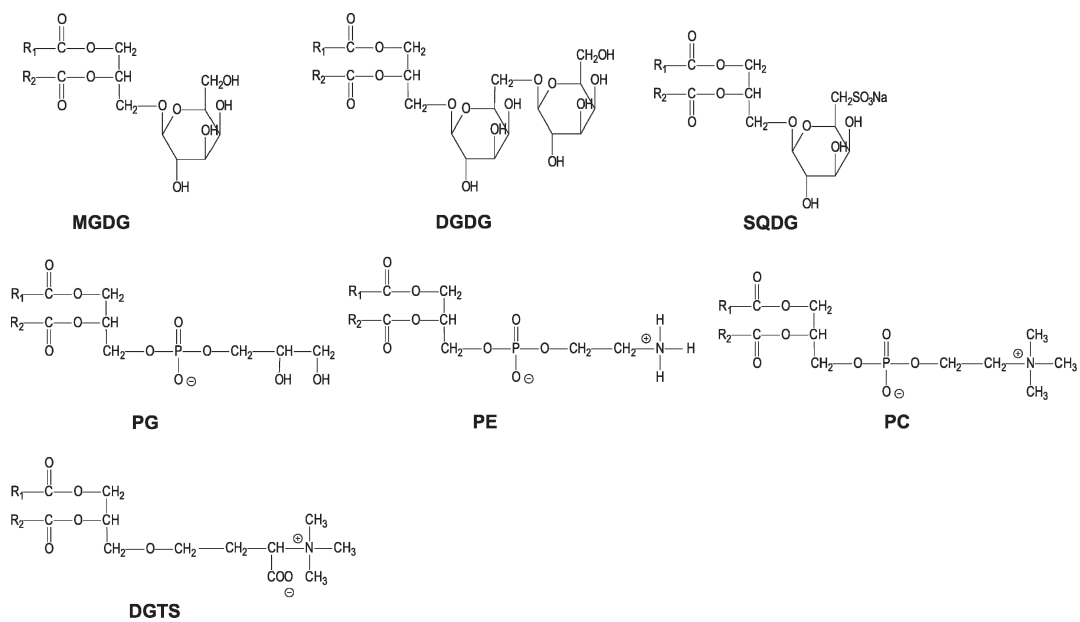

Fig. 1. Structures of the most important lipids found in the different algal groups. R1 and R2 represent varying acyl residues. Abbreviations: MGDG: monogalactosyl-diacylglycerol, DGDG: digalactosyl-diacylglycerol, SQDG: sulfoquinovosyl-diacylglycerol, PG: phosphatidyl-glycerol, PE: phosphatidyl-ethanolamine, PC: phosphatidyl-choline, DGTS: 1,2-diacylglyceryl-3-O-4′-(N,N,N-trimethyl)-homoserine.

ronment. DGTS is also a typical lipid of primitive vascular plants, such as ferns and mosses, where it also serves as a replacement for PC (Sato and Furuya, 1983). Recent investigations of the lipid composition of the green alga *C. reinhardtii* using MALDI-TOF MS (Vieler et al., 2007) have supported the existing data that PC and phosphatidylserine are not present in the lipid membranes, and that DGTS represents a major membrane component of *C. reinhardtii*. In addition to the typical thylakoid membrane lipids MGDG, DGDG and SQDG, an additional lipid, acylsulfoquinovosyldiacylglycerol, is present in *C. reinhardtii* (Riekhof et al., 2003).

Other Algal Groups

In general, the other algal groups possess the same phospho- and glycolipids as do the Chlorophyta (Fig. 1). Diatoms belonging to the group Heterokontophyta contain high amounts of the four chloroplast lipids, MGDG, DGDG, SQDG and PG, often accompanied by PC and phosphatidylsulfocholine (Harwood and Jones, 1989). In contrast to vascular plants, and even to green algae, the concentration of the sulfolipid SQDG is increased in diatoms. This has been corroborated by a recent study (Vieler et al., 2007) that showed that, in the diatom *Cyclotella meneghiniana*, the content of acidic lipids is significantly higher than in the green alga *C. reinhardtii* and amounts to 40% of the total lipids. Thus, the lipid composition of diatoms more closely resembles that of the cyanobacteria (see also Chapter 8) than that of vascular plants. The high levels of SQDG found in diatoms evidently lead to a higher overall charge of the thylakoid membrane in diatoms compared to that of green algae. Taking into account the similarity of membrane proteins, it will be an interesting topic for future experiments to address the question of why such an increase in the content of anionic thylakoid lipids is needed in diatoms, and how this affects the lipid protein interaction and the overall function of the thylakoid membrane.

Brown algae also possess the same glycerol-based lipids as vascular plants. However, the thylakoid-membrane lipids MGDG, DGDG, SQDG and PG may be accompanied by novel glyco-, phospho- and sulfolipids, as found in the brown algae *Fucus serratus*, *Fucus vesiculosus* and *Pelvetia canaliculata* (Liem and Lauer, 1976a, b). These novel polar lipids have a complex structure and are species-characteristic. Brown algae also contain significant amounts of betaine lipids (Eichenberger, 1993). In contrast to green algae, DGTS is exchanged by diacylglycerolhydroxymethylalanine (DGTA), which replaces PC as zwitterionic membrane compound. The algal species *Cryptomonas* (Chryptophyta) and *Ochromonas* (Chrysophyceae) were shown to produce both betaine lipids DGTS and DGTA, respectively (Eichenberger, 1993). As in diatoms, SQDG is present in significantly higher concentrations than in vascular plants.

Red algae generally have higher amounts of phosphoglycerides compared with green algae and also contain unusual glycolipids, with the sugar moieties mannose and rhamnose found in *Polysiphonia lanosa* and *Chondrus crispus* (Harwood, 1998).

2 Fatty Acids

Chlorophyta

The fatty acid composition of freshwater green algae is comparable to that of vascular plants and consists primarily of C16 and C18 fatty acids with a high degree of unsaturation (Watanabe et al., 1983; Ahlgren et al., 1992). However, freshwater algae contain higher amounts of C16 and lower amounts of C18 fatty acids compared to plants. Polyunsaturated fatty acids (PUFAs) exceeding 18C atoms are generally found in marine green algae that also exhibit a higher diversity in their fatty acid profile (Ahlgren et al., 1992; Mikhailova et al., 1995). Most green algae resemble the so-called 16:3 green plants, meaning that their galactolipids contain one C18 and one C16 fatty acids (Eichenberger, 1993; Cho and Thompson, 1987). This is typical for the prokaryotic pathway of MGDG and DGDG synthesis that leads to the formation of MGDG and DGDG, with 18C fatty acids esterified to the *sn*-1 position and 16C fatty acids located at the *sn*-2 position. Other green algae, including *Chlorella kessleri* (Sato et al., 2003), for example and other vascular plants, also use the eukaryotic pathway for MGDG and DGDG synthesis, leading to the formation of MGDG and DGDG with C18 fatty acids targeted to both the *sn*-1 and *sn*-2 positions.

Other Algal Groups

Long-chain PUFAs are found in diatoms, where, for example, in *Skeletonema costatum*, eicosapentaenoic acid (EPA, C20:5) is found in addition to 16:3 and 16:4 fatty acids (Berge et al., 1995). EPA is located primarily in the polar lipids, whereas docosahexaenoic acid (DHA, C22:4) is concentrated in phospholipids. Another diatom, *Phaeodactylum tricornutum*, contains EPA and hexadecenoic acid as major fatty acids (Tonon et al., 2002). A recent study (Vieler et al., 2007), although unable to discriminate between the two fatty acids bound to the individual lipid classes, provided a detailed analysis of the fatty acid composition of the different lipids in the diatom *C. meneghiniana*. In this alga, PC is composed of 32:4, 36:8 and 38:9, whereas SQDG contains fatty acids of shorter length and consists of 30:0 and 32:1. MGDG is composed of 32:6 and 36:8 and several minor MGDG species. DGDG is not as heterogeneous as MGDG and consists of 36:7 and small amounts of 32:2. Red algae also accumulate large amounts of EPA, accompanied by arachidonic acid (AA, C20:4; Shiran et al., 1996), which is also a typical long-chain fatty acid of the brown algae. In the red alga, C16 and C18 polyunsaturated fatty acids of MGDG and DGDG are replaced by EPA or AA. As red algae, such as *Porphyridium cruentum*, use both the prokaryotic and eukaryotic pathways of galactolipid synthesis, either MGDG/DGDG with EPA or AA at the *sn*-1 position and C16 at the *sn*-2 position, or MGDG/DGDG with EPA or AA at both positions exist (Khozin et al., 1997).

3 Unusual Lipids

Green algae contain unusual hydrocarbons and ether lipids (Metzger and Rager, 2002) in addition to algaenan (Allard and Templier, 2000), an insoluble cell wall component. Algaenan is composed of very long-chain fatty acids, that is, C30 to C34 mono- and diunsaturated fatty acids and may provide protection against different pathogens, such as bacteria or algal viruses. Long-chain alkenones (C35-C40) have been detected in the haptophyte *Chrysotila lamellose* (Rontani et al., 2004).

Halogenated fatty acids play an important role in different algal groups, mainly in the marine red and brown algae (Hewson and Hager, 1980; Dembitsky and Srebnik, 2002). Chlorosulfolipids can be found in a great number of species, including both freshwater and marine green, red and brown algae. They can accumulate to significant concentrations based on the total lipid content, and represent one of the major lipid components in the chrysophyte alga *Ochromonas danica* (Dembitsky and Srebnik, 2002). Brominated fatty acids have been isolated from various red algae (Hewson and Hager, 1980), where they represent a significant fraction of the total fatty acids. Red algae may also contain iodinated acetic and acrylic acids. Halogenated fatty acids are thought to provide protection against pathogen attack due to their antiviral and antifungal properties (Dembitsky and Srebnik, 2002). The enzymes involved in halogenation are described below in Section III.A.1.

Additional unusual lipids that can be found in Euglenophyta, Rhodophyta and diatoms include unusual sulfur-containing lipids, such as phosphatidylsulfochinoline in *Euglena gracilis* (Harwood and Jones, 1989). Different species of brown algae contain a novel phospholipid, phosphatidylhydroxyethylglycine, which contains AA and EPA as fatty acid residues (Eichenberger et al., 1995).

4 Oxylipins

Hydroxylated fatty acids and oxygenated derivatives of fatty acids are termed oxylipins. Oxylipins have been found in a variety of macrophytic algae belonging to the groups Chlorophyta, Rhodophyta and Phaeophyceae (for reviews, see Gerwick, 1994; Guschina and Harwood, 2006). Work in recent years has also concentrated on oxygenated fatty acids from diatoms, and several oxylipins have been isolated and characterized. Oxylipins are also found in vascular plants that use mainly linoleic and linolenic acid, as precursors that are metabolized at the C-9 and C-13 positions (Blee, 1998). A similar pattern of oxylipin production has been observed in green macrophytic algae (Bernart et al., 1993). Brown algae also use C18 fatty acids in addition to C20 fatty acids as precursors (Proteau and Gerwick, 1992), whereas the red algae metabolize mainly C20 fatty acids (Jiang and Gerwick, 1991). In red algae, introduction of oxygen takes place via a 12-lipoxygenase, and in the brown algae,

lipoxygenases normally show specificity for C6 (Jiang and Gerwick, 1991; Proteau and Gerwick, 1992). Oxylipins were first discovered in the Rhodophyta, and many different oxylipin species have been described. *Gracilariopsis lemaneiformis*, for example, synthesizes 13 different oxylipin compounds from 18C and 20C fatty acids via lipoxygenase activity at C8, C9 and C12 (Jiang and Gerwick, 1991). The oxylipins include C18 and C20 hydroxy and dihydroxy acids, which contain diene and keto-diene groups. The oxygenated fatty acids are also incorporated as acyl groups into MGDG and DGDG of *G. lemaneiformis*. Other red algal species contain oxylipins of the eicosanoid family, while in the red alga *Rhodymenia pertusa*, a 5-lipoxygenase introduces oxygen into both AA and EPA (Jiang et al., 2000). Additional red algal oxylipins have been isolated from the calcareous *Lithothamnion corallioides* (Guerriero et al., 1990). Interestingly, the tropical red alga *Polycavernosa tsudai* contains macrolide disaccharides whose macrolide portions show structural similarity to oxylipins (Yotsu-Yamashita et al., 1993). These macrolide disaccharides have been shown to be of high toxicity. Synthesis of jasmonic acid and prostaglandins has also been reported for red algal species. Jasmonic acid production was found in the macrophyte *Gelidium latifolium* (Krupina and Date, 1991), and prostaglandins are formed by *Gracilaria verrucosa* (Gregson et al., 1979). With respect to the function of oxylipins in red algae, there is preliminary evidence to suggest that they may play roles comparable to those in the metabolism of mammals, where they are responsible for cellular communication, innate immunity and cell homeostasis. A role of oxylipins in the defense mechanisms of *Chondrus crispus* has been described (Bouarab et al., 2004). Brown algal oxylipins include ether oxylipins, which are, for example, synthesized by the intertidal kelp *Cymathere triplicata* (Proteau and Gerwick, 1992) and hydroxy acids, divinyl ethers and aldehydes formed by different *Laminaria* species (Proteau and Gerwick, 1993; Boonprab et al., 2003). Divinyl ethers, hydroxy acids and aldehydes are formed from C18 and C20 fatty acids through the action of a C6 lipoxygenase, which leads to the production of hydroperoxides that are later either reduced or converted to the respective divinyl ethers. In brown algae, oxylipins also play an important role as precursors in the synthesis of gamete-attracting substances, such as ectocarpene (Stratman et al., 1993). Several species, including *Ectocarpus siliculosus* and *Sphacelaria rigidula*, use AA or EPA for the synthesis of the C11-pheromones via the formation of oxylipins as intermediate products in the biosynthesis pathway. Oxylipins in brown algae may also act as bioactive substances, a function that has also been described for oxylipins from diatoms (Adolph et al., 2004; Ribalet et al., 2007, 2008; Taylor et al., 2007). Diatoms synthesize bioactive aldehydes from 16C PUFAs or EPA, which are liberated from glycolipids and oxygenated by the action of lipoxygenases (Pohnert, 2002; D'Ippolito et al., 2004). Diatom cytotoxic oxylipins were first described for *Thalassiosira rotula* (Miralto et al., 1999). Several different oxylipins, such as 2,4-heptadienal, 2,4-octadienal, 2,4,7-octatrienal, 2,4-decadienal, 2,4,7-decatrienal and oxoacids with an aldehyde structure similar to oxylipins have been described in parallel for both marine and freshwater diatoms (Pohnert, 2005; Wichard and Pohnert, 2006). The diatom oxylipins exhibit activity against phytoplankton (Casotti et al., 2005), fungi (Adolph et al., 2004) and have a detrimental effect on egg hatching success and cell division of marine invertebrate embryos (Ianora et al., 2006). Recent studies have shown that, among the diatom oxylipins, 2,4-decadienal is the most toxic product for the marine copepod *Tisbe holothurie* (Taylor et al., 2007). The effects of oxylipins on bacteria are also under investigation. While former studies have reported that diatom oxylipins reduce the growth of pathogenic and non-marine bacteria (Bisignano et al., 2001; Adolph et al., 2004), a recent publication describing the effects of decadienal, octadienal and heptadienal on 33 species of marine bacteria has offered a more detailed view (Ribalet et al., 2008). In this study, a concentration-dependent reduction of growth was observed for 16 bacterial strains, while 12 species were unaffected and two strains even showed a stimulation of growth. Bacterial strains that were isolated from a bloom of the oxylipin-producing diatom *Skeletonema marinoi* exhibited remarkable resistance against oxylipin exposure. Diatom aldehydes have also been described as chemical signals during stress or unfavorable environmental conditions (Casotti et al., 2005). The production of oxylipins in diatoms is not restricted

to marine species, but can also be found in the freshwater diatoms as a response to abiotic stress. Oxylipins are also present in haptophytes, where a recent study revealed the presence of the toxic 2,4-decadienal in the phytoplankton *Phaeocystis pouchetii* (Hansen et al., 2004).

B Effects of Abiotic Stress on the Lipid Composition

Marine and freshwater algae can experience drastic changes in their environmental temperature, light intensity and nutrient supply. This holds true for the macrophytic algae, but presents an even greater challenge for the species belonging to the phytoplankter. In general, phosphate limitation leads to changes in the ratio of membrane phospolipids to glycolipids. Under low phosphate concentrations, an increase in the content of glycolipids and betaine lipids can be observed, accompanied by a concomitant decrease of phospholipid levels. Phosphate limitation may also affect the fatty acid composition and can lead to a higher degree of unsaturated fatty acids, as observed in the green alga *Chlorella kessleri* (El-Sheek and Rady, 1995). Sulphate limitation also leads to an imbalance in the membrane lipid composition, because SQDG levels are strongly reduced under these conditions. To compensate for the loss of the anionic lipid, a complementary increase in PG occurs, and maintains constant levels of PG and SQDG (Sato et al., 2000). Changes in the composition of anionic sulfolipids and phospholipids that occur in different algae subjected to either sulphate or phosphate limitation were first described for vascular plants (Essigmann et al., 1998) and bacteria (Benning et al., 1993; Güler et al., 1996). Studies on *C. reinhardtii*, *Arabidopsis thaliana* and *Synechocystis* sp. PCC6803 mutants have provided the information that PG plays an important role in the assembly of trimeric PS I in the cyanobacteria (Sato et al., 2004) and in the dimerization of PS II (Gombos et al., 2002; Domonkos et al., 2008). SQDG is required for the functionality of the PS II donor side (Minoda et al., 2003) and PS II dimerization (Loll et al., 2007; see also Section II. C. 1). At present, however, it is not clear what role the anionic SQDG plays in the organization of the thylakoid membrane structure, but its high concentration in diatom membranes and the need for a compensation of SQDG losses suggest that it is important not only for the function of the PS II donor side, but also for the provision of larger membrane domains exhibiting a negative surface charge (see also Apostolova et al., 2008). Nitrogen starvation leads to a significant increase in the total lipid content of the cells, as observed in the green algae *Chlorella vulgaris* and *Scenedesmus obliquus*, which both accumulate high levels of triglycerides containing mainly 16:0 and 18:1 fatty acids (Piorreck et al., 1984). The excessive lipids accumulated under nitrogen starvation can be regarded as storage lipids, which remain in the cell until environmental conditions are again favorable for further cell division. In a recent study, the diatom *P. tricornutum* was investigated under two light climates in a nitrogen-limited culture medium (Jakob et al., 2007). In both cultures, grown under fluctuating and non-fluctuating light conditions, nitrogen limitation led to a strong reduction of the overall protein content of the cells, accompanied by concomitant increases in the carbohydrate content. The overall lipid concentration in the *P. tricornutum* cells was, however, unaffected by the nitrogen starvation. It also contributed to a comparable extent to the complete biomass of the cells grown under fluctuating and non-fluctuating light conditions. Temperature effects on the lipid and fatty acid composition have been studied in various microalgae from all major groups. Although it is generally believed that a decrease in the temperature leads to a higher degree of fatty acid unsaturation in order to keep the membranes in a fluid state, the observed changes are often more complex. Algal species that react with changes in the degree of fatty acid unsaturation include *Chlorella vulgaris*, in which a decrease in the content of polyunsaturated fatty acids was observed at elevated temperatures (Sushchik et al., 2003), and the diatom *Chaetocerus* sp., whose main lipid EPA content decreases with a concomitant increase of the temperature (Renaud et al., 2002). Ice algae, which are adapted to extreme temperatures, are dominated by different diatom species. The lipid composition of the diatom species belonging to the ice algae (*Nitzschia frigida* and *Melosira arctica*) is comparable to other diatoms, although higher amounts of triacylglycerols are generally found in the ice algae. In *Nitzschia frigida*, for example, it has been shown that the neutral lipids

dominate over glycolipids and phospholipids (Henderson et al., 1998). In spring samples, the major fatty acids of the neutral lipid fraction are 16:0, 16:1 and 20:5, while the glycolipid fraction contains high levels of 20:5 and 16:4 unsaturated fatty acids. The phospholipids of *N. frigida* are characterized by higher levels of 22:6 compared to the other lipid fractions, but contain 20:5 fatty acids as their main PUFA. Autumn samples show a greater degree of saturation in the neutral lipids, but are otherwise comparable to the spring samples. The diatom *Melosira arctica* seems to contain reduced amounts of triacylglycerols compared with *N. frigida*, reflected in the fatty acid composition as a higher concentration of C16 PUFAs and EPA, which are components of the polar lipids (Falk-Petersen et al., 1998). Ice algae have also been used to study the effects of different light intensities on the lipid composition. Here, it has been shown for the diatom species *Fragilariopsis curta*, *Navicula gelida* and *Nitzschia medioconstricta* that low light illumination in combination with low temperatures leads to a significant increase of EPA bound to plastidic MGDG, reflecting an increase in the MGDG concentration in relation to other lipids of the thylakoid membrane (Mock and Kroon, 2002). It has been hypothesized that, in the presence of increased concentrations of membrane proteins, fewer bilayer lipids (i.e., DGDG) are needed to stabilize the bilayer structure of the membrane. Various other studies analyzing the effects of different intensities of light during growth on the lipid composition of algae have been performed, and have included algae of all major groups. In general, high intensities of light during growth lead to an accumulation of triacylglycerol (TAG), which contains higher levels of saturated fatty acids. Low light intensities, on the other hand, induce an increase in the content of polar lipids (MGDG and DGDG) accompanied by a concomitant increase in the PUFA content. These increases are, however, due to a proliferation of thylakoid membranes in reaction to the limiting light conditions and are therefore not directly comparable to the stress-induced accumulation of TAGs. Increased TAG levels under high light conditions and increased amounts of thylakoid membrane lipids under low light illumination have been shown for green algae (*Cladophora* sp.), for diatoms (*Thalasiosira pseudonana*) and for brown and red algae (Napolitano, 1994; Brown et al., 1996).

C Functions

1 Lipids as Structural Components of the Photosystems

Recent advances in the elucidation of the molecular structures of both PS I and PS II have pointed to an important role of membrane lipids in the establishment and maintenance of the higher order structures of the photosynthetic pigment protein complexes (for details, see also Chapter 10). In the PS II of the cyanobacterium *Thermosynechococcus elongatus*, lipids have three different functions (Loll et al., 2007). First, they form a lipid belt around the D1 and D2 protein that provides a flexible environment for the exchange of the D1 protein during its turnover and repair cycle. The lipids involved in this lipid belt are the thylakoid membrane lipids MGDG, DGDG, SQDG and PG. Second, three molecules of lipids (one molecule of MGDG and two molecules of SQDG) are present at the dimerization interface, where they mediate the interaction of the PS II monomers and help to establish the dimer conformation that represents the native conformation of PS II in the thylakoid membrane. Third, several lipids are seen in close proximity to the binding pocket of Q_B at PS II, where they form a diffusion pocket for Q_B. This is thought to facilitate the binding/detachment of Q_B at PS II and the diffusion to and exchange with the membrane located plastoquinone pool. PS I of *T. elongatus* contains four lipid molecules per PS I trimer (three molecules of PG and 1 molecule of MGDG) that are likely to support the electron transfer steps located at the PS I core complex (Jones, 2007). PG, which is located at the monomer–monomer interface, may play a role in the oligomerization of PS I and in the stabilization of the oligomers. In vascular plants, it is suggested that PG plays a similar role in the dimerization of PS II (Kruse et al., 2000). Although the information regarding the roles of lipids in the PS structures stems from data on cyanobacterial reaction centers and antennae, it is reasonable to believe that lipids in algae serve comparable functions with respect to the diffusion of plastoquinone, the turnover of D1 protein and the oligomerization of PS I and PS II.

This assumption is also supported by a comparison of the PS II structures of the cyanobacterium *Synechococcus elongatus*, the green alga *C. reinhardtii* and spinach (Nield et al., 2000), which showed great similarity related to overall shape and size of the dimeric PS II core complexes. However, the lipid composition of the different photosystems II varies such that different lipids may serve the functions described in detail above. Crystallization of photosynthetic membrane proteins from different algae, in conjunction with x-ray crystallography or electron microscopy, needs to be performed to analyze the structural role of lipids in algal photosystems. However, some data on the role of lipids in the establishment of membrane protein structure exist in algae. In *C. reinhardtii*, it has been shown that lipids are essential for the reconstitution and folding of LHCII and that the trimerization of the LHCII depends on the presence of PG (Garnier et al., 1990; Dubertret et al., 1994). This is consistent with recent data on the molecular structure of the spinach LHCII, where it was shown that PG is located at the monomer–monomer interface and mediates the LCHII trimerization (Liu et al., 2004). For the alga *Dunaliella salina* it has also been found that the plastidic ATP synthase is enriched in bound SQDG (Pick et al. 1985). Future measurements may also reveal whether the significant differences in the lipid composition of vascular plants and diatoms are responsible for the differences in their thylakoid membrane arrangement. Diatom thylakoid membranes are arranged in stacks of three and are only loosely appressed (Pyszniak and Gibbs, 1992), whereas the thylakoids of vascular plants exhibit high heterogeneity with a differentiation into grana and stroma membranes. Interestingly, in vascular plants, the LHCII, which is the driving force in the process of grana stacking, interacts with MGDG. MGDG, which forms inverted hexagonal structures in aqueous solutions (Fig. 2), is forced into a bilayer structure by this interaction (Simidjiev et al., 2000). At present, nothing is known about a possible interaction between diatom antenna complexes, the fucoxanthin–chlorophyll-binding proteins, and lipids, but the significantly higher concentration of the bilayer-forming lipid SQDG in thylakoid membranes of diatoms (Vieler et al., 2007) points to alterations in the lipid–protein interplay. Future measurements will be required to reveal how bilayer and inverted hexagonal phase-forming lipids interact with the respective membrane proteins in different algae and how this affects the three-dimensional structure of the thylakoid membrane.

2 Role of Lipids in Xanthophyll Cycling

The xanthophyll cycles of vascular plants and algae are important photoprotective mechanisms that prevent the overexcitation of PS II under conditions of high light illumination (for review see the book by Demmig-Adams et al., 2006). Two different xanthophyll cycles exist in the different algal groups: the violaxanthin cycle of green and brown algae, which is also found in vascular plants, and the diadinoxanthin cycle of the algal classes Bacillariophyceae, Chrysophyceae, Xanthophyceae, Haptophyceae and Dinophyceae (for a review see Wilhelm et al., 2006). The de-epoxidation reactions of the diadinoxanthin and the violaxanthin cycle, which lead to the photoprotective pigments diatoxanthin and zeaxanthin, are catalyzed by the enzymes diadinoxantin de-epoxidase (DDE) and violaxanthin de-epoxidase (VDE), respectively. Both de-epoxidases require the presence of the major thylakoid membrane lipid MGDG for activity. MGDG is needed for the solubilization of the hydrophobic substrates of the de-epoxidases, that is, diadinoxanthin and violaxanthin (Goss et al., 2005, 2007). Due to greatly increased solubility of diadinoxanthin in MGDG compared with violaxanthin, the MGDG concentrations for optimal DDE activity are much lower than those needed for full activation of VDE. In addition to its role in pigment solubilization, MGDG provides another essential feature needed for xanthophyll de-epoxidation. MGDG belongs to the so-called non-bilayer lipids, which, due to their small head-group area and critical packing parameter value higher than one, form inverted hexagonal structures (H_{II} phases) in an aqueous medium (Fig. 2). These three-dimensional structures are required for efficient violaxanthin and diadinoxanthin de-epoxidation (Latowski et al., 2002, 2004; Goss et al., 2005, 2007). It has been proposed that the H_{II} phases enable the binding of VDE and DDE to the thylakoid membrane after the pH-dependent activation of the enzymes has taken place. Although the existence of

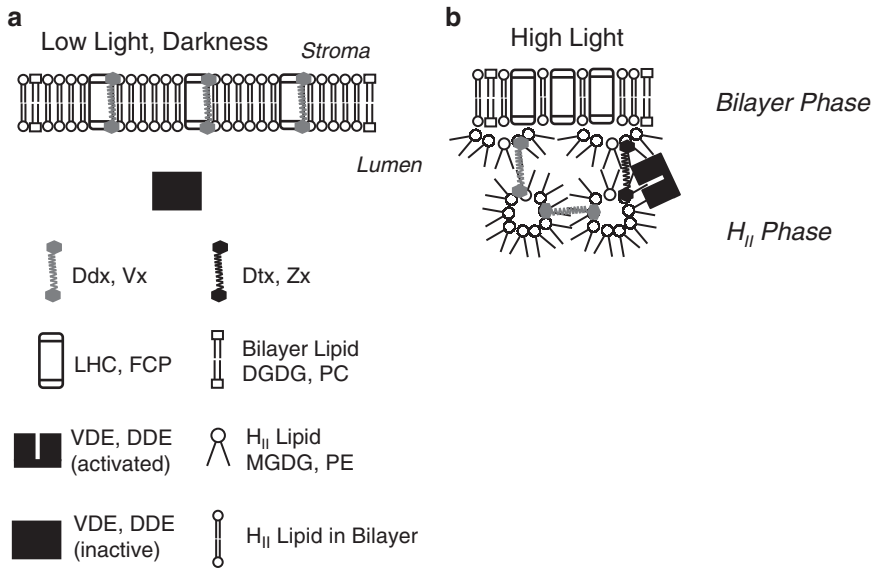

Fig. 2. Model depicting the lipid arrangement of the thylakoid membrane during low light or darkness (a) and high light illumination (b). During low-light illumination or darkness MGDG, due to its interaction with the light-harvesting complexes (LHC), is forced into a membrane bilayer structure. High-light illumination leads to an aggregation of LHC and to a sequestering of MGDG from the membrane bilayer. Due to its overall conical shape MGDG forms inverted hexagonal phases in the aqueous medium of the thylakoid lumen. DGDG, which remains in the membrane, has a cylindrical shape and forms bilayer structures (for a more detailed view on the shape-structure concept of lipids refer to Van den Brink-van der Laan et al., 2004). The H_{II} phases, which stay in direct contact with the membrane bilayer, are the lipid phases where the xanthophyll cycle is taking place. They have a high capacity for diadinoxanthin (Ddx) and violaxanthin (Vx) solubilization and provide the docking site for the xanthophyll cycle enzymes Ddx and Vx de-epoxidase (DDE, VDE). In bilayer phases both pigment solubility and enzyme activity are, on the other hand, severely restricted.

MGDG-induced H_{II} phases in thylakoid membranes has been indicated by various experimental methods (Gounaris et al., 1983; Haranczyk et al., 1995), their precise arrangement is still under debate. It has been suggested that the non-bilayer lipids are forced into a bilayer structure in the native thylakoid membrane due to interactions with the main light-harvesting complex of photosystem II, the LHC II (Simidjiev et al., 2000). Under certain conditions, such as structural rearrangements of proteins, non-bilayer lipids might be sequestered from the membrane and form H_{II} phases that are attached to the surface of the thylakoid bilayer (Garab et al., 2000). These inverted hexagonal phases, which would provide the docking sites for DDE and VDE, may stay in direct contact with the bilayer, so that rapid and direct interactions between the two lipid phases are possible (Fig. 2). A recent study employing different phospholipids with different three-dimensional structures has added new information to the topic of the lipid dependence of xanthophyll de-epoxidation (Vieler et al., 2008). In this study, it was shown that the phase transition from the gel (L_β) to the liquid crystalline phase (L_α) strongly enhances both the solubilization of the xanthophyll cycle pigments and the activity of the respective xanthophyll de-epoxidases. At higher temperatures, the L_α phase provides the ideal environment for enzyme activity, whereas at lower temperatures, the H_{II} phase is better suited to maintain a high level of de-epoxidase activity.

III Lipids in Lichens

Lichens consist of a symbiotic association between a fungal partner called mycobiont, which is generally a representative of the ascomycetes, and an alga called photobiont, which belongs either to the eukaryotic green algae or to the

procaryotic cyanobacteria. The symbiotic interaction between partners leads not only to a completely different morphology of the mycobiont thallus, but also to different biosynthetic capacity. Most lichens contain secondary products that cannot be synthesized by the fungal or algal partner alone (Ahmadjijan, 1993 and Cordereiro et al., 2004). However, many studies have been performed using isolated mycobionts, and provide evidence for an intimate metabolic relationship, not only between the partners but also between metabolic pathways. A recent comparison of the fatty acid composition of the separately cultivated symbionts of the lichen *Teloschistes flavicans* showed significant differences between the symbionts and the intact lichen grown under similar conditions (Reis et al., 2005). This suggests that the lipid metabolism in both partners is under the influence of the symbiosis. In addition, it has been shown that the synthesis of phenolic secondary metabolites is closely linked to fatty acid metabolism. The enormous demand in NADH of the fatty acid synthase is covered by the glycolytic degradation of sugars (Molina et al., 2003).

A better understanding of this metabolic network is of high commercial and scientific interest for several reasons.

- Lichens can survive in extreme environments. Their adaptation potential to extreme temperature and drought reflects a unique potential to adjust the lipid metabolism to these conditions. Because lipid metabolism is suggested to be the crucial key factor determining plant survival under high temperature and drought (Piervittori et al., 1994), lichens are used as model organisms to understand the biochemical strategies to cope with these conditions.
- Lichens contain secondary products that have powerful protective potential against oxidative stress. Alcoholic extracts of lichens can prevent the outbreak of skin cancer and can inhibit biochemical reactions known be involved in signal transduction leading to uncontrolled cell division. Recent research has revealed evidence that phenolic compounds are the main active compounds; however, their biological efficiency can be modulated by the biochemical structure of the lipids where the phenolic compounds are embedded.

Lipids of lichens have attracted scientific interest since the early 1980s. Dembitsky (1992) summarized the results obtained from GC/MS analysis of lipid fractions from a wide variety of lichen species. Here, we concentrate our efforts on providing an update to this information, reporting on new lipids isolated from lichens and new biochemical mechanisms or enzymes involved in lipid modification.

A Biochemistry

1 Halogenated Lipids

Lichens produce a wide variety of halogenated lipids (Hunek and Yoshimura, 1996; Dembitsky and Srebnik, 2002). Most are brominated C18 fatty acid methylesters with different numbers of conjugated double or triple bonds carrying one or two bromine residues at the end of the fatty acid chain. They have been isolated from the aerophytic lichens *Cladonia fucata*, *Lacanor fructulosa*, *Parmelia linctina and P. comtseliadalis* (Rezanka and Dembitsky, 1999a). The contents of these brominated lipids in lichens are relatively low, ranging from 10 to 400 μg g^{-1} dry weight (Rezanka and Dembitsky, 1999b). Brominated acetylenic acids and their methylesters possess unique biological activities as there are cytotoxic, ichthyotoxic and antimicrobial potentials due to their ability to block H or K-ATPases and the HIV protease (see Ichiba et al., 1994). Biohalogenation is catalyzed by the activity of haloperoxidases, which have been isolated from many organisms. Plat et al. (1987) reported that bromoperoxidase from the lichen *Xanthoria parietina* catalyses the bromination of an organic compound in the presence of a peroxide, such as H_2O_2. Haloperoxidases have been intensively studied as a result of their technical interest. The gene for the bromoperoxidase has been cloned and sequenced from the red alga *Corallina officinalis* (Coupe et al., 2007) and the crystal structure has been resolved with 2 Å resolution (Isupov et al., 2004; Littlechild and Garcia-Rodriguez, 2003). In the brown alga *Laminaria digitata*, it was shown that the gene for the bromoperoxidase is highly expressed under stress conditions (Roeder et al., 2005). Manley and Barbero (2001) showed that the formation of halogenated products is directly linked to photosynthesis. Haloperoxidases depend on the presence of relatively high internal concentrations of hydrogen peroxide, which is formed by the action of the superoxide dismutase that converts the PS I-generated superoxide anion into hydrogenperoxide.

Most haloperoxidases have broad specificity to substrates. They can catalyze not only halogenation, but also sulfoxidation (Ten Brink et al.)

2 Betaine Lipids

In contrast to vascular plants, lichens synthesize a betaine lipid DGTS (Künzler and Eichenberger, 1997), as do ferns and mosses. Diatoms and brown algae synthesize another betaine lipid DGTA (Müller and Eichenberger, 1994). By contrast, Chrysophytes and Cryptophytes synthesize both DGTA and DGTS. Thus, the composition of betaine lipids has been proposed to serve as taxonomic marker (Müller and Eichenberger, 1994). DGTS shares structural similarities with PC. Because the content in the bilayer-forming PC can be extremely low when DGTS is present, it has been hypothesized that it may replace it under certain conditions.

3 New Galactolipids

Lichens behave in a very unusual way with respect to galactolipids. MGDG and DGDG, which are essential for the function of photosynthetic membranes in most photosynthetic organisms, are absent in *Pamelia saxatilis* and *Evernia prunasti*, although both lichens are photosynthetically active (Derten et al., 1977). This is in accordance with more recent data that, in lichens, the proportion of glycolipids to the total lipids is far below that known for other photosynthetic organisms. In most cases, the percentage of glycolipids to total lipids varies between 30% and 8% in the case of a Peltigera species (Dembitsky, 1992). The sugars bound to glycerol are mainly glucose, galactose and mannose, and pentoses have also been identified in a few species (Solberg, 1970). Surprisingly, the cyanobacterial photobiont Sytonema of the lichen *Dictyonema glabratum* can synthesize a glycolipid with β-galactofuranose, which is normally restricted to fungal cells (Sassaki et al.). From the fungal partner of this lichen, the glycolipid O-α-D-Galp-(1→6)-O-α-D-Galp-(1→6)-O-β-D-Galp-(1↔1)-2- and -3-monoacyl-D-glycerol was isolated, and its chemical structure was observed that has not been previously found in nature (Sassaki et al., 2001). It is also interesting to note that the fatty acid components of glycolipids from lichens are extremely highly unsaturated (up to 80%) and that molecules with a chain length above 20 C-atoms can reach values of 90% (Derten et al., 1977).

4 n-Alkanes

Lichens can survive in extreme hot and dry environments. To avoid water loss, the surface of lichens can be extremely hydrophobic. *n*-Alkanes are found on the surface of lichens and of fungi with chain lengths between 8 and 30 C-atoms. Some photobionts (e.g., Trebouxia) are also found in nature without fungal partners as aerophytic alga forming green mats or biofilms. Here again, the surface can be highly waterproof due to the existence of *n*-alkanes. Torres et al. (2003) showed that the amount of *n*-alkanes depends on the environmental conditions, and that the mycobiont can accumulate *n*-alkanes up to a concentration of 200 mg g^{-1} dry weight. The enzymes responsible for the synthesis of these *n*-alkanes are not known, and a much better understanding of chain elongation and metabolic flux regulation from photosynthates into *n*-alkanes would be highly desirable in the context of biofuels.

B Functions

1 Lipids under Dehydration

Due to their extreme tolerance against dehydration, lichens have been intensively analyzed with respect to changes in the lipid composition during de- and rehydration. From these experiments, data have shown that extrachloroplastic lipids respond more strongly to changes in the water content than the lipids involved in photosynthesis. The glycolipids of the phycobiont remain relatively constant during dehydration and increase when rehydration begins. By contrast, the de novo synthesis of neutral lipids is decreased after long-term dehydration. These data suggest that drought tolerance depends primarily on the phycobiont, and, to a far lesser extent, on the mycobiont (Kotlova and Sinyutina 2005). It is well known that lichens can restore respiration activity within a few seconds, and photosynthesis within minutes, even after extreme desiccation. The mechanism to protect the membrane lipids against degradation and to preserve the lipid–protein organization seems to be the accumulation of compatible solutes, such

as polyols (Aubert et al., 2007). A crucial parameter for dehydration resistance is the capacity to seal disrupted membranes, which may result from lipid peroxidation and de-esterification. It has been observed that an increase in the precursors of PC is due to the reorganization of the structural integrity of the tonoplast and plasmalemma membranes (van der Rest et al., 2002).

2 Air Pollution and Lipid Composition

Lichens are often used as bioindicators for air pollution (Seaward, 2004). Transplantation experiments from clear air regions to polluted areas always lead to the observation that the photosynthetic membranes degraded, and the cytoplasmic membrane became leaky, followed by severe losses of potassium ions (Garty et al., 2000). The underlying mechanism is that air pollutants, that is, heavy metals, ozone, or sulphoxide, induce oxidative stress, which degrades the membrane lipids if the antioxidant potential is exhausted (Deltoro et al., 1999; Weissman et al., 2006). The effect of fumigation on the biochemistry of lipids is not yet well understood. Due to the different fumigation resistance of the species, lipids respond heterogeneously with respect to relative changes in the ratio of phospholipids to galacto- and neutral lipids. However, a general increase in fatty-acid unsaturation has been observed in response to SO_2 exposure, and is probably significant for adaptation.

3 Other Functions

Many bioactive metabolites have been isolated from lichens. Most are very hydrophobic and soluble only in strong organic solvents. *Myelochrea leucotyliza* can, for example, produce lipid bodies that fill the entire inner space of the thallus with a contribution of 30% to the total dry weight (Arakawa-Kobayashi et al., 2004). The lipid is formed solely by the fungal partner and is excreted by an unknown mechanism. It has been identified to be atronorin, which belongs to the polyketides and is synthesized in the cytosol by the polyketide synthase. Since this enzyme plays a crucial role in the synthesis of many lichen-derived bioactive molecules that act as antioxidants or anti-inflammatory agents, its gene has been recently cloned and sequenced from the lichen *Xanthoparmelian semiviridis* (Chooi et al., 2008). Phenolic fractions from lichens can act as ecologically important infochemicals. Mixtures containing antranol and antronorin can inhibit seed germination. Despite their hydrophobic nature, the compounds can be washed out by rainwater and accumulate in the cell walls of seeds, where they can resist microbial degradation and act as allelochemicals. Because the efficiency of these extracts differs, when the lichens are harvested in winter or summer, there is some indication that the biosynthesis is under active metabolic control (Toledo-Marante et al., 2003).

IV Lipids in Mosses

Mosses have attracted new scientific interest by biochemists and molecular biologists since the complete genome sequence of *Physcomitrella patens* has been published. Here, mosses are assumed to represent the transition from the aquatic environment to land life; thus, metabolic pathways that are essential for survival under stress conditions on land, that is, short-term variations in temperature and hydration, had been acquired at this stage (Rensing et al., 2007). Because the molecular mechanisms to respond to these new conditions of land life gave rise to the development of plant hormones, mosses show responsiveness similar to vascular plants to auxin, cytokinin and abscisic acid. However, mosses allow easy accessibility of living cells to direct observation and manipulation because of their simplified developmental stages, like the haploid filamentuous protonema stage, which can be easily genetically modified and grown in large photo-bioreactors (Cove et al., 2006). This ability is of even greater interest for lipid biochemistry, as bryophytes synthesize polyunsaturated long chain fatty acids (PUFA) up to a chain length of 26 carbon atoms. Hartmann et al. (1986) have shown that protonemata from *Leptobryum pyriforme* contains up to 20% AA and 7% of EPA acids of total lipids. Therefore, lipid research on bryophytes has concentrated on three different aspects:

- Widening the spectrum of PUFA species synthesized from bryophytes and optimization of the culture conditions to establish high yield production (Hansen and Rossi, 1991; Chiou et al., 2001)

- Cloning and sequencing of genes involved in the synthesis of PUFAs (Zank et al., 2002a)
- Understanding the metabolic regulation of those genes, which encode key enzymes in PUFA synthesis (Zank et al., 2002a)

A New Polyunsaturated Fatty Acids from Bryophytes

In addition to the major constituents of PUFAs, AA and EPA, many other fatty acids have been isolated from mosses. In neutral, as well as in glycolipid and the phospholipid fractions, long chain fatty acids with chain lengths up to 26 carbon atoms could be found. The degree of unsaturation ranged from monoenoic (mainly in position 2) to dienoic (in the positions 3, 4 or 5) fatty acids, including species with one additional triple bond at various positions in both the monoenoic and the dienoic fraction. The production of acetylenic fatty acids in mosses has been described by Kohn et al. (1994). Interestingly, acetylenic PUFAs were not found in glycolipids (Dembitsky et al., 1993a). The same author found that 72% of the fatty acids in the neutral fraction from the moss *Dicranum polysetum* was acetylenic, and very unusual fatty acids were identified, for example., 9a,12–18:2, 6a9,12,15–18:4, 8a11,14–20:3 and finally 5a8,11,14–20:4 (Dembitsky et al., 1993b). Recently, Stumpe et al. (2006) showed that the moss *Physcomitrella* produces oxylipins from its PUFA reservoir leading to metabolites typical for plants, algae, animals and fungi. *Physcomitrella* uses AA as a starter substance for a wide variety of metabolites that might be responsible for the mosses' resistance to herbivores and pathogens.

B New Enzymes for Bryophyte Lipid Metabolisms

A Δ6-acyl-group desaturase has been identified by Girke et al. (1998) by targeted gene disruption. The corresponding enzyme, which catalyzes the formation of triple bonds, has been described as a bio-functional acetylenase/desaturase by Sperling et al. (2000). Later, Zank et al. (2002b) isolated and characterized the PSE1 gene from *Physcomitrella patens* that encodes an enzyme, which catalyzes the Δ6-elongation and Δ5-desaturation; this sequence of reaction converts polyunsaturated C18 fatty acids to C20 PUFAs. Interestingly, expression of this gene in *S. cerevisiae* led to the formation of C20 PUFAs from exogenously added polyunsaturated C18 fatty acids, providing clear evidence that transgenic cells can be used to produce long chain polyunsaturated fatty acids by a biotechnological approach. Mutants of *P. patens* where the PSE1 gene was inactivated did not show any phenotype that might indicate that PUFAs are not essential for moss development under fermenter conditions. From the same species, Kaewsuwan et al. (2006) identified a Δ5−desaturase gene that catalyzes the reaction from di-homo-linolenic acid to AA and from ω-6 eisosadienoic acid to ω-3 EPA, respectively. Inactivation of this gene led to a severe disturbance of the fatty acid composition in the mutant. The scheme of Fig. 3 shows which fatty acids are synthesized in this moss and which enzymes are needed.

A very unique lipid-metabolizing plastid-targeted enzyme was identified in an EST library from *Physcomitrella patens*. It showed high sequence homology to type 2 lipoxygenase from plants. However, the substrate specificity was very unusual: the preferential substrates were AA and EPA acids, with the enzyme exhibiting a high activity as hydroperoxidase and fatty acid chain cleaving lyase. Therefore, this enzyme can produce a relatively complex set of different derivatives of AA and EPA, which can be used in parallel for signaling and pathogen defense (Senger et al., 2005).

C Lipid Metabolism under Stress

Temperature acclimation of mosses induces changes in the degree of fatty-acid unsaturation and the length of fatty-acyl chain in a complex manner, with diverse reaction patterns in different moss species (Koskimies and Nyberg, 1991). However, the situation is much clearer for drought stress. Under desiccation conditions, most vascular plants decrease the content in glyco- and phospholipids, whereas the content of neutral lipids is increased. This is consistent with result of the gene expression that shows that the m-RNAs for phospholipase D and galactolipase are increased (El-Hafid et al., 1989; El Maarouf et al., 1999). In addition, the degree of fatty-acid unsaturation was found to be reduced (Pham Thi et al., 1987). This pattern was also

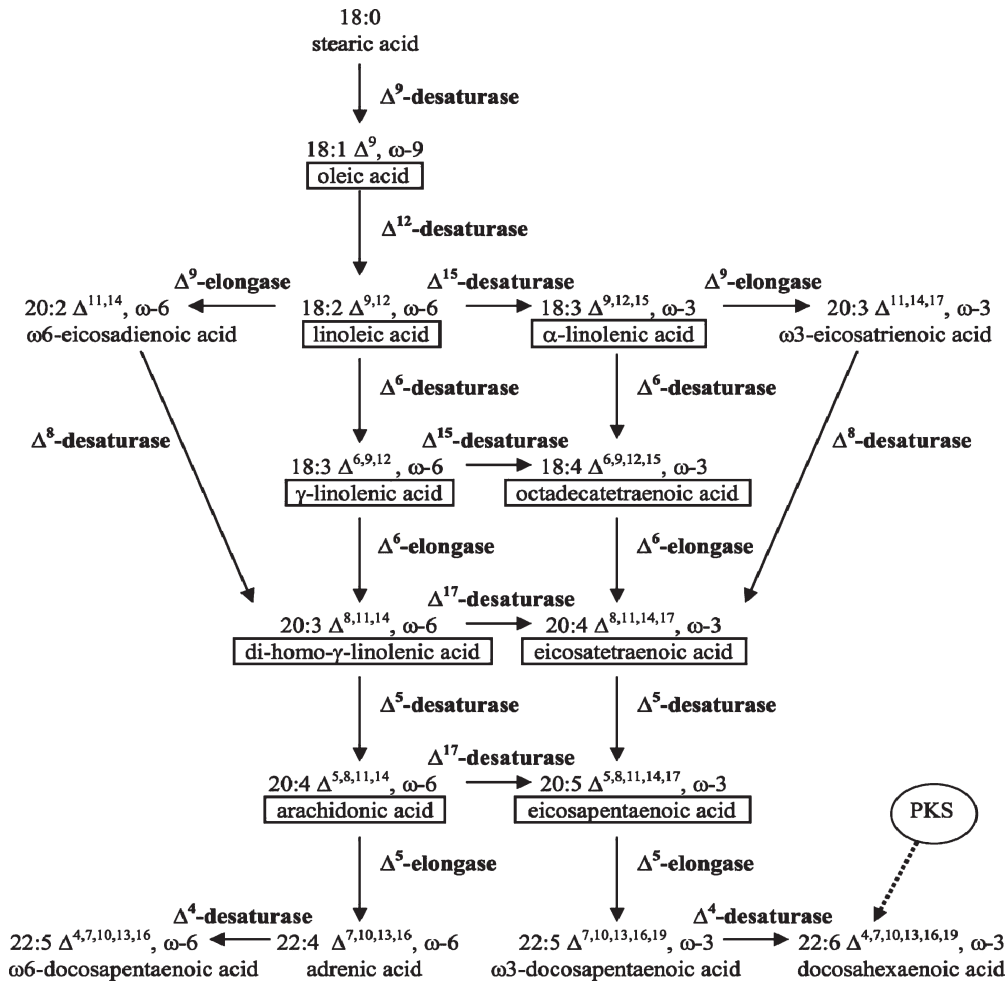

Fig. 3. Biosynthesis pathway of long-chain PUFAs in lower eukaryotes. The common pathway for synthesis of ω-6 and ω-3 long-chain PUFAs is shown in bold arrows, and the alternative prokaryotic polyketide synthase (PKS) pathway that exists in some organisms is indicated with a *dashed arrow*. The boxes indicate the fatty acids detected in the moss *P. patens* (adapted from Kaewsuwan et al. (2006) with permission from Journal of Biological Chemistry).

confirmed for the moss *Atrichum androgynum*, and the results clearly indicate a similar gene expression that regulates the function of abscisic acid in mosses and vascular plants (Guschina et al., 2002). The same author showed in another moss species, *Dicranum scoparia*, that the lipid metabolism is under the control of light, because the ratio of total to polar lipids is constant in the light but declines in the dark. Also, the activity of the Δ6-desaturase was modulated by ROS and light, which might be the reason that under stress conditions acetylenic triacylglycerols can accumulate (Guschina et al., 2002).

V Conclusions

Although the lipid metabolism of algae, lichens and mosses shows an acclimation pattern similar to that of vascular plants, due to the existence of a much broader spectrum of unusual long chain polyunsaturated lipids, these organisms manage their acclimation by the use of a far greater variety of lipids and lipid-modifying enzymes. The greater metabolic diversity, together with much easier handling in photobioreactors provides an excellent perspective for genetically modified algae and moss species to produce high value

PUFAs with a high efficiency. Furthermore, the sequence information from the complete genome of *C. reinhardtii*, *P. tricornutum*, *Thallassiosira pseudonana* and the moss *Physcomitrella patens* will open up new perspectives in the understanding of the enzymes responsible for the biosynthesis of the exotic lipids found in algae, lichens and mosses. Transformation of different algal and moss species in combination with the generation of new specific lipid-deficient mutants will provide further information about the function of membrane lipids in the lower eukaryotic plants. New studies on lipid protein interactions and the structural roles of membrane lipids seem especially rewarding in algae, taking into account the high diversity of antenna complexes and proteins exhibited by the different algal groups.

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Chapter 7

Molecular Genetics of Lipid Metabolism in the Model Green Alga *Chlamydomonas reinhardtii*

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Summary

Research focusing on microalgae is currently experiencing a renaissance due to the potential of microalgae for providing biofuels without competing with food crops. Despite this potential, our knowledge of neutral and membrane lipid metabolism in microalgae is very limited, and opportunities to explore lipid metabolism in microalgae and contrast it to plant lipid metabolism abound. The unicellular green alga *Chlamydomonas reinhardtii* is currently the best genetic and genomic model for microalgal lipid research. This chapter summarizes the current knowledge of lipid metabolism in this alga. *Chlamydomonas* lipid metabolism differs in some aspects from that of seed plants. For example, *Chlamydomonas* lacks phosphatidylcholine and has in its place the betaine lipid diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine. This has important implications for lipid trafficking and lipid modification.

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These distinct aspects of algal lipid metabolism combined with the lower number of genes involved in lipid metabolism in *Chlamydomonas* provide several opportunities for basic research aimed at a more in-depth understanding of lipid metabolism in eukaryotic photosynthetic organisms in general.

I Introduction

Lipid biosynthesis in plants has been studied for decades and our current molecular understanding of lipid metabolism in plants is substantial. Genes encoding enzymes of glycerolipid biosynthesis and fatty acid desaturation have been identified by genetic and biochemical means (Ohlrogge and Browse, 1995; Joyard et al., 1998; Frentzen, 2004; Benning and Ohta, 2005; Holzl and Dörmann, 2007), and the first examples of components involved in lipid trafficking between the plastid and the endoplasmic reticulum (ER) are being discovered (Jouhet et al., 2007; Benning, 2008). Annotation of the *Arabidopsis* genome sequence (The Arabidopsis Genome Initiative, 2000) has led to the identification of novel genes, which likely encode proteins involved in lipid biosynthesis, trafficking, and catabolism (Beisson et al., 2003).

Like *Arabidopsis*, the eukaryotic green alga *Chlamydomonas reinhardtii* is a well established model for the study of different processes of general relevance, such as photosynthesis (Niyogi, 1999) and post-transcriptional gene silencing (Wu-Scharf et al., 2000). Beyond these, *Chlamydomonas* research has provided substantial insights into processes more specific to unicellular algae, e.g., phototaxis and flagellar function (Silflow and Lefebvre, 2001), nutrient acquisition (Davies et al., 1994, 1996, 1999), and microalgal metabolism (Grossman et al., 2007). The recent completion of the *Chlamydomonas* genome sequence (Merchant et al., 2007), as well as the development of insertional mutagenesis (Tam and Lefebvre, 1993), RNA interference (RNAi) methods (Fuhrmann et al., 2001; Sineshchekov et al., 2002), and a molecular map (Kathir et al., 2003) make *Chlamydomonas* an attractive model to study gene function by genetic or direct molecular analysis. Preliminary annotations of lipid genes present in the genome of *Chlamydomonas* were recently published (Riekhof et al., 2005b; Riekhof and Benning, 2008). Based on these attributes, *Chlamydomonas* has great promise for the analysis of the biosynthesis and physiological functions of different lipids.

Availability of a suitable microalgal model system is timely, as microalgae are increasingly discussed as a biomass resource for the production of biofuels that does not have to compete with the agricultural production of food crops (Hu et al., 2008). While *Chlamydomonas reinhardtii* itself is not a candidate species for the commercial production of biofuels, it still is the best studied microalga at the genetic and genomic level. Moreover, *Chlamydomonas* is related to other unicellular green algae that are commercially used, e.g., *Dunalliella salina*, and *Chlamydomonas* has been reported to accumulate triacylglycerols (TAGs) under conditions of nutrient deprivation (Weers and Gulati, 1997) or high light (Picaud et al., 1991). *Chlamydomonas* also synthesizes TAGs from lipids supplied in the medium (Grenier et al., 1991). To fill in the gaps in knowledge, efforts are currently underway in our lab to genetically dissect the biosynthesis of TAGs and its regulation in *Chlamydomonas*, and to identify genes that might be useful for the engineering microalgal production strains.

Abbreviations: ACP – Acyl carrier protein; CDP-DAG – CDP-diacylglycerol; DAG – Diacylglycerol; DGTS – Diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine; DGDG – Digalactosyl-diacyl-glycerol; ER – Endoplasmic reticulum; FAS – Fatty acid synthase; MGDG – Monogalactosyldiacylglycerol; PA – Phosphatidic acid; PC – Phosphatidylcholine; PE – Phosphatidylethanolamine; PG – Phosphatidylglycerol; PI – Phosphatidylinositol; PS – Phosphatidylserine; PUFA – Polyunsaturated fatty acid; RNAi – RNA interference; SQDG – Sulfoquinovosyldiacylglycerol; TAG – Triacylglycerol.

II General Differences in Lipid Metabolism between *Chlamydomonas* and Seed Plants

As elaborated below, many aspects of lipid metabolism follow common pathways that were presumably established during the evolution of chloroplasts of green algal and plant ancestors (Reyes-Prieto et al., 2007). However, at least

two possibly related aspects of lipid metabolism in *Chlamydomonas* differ from lipid metabolism in seed plants (Fig. 1). Most prominently, *Chlamydomonas* is unable to synthesize the otherwise common phosphoglycerolipid phosphatidylcholine (PC). Instead, it produces the non-phosphorus betaine lipid diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS) (Eichenberger and Boschetti, 1977). This lipid is similar in structure and function to PC (Fig. 2) and is thought to substitute for PC in *Chlamydomonas* (Sato and Murata, 1991; Sato, 1992; Moore et al., 2001). Interestingly, PC is central to lipid metabolism in developing seeds or leaves where it serves as substrate for fatty acid modifying enzymes, such as desaturases (Browse and Somerville, 1991; Ohlrogge and Browse, 1995; Wallis and Browse, 2002), or possibly as the lipid transferred between the ER and the plastid (Jouhet et al., 2007; Benning, 2008).

Precursors of thylakoid lipid biosynthesis in many plants are derived from two pathways (Mongrand et al., 1998), the plastid and the ER

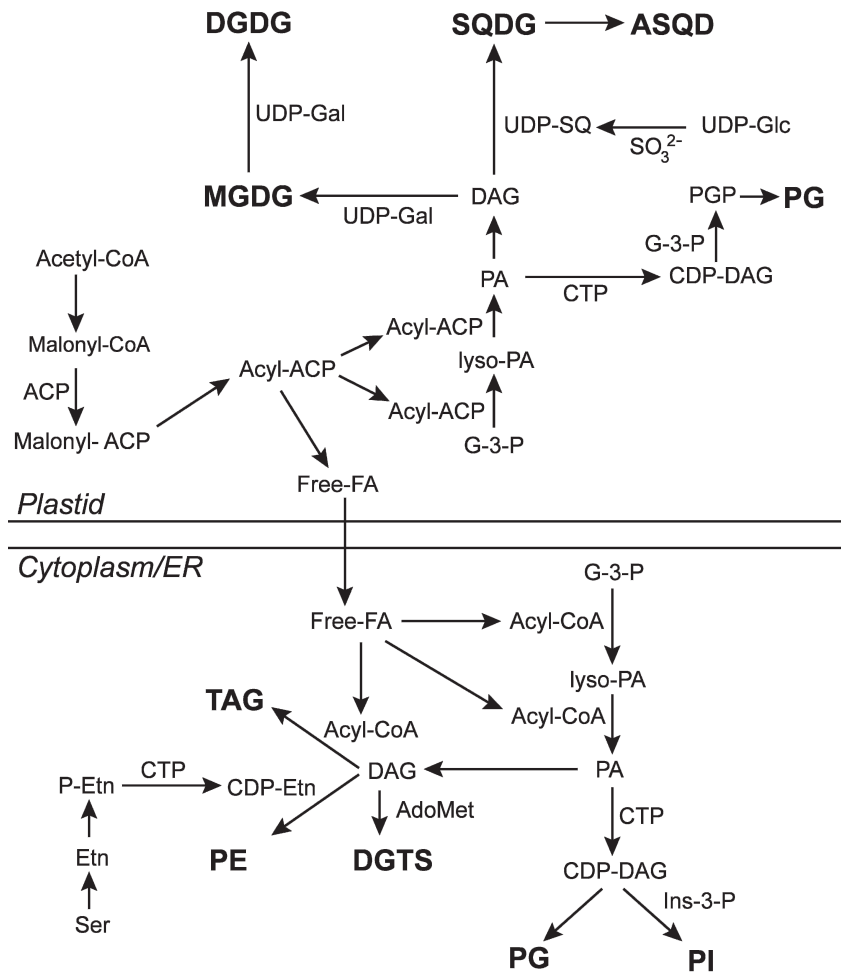

Fig. 1. Overview of glycerolipid biosynthesis in Chlamydomonas. Endproducts are shown in bold. Abbreviations: ACP, acyl carrier protein; AdoMet, S-adenosylmethionine; ASQD, 2′-O-acyl-sulfoquinovosyldiacylglycerol; CDP, cytidine 5′-diphosphate; CoA, coenzyme A; CTP, cytidine 5′-triphosphate; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceryl-N,N,N-trimethylhomoserine; Etn, ethanolamine; FA, fatty acid; G3-P, glycerol 3-phosphate; Glc, glucose; Ins-3-P, inositol 3-phosphate; MGDG, monogalactosyldiacylglycerol; P-Etn, phosphoethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PI, phosphatidylinositol; PA, phosphatidic acid; Ser, serine; SQ, sulfoquinovose; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol; UDP, uridine 5′-diphosphate (modified with permission from Fig. 1 in Riekhof et al., 2005b).

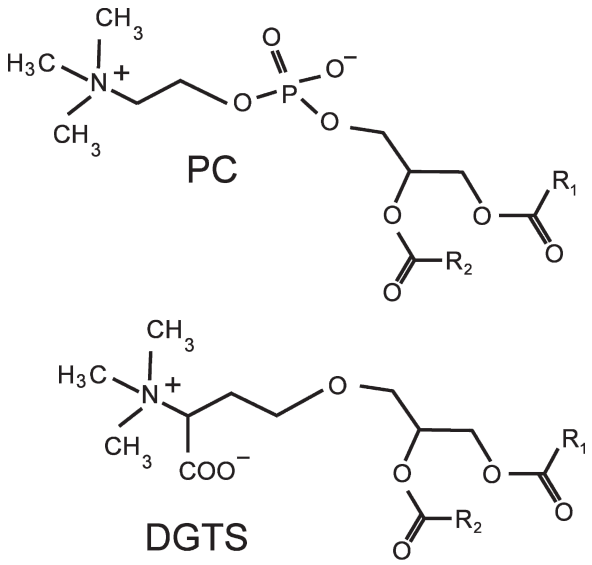

Fig. 2. Structural similarity between phosphatidylcholine (PC) and betaine lipid (DGTS).

pathways. This two pathway hypothesis was formulated by Roughan and coworkers based on labeling experiments (Roughan et al., 1980; Roughan and Slack, 1982) and later confirmed by mutant analysis in *Arabidopsis* (Browse and Somerville, 1991; Wallis and Browse, 2002). Thylakoid lipid molecular species derived from either of the two pathways can be distinguished based on their fatty acid composition (Heinz and Roughan, 1983), and fluxes through the two pathways have been determined (Browse et al., 1986). While a large number of plant species have lost the ability to de novo assemble thylakoid lipids, such as the dominant galactoglycerolipids in the plastid, nearly all reported plant species derive at least a fraction of their thylakoid lipids from precursors assembled at the ER (Mongrand et al., 1998) requiring import of lipid precursor into the plastid. However, detailed compositional analysis of lipids and labeling studies suggest that in *Chlamydomonas* all thylakoid lipids are assembled de novo in the plastid (Giroud et al., 1988). Thus, it is possible that the lack of PC and the lack of trafficking of lipid precursors from the ER to the plastid in *Chlamydomonas* are related if PC is a critical intermediate in ER-to-plastid lipid trafficking. Because in the betaine lipid, DGTS, the head group moiety is ether-linked to the diacylglyceryl moiety (Fig. 2), *Chlamydomonas* might lack an enzyme to break this ether linkage. This ether linkage is more stable than the phosphate ester linkage in phosphoglycerolipids. Therefore, the conversion of DGTS into the galactoglycerolipid precursor diacylglycerol might not be possible in *Chlamydomonas*.

Aside from the betaine lipid, *Chlamydomonas* and many other microalgae contain a rich set of polyunsaturated fatty acids (Fig. 3) not present in most seed plants, which will be discussed in detail below.

III Membrane Glycerolipid Biosynthesis

A Fatty Acid Synthesis and Incorporation into Glycerolipids

De novo synthesis of fatty acids is localized to the chloroplast of *Chlamydomonas* cells (Sirevag and Levine, 1972). The common ancestral origin of green algal and seed plant plastids is particularly apparent in many homologous components of the fatty acid biosynthetic machinery. For example, bioinformatic analysis of the *Chlamydomonas* genome has identified genes for the full suite of enzymes required for the conversion of acetyl-CoA to acylated-acyl carrier protein (ACP), including the multimeric bacterial-type acetyl-CoA carboxylase and fatty acid synthase complexes (Riekhof et al., 2005b; Riekhof and Benning, 2008). These enzymes are essential for fatty acid biosynthesis in plants (and presumably algae), which predominantly produce 16:0-ACP and 18:1-ACP as the result of desaturation of 18:0-ACP by a soluble stearoyl-ACP Δ9 desaturase (Browse and Somerville, 1991; Shanklin and Somerville, 1991). In plants, fatty acids are incorporated directly into chloroplast membrane glycerolipids by stepwise acylation of glycerol 3-phosphate to form phosphatidic acid (*sn1*–18:1, *sn2*–16:0-PA) by glycerol 3-phosphate:acyl-ACP acyltransferase (GPAT), which shows substrate specificity for 18:1-ACP, and then by lysophosphatidate:acyl-ACP acyltransferase (LPAT, 16:0-ACP specific) (Kunst et al., 1988; Browse and Somerville, 1991; Murata and Tasaka, 1997; Kim and Huang, 2004; Xu et al., 2006).

Fatty acids are also assembled into glycerolipids at the ER where isoforms of the plastid

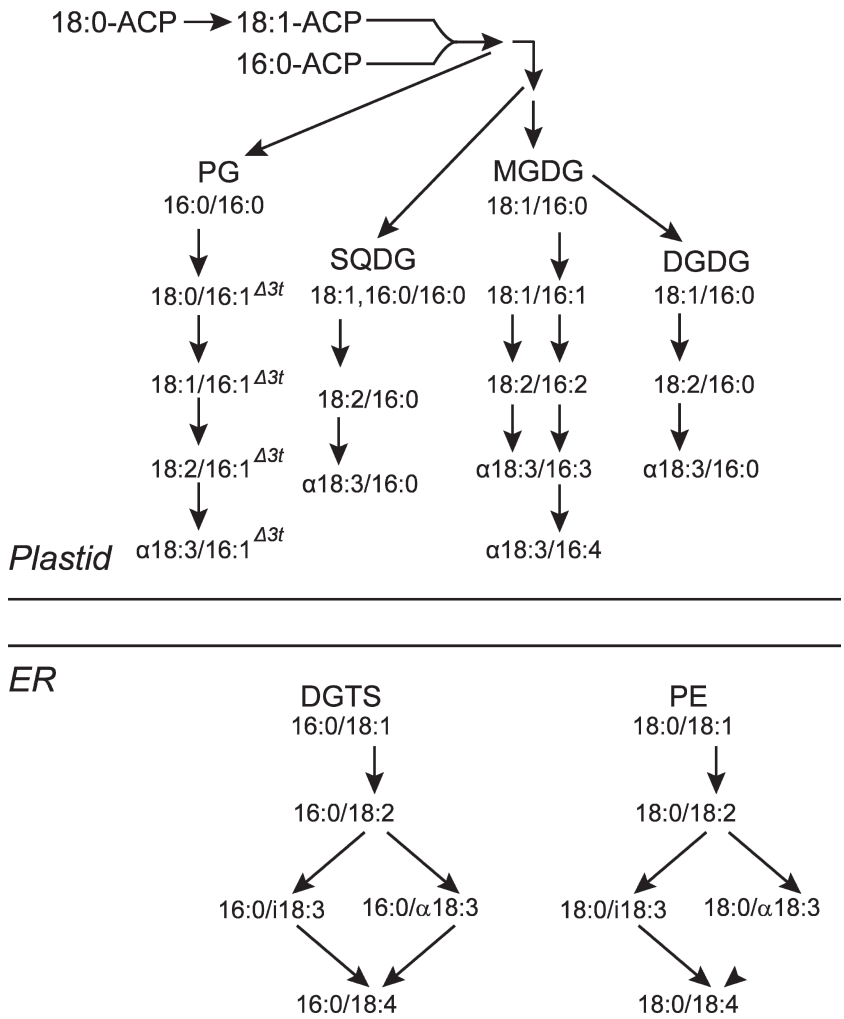

Fig. 3. Overview of acyl-chain desaturation in Chlamydomonas. Glycerolipid abbreviations are the same as those in Fig. 1. Fatty acids are referred to by the standard abbreviation "carbon atoms:double bonds." Fatty acids at the sn-1 and sn-2 positions of the glyceryl moiety are indicated. Double bond positions within the fatty acid chain and/or common names of the fatty acids are as follows: 16:0, palmitic acid; $16:1^{\Delta 7}$, plamitoleic acid or $16:1^{\Delta 3t}$ in plastidic PG; $16:2^{\Delta 7,10}$; $16:3^{\Delta 7,10,13}$; for 16:4 the double bond position is not known; 18:0, stearic acid; $18:1^{\Delta 9}$, oleic acid or in some lipids $18:1^{\Delta 11}$, vaccenic acid; $\alpha 18:2^{\Delta 9,12}$, αlinoleic acid; $\alpha 18:3^{\Delta 9,12,15}$, αlinoleic acid; $i18:3^{\Delta 5,9,12}$, pinolenic acid; $18:4^{\Delta 5,9,12,15}$, coniferonic acid. The predominant molecular species of SQDG is 16:0/16:0 (modified with permission from Fig. 2 in Riekhof et al., 2005b).

acyltransferases are present and have been characterized in *Arabidopsis* (Zheng et al., 2003; Kim et al., 2005). Putative orthologs of the plant GPAT and LPAT genes are annotated in the final *Chlamydomonas* genome draft (Riekhof et al., 2005b; Riekhof and Benning, 2008). Candidates for the plastid PA phosphatase, which produces the diacylglycerol precursors for the biosynthesis of non-phosphorus lipids in the plastid, have been recently identified in *Arabidopsis* (Nakamura et al., 2007). However, there is currently no good candidate in the *Chlamydomonas* genome predicted to encode this enzyme (Riekhof et al., 2005b; Riekhof and Benning, 2008).

B Chloroplast Membrane Lipids

The overall structural organization of membranes in the chloroplast of *Chlamydomonas* and seed plant chloroplasts is essentially identical, where the inner and outer envelope membranes enclose an extensive thylakoid membrane system in which the

photosynthetic apparatus is embedded. Genetic studies of *Arabidopsis* have identified many of the genes responsible for the biosynthesis of chloroplast membrane lipids, and have revealed the essential role that lipid composition plays in optimal photosynthetic function (Vijayan et al., 1998; Dörmann and Benning, 2002; Wallis and Browse, 2002; Benning and Ohta, 2005). Though the genetic study of glycerolipid metabolism in *Chlamydomonas* has far fewer documented examples, detailed biochemical analysis of this alga's lipid composition has long confirmed the presence of the major chloroplast membrane lipids found in land plants – including the galactoglycerolipids mono- and digalactosyldiacylglycerol (MGDG and DGDG), sulfoquinovosyldiacylglycerol (SQDG), and the phosphoglycerolipid phosphatidylglycerol (PG) (Giroud et al., 1988).

As in plants, galactoglycerolipids are the predominant membrane glycerolipid class in *Chlamydomonas*, where they make up a majority of the chloroplast membrane lipids (Janero and Barrnett, 1981a; Giroud et al., 1988). In *Arabidopsis*, the bulk of galactolipid biosynthesis involves two enzymatic steps, whereby MGDG is formed from diacylglycerol (DAG) and UDP-galactose (UDP-Gal) substrates by MGDG synthase (MGD1), and DGDG is formed from MGDG and UDP-Gal by DGDG synthase (DGD1) (Benning and Ohta, 2005). Genes encoding MGDG and DGDG synthases have been identified in the *Chlamydomonas* genome as orthologs of the *Arabidopsis* genes *MGD1* and *DGD1*, respectively (Riekhof et al., 2005b; Riekhof and Benning, 2008). *MGD1* and *DGD1* are single-copy genes in *Chlamydomonas*, which differs from that of the *MGD1*, *2*, *3* and *DGD1*, *2* paralogs found in the *Arabidopsis* genome. Molecular analysis of the *Arabidopsis MGD2*, *3* and *DGD2* genes has revealed their role in a galactolipid biosynthetic pathway that is transcriptionally induced during phosphate deprivation, and is proposed to provide galactolipids for extraplastidic membranes (Härtel et al., 2000; Kelly and Dörmann, 2002; Jouhet et al., 2004). The apparent lack of this induced galactolipid pathway in *Chlamydomonas* suggests a distinct lipid metabolic response to phosphate limitation, or a lack of need for one; however, to date the galactolipid biosynthetic genes of *Chlamydomonas* have not been studied in detail at the molecular level to test these hypotheses.

The sulfolipid sulfoquinovosyldiacylglycerol (SQDG) has long been studied in the context of its role in photosynthetic membranes, not only due to its prevalence in photosynthetic eukaryotes and prokaryotes, but also because of its association with photosynthetic pigment–protein complexes (Menke et al., 1976; Gounaris and Barber, 1985; Pick et al., 1985; Stroebel et al., 2003). However, the more recent discovery of SQDG and/or the genes and enzymes involved in SQDG biosynthesis in non-photosynthetic bacteria as summarized in (Cedergren and Hollingsworth, 1994; Benning et al., 2008), has clearly indicated that the role of sulfolipids is not limited to the function of photosynthetic membranes. The biosynthesis of SQDG is carried out in two enzymatic steps in *Arabidopsis* by SQD1, which catalyzes the formation of UDP-sulfoquinovose from UDP-Glc and sulfite, and SQD2, which transfers the sulfoquinovose moiety from UDP-sulfoquinovose to DAG, forming SQDG (Essigmann et al., 1998; Sanda et al., 2001; Yu et al., 2002). A single copy ortholog of *SQD1* is present in *Chlamydomonas*, and two possible orthologs of *Arabidopsis SQD2* are found in the genome (Yu et al., 2002; Riekhof et al., 2003). Recently, a *Chlamydomonas* mutant deleted in *SQD1* (Δ*sqd1*) and completely lacking sulfolipid has been studied (Riekhof et al., 2003). Phenotypic analysis of Δ*sqd1* revealed a reduced growth rate during phosphate-limiting conditions, under which the SQDG level was found to double in wild-type cells. This is similar to what has been observed in sulfolipid-deficient mutants in other organisms, such as *Arabidopsis*, which showed impaired growth after severe phosphate limitation (Yu et al., 2002), and in the photosynthetic purple bacterium *Rhodobacter sphaeroides* (Benning et al., 1993). In both *Chlamydomonas* and *Arabidopsis*, the increase in SQDG levels under phosphate-limiting conditions is accompanied by a decrease in PG, resulting in little net change in the amount of anionic glycerolipids. These results suggest a role for SQDG in partially replacing PG during phosphate limitation in order to maintain thylakoid membrane function (Riekhof et al., 2003). However, during sulfur (S) limitation a large decrease in SQDG and concomitant increase in PG has been observed in *Chlamydomonas* (Sugimoto et al., 2008), and SQDG was shown to be a major

internal S-source for protein synthesis in the early phases of the S-starvation response (Sugimoto et al., 2007).

In addition, Δ*sqd1* showed sensitivity to a photosystem II inhibitor under normal growth conditions (Riekhof et al., 2003). This is consistent with another *Chlamydomonas* SQDG-deficient mutant, *hf-2*, which was first discovered as a high chlorophyll fluorescence mutant, and was later found to be impaired in photosystem II stability and showed increased sensitivity to a PSII inhibitor, which could be partially restored by SQDG addition (Sato et al., 1995a, b; Minoda et al., 2002, 2003). However, whether the *hf-2* mutant is impaired in growth during phosphate limitation has not been reported, nor has the exact molecular defect in this mutant been determined. Interestingly, detailed biochemical analysis of the *Δsqd1* mutant also led to the discovery of the novel sulfolipid derivative, 2′-*O*-acylsulfoquinovosyldiacylglycerol (ASQD), which was also not produced in *Δsqd1* (Riekhof et al., 2003). Due to the loss of both sulfolipids in *Δsqd1*, the specific roles played by SQDG and ASQD in *Chlamydomonas* and phenotypes associated with *Δsqd1* can only be fully interpreted after the identification and characterization of the acyltransferase catalyzing ASQD production has been undertaken.

Phosphatidylglycerol (PG) is presumably the only major phospholipid component in thylakoid membranes of seed plants, and biochemical analysis of thylakoid lipid composition has confirmed this to be the case in *Chlamydomonas* (Janero and Barrnett, 1981b; Mendiola-Morgenthaler et al., 1985). While the gene encoding the final enzyme in PG biosynthesis, phosphatidylglycerolphosphate (PGP) phosphatase, remains unknown in plants and algae (Beisson et al., 2003), the putative genes encoding the enzymes that catalyze the formation of the two intermediates, CDP-DAG synthetase and phosphatidylglycerolphosphate synthase, have been identified (Riekhof et al., 2005b; Riekhof and Benning, 2008), but not yet confirmed. While neither the single gene encoding the CDP-DAG synthetase or the two putative plastid paralogs encoding phosphatidylglycerolphosphate synthase have been studied at the molecular/genetic level, PG deficient mutants, *mf 1* and *mf 2*, have been isolated and studied in great biochemical detail (Garnier et al., 1987; Maroc et al., 1987; Garnier et al., 1990; Maanni et al., 1998; Dubertret et al., 2002; Pineau et al., 2004). The *mf 1*, *2* mutants were first isolated as low fluorescent strains lacking functional Photosystem II (PS II), as well as an oligomeric form of the light-harvesting chlorophyll antenna (CPII) (Maroc et al., 1987; Dubertret et al., 1994). It was also shown that both *mf 1* and *mf 2* contained approximately 30% of wild-type PG levels and lacked Δ3-*trans*-hexadecenoic acid ($16{:}1^{\Delta 3trans}$ [carbons:double bonds$^{\Delta positions}$]) (Maroc et al., 1987; Dubertret et al., 1994), a fatty acid that is specifically esterified to chloroplastic PG in both *Arabidopsis* and *Chlamydomonas* (Browse et al., 1985; Garnier et al., 1987; Giroud et al., 1988). Addition of a preparation of spinach leaf PG containing $16{:}1^{\Delta 3trans}$ to *mf-2* cells restored the ability to form oligomeric CP II, while 18:0 PG additions did not, and 16:0 PG did so only weakly so (Garnier et al., 1990; Dubertret et al., 1994). A PG-deficient mutant in *Arabidopsis*, *pgp1*, which is defective in the chloroplastid isoform of PGP synthase has been found to be photosynthetically impaired with decreased quantum yield through PSII, but did not lack $16{:}1^{\Delta 3trans}$ PG (Xu et al., 2002; Hagio et al., 2002; Babiychuk et al., 2003). Similarly, two *Synechocystis* PG deficient mutants showed altered PSII activity and required exogenous PG for phototropic growth (Hagio et al., 2000; Sato et al., 2000).

Taken together with the contrasting findings from analyses of the *Arabidopsis fad4* mutant, which lacks $16{:}1^{\Delta 3trans}$, but is otherwise not affected in chloroplast PG content and also shows no apparent photosynthetic defects (Browse et al., 1985), it can currently only be concluded that in general PG plays an important role in photosynthetic membrane biogenesis and function, and it seems possible that the $16{:}1^{\Delta 3trans}$ PG form could be essential in some organisms (e.g., *Chlamydomonas*), but is of conditional importance or dispensable in others. It is certain however, that the elucidation of the exact molecular defects in the *Chlamydomonas mf 1*, *2* mutants, and the identification of the genes encoding FAD4 activity as well as the elusive plant/algal PGP phosphatase that catalyzes the final step in PG biosynthesis, will be prerequisite to gaining a better understanding of the roles PG plays in the photosynthetic membranes in various species.

C Extrachloroplastic Membrane Lipid Metabolism

In eukaryotic photoautotrophs the bulk of extraplastidic membrane glycerolipids is assembled in the ER from acyl-CoA thioesters, which are formed from free fatty acids after their liberation from acyl-ACPs in the plastid (see Fig. 1). While other extraplastidic sites for lipid synthesis are known (e.g., mitochondria), the ER localized pathway is predominant, and in most plants the ER lipid assembly pathway significantly contributes to thylakoid membrane biogenesis. As such, a discussion of the analogous pathways in *Chlamydomonas* is merited. As mentioned above, *Chlamydomonas* lacks the capability for PC biosynthesis (Giroud et al., 1988) and genes predicted to encode enzymes involved in PC biosynthesis are not present in its genome (Riekhof et al., 2005b; Riekhof and Benning, 2008). Instead, it contains the non-phosphorous zwitterionic betaine lipid DGTS (Fig. 2) in its membranes (Eichenberger and Boschetti, 1977; Janero and Barrnett, 1982), which has similar biophysical properties to PC (Sato and Murata, 1991). DGTS has also been found in other algal species, e.g. (Eichenberger, 1982), prokaryotes like the purple bacterium *Rhodobacter sphaeroides*, e.g. (Benning et al., 1995; Hofmann and Eichenberger, 1996), and in non-seed plants, such as ferns, e.g. (Sato and Furuya, 1983; Eichenberger, 1993), but appears to be absent in seed plants. Labeling studies suggest that the biosynthesis of DGTS is similar in all organisms studied (Sato, 1988, 1991; Sato and Kato, 1988; Vogel and Eichenberger, 1992; Hofmann and Eichenberger, 1996). It begins with the transfer of the 3-amino 3-carboxypropyl residue from *S*-adenosylmethionine (AdoMet) to DAG catalyzed by AdoMet:DAG 3-amino-3-carboxypropyltransferase activity followed by successive methylation of the amino group by an AdoMet-dependent *N*-methyltransferase (Fig. 1). The two genes encoding these catalytic activities, *btaA* and *btaB*, were first identified in *R. sphaeroides* (Klug and Benning, 2001; Riekhof et al., 2005a).

More recently, a single gene sufficient for DGTS biosynthesis in *Chlamydomonas*, *Bta1*, was identified in the genome (Riekhof et al., 2005b). The encoded protein Bta1 is similar in its N-terminal domain to bacterial BtaB and in its C-terminal domain to BtaA and the predicted catalytic function of each Bta1 domain was confirmed by mutagenesis (Riekhof et al., 2005b). The bifunctionality observed in Bta1 as a fusion of two prokaryotic enzymes active in the same pathway into a single polypeptide is a common theme in plants (Moore, 2004), and could represent an improvement in DGTS biosynthesis by eliminating the need for coordinated regulation of two independent gene products or permitting substrate channeling. In addition, the presence of DGTS and concomitant lack of PC are perhaps related to the absence of additional galactolipid biosynthetic pathways seen in seed plants, e.g., MGD2, MGD3 and DGD2 in *Arabidopsis* (Benning and Ohta, 2005). As this alternative galactolipid pathway is believed to be involved in replacing phospholipids in extraplastidic membranes with DGDG during phosphate deprivation, the constitutive replacement of PC with the non-phosphorous DGTS has perhaps obviated the need to replace PC by extraplastidic DGDG. Interestingly, DGTS was tentatively identified as a component of purified *Chlamydomonas* thylakoids (Janero and Barrnett, 1981b, 1982) and chloroplast envelope membranes (Mendiola-Morgenthaler et al., 1985). However, whether DGTS is indeed present in the chloroplast membranes of *Chlamydomonas* or plays a specific function in thylakoids remains to be confirmed.

Phosphatidylserine (PS) is a minor component of extraplastidic membranes of plants and can be decarboxylated to phosphatidylethanolamine (PE) in plants and other organisms (Vance and Steenbergen, 2005; Nerlich et al., 2007). However, PS is absent in *Chlamydomonas* membranes (Giroud et al., 1988) and genes encoding the phosphatidylserine synthase or relevant phospholipid base exchange enzymes were not detected in the genome (Riekhof et al., 2005b; Riekhof and Benning, 2008). As such, the biosynthesis of PE, which is a known component of extraplastidic membranes in *Chlamydomonas*, is likely only carried out by a single pathway (Fig. 1). Genes encoding a serine decarboxylase which produces ethanolamine, an ethanolamine kinase and CTP:phosphoethanolamine cytidylyltransferase the combined activities of which produce CDP-ethanolamine, and a CDP-ethanolamine:DAG ethanolamine phosphotransferase, which produces PE, have been identified in the genome (Riekhof et al., 2005b; Riekhof and Benning, 2008).

Recently, the gene encoding the CTP: phosphoethanolamine cytidylyltransferase has been characterized by heterologous expression in *Escherichia coli*, and the expression of the respective gene was found to be up-regulated during the reflagellation of *Chlamydomonas* cells (Yang et al., 2004). Phosphatidylinositol (PI) is a minor component of *Chlamydomonas* membranes, and genes required for its biosynthesis, including inositol-3-phosphate synthase and CDP-DAG:inositol phosphotransferase, are present in the genome (Riekhof et al., 2005b; Riekhof and Benning, 2008). The PI biosynthetic enzymes of *Chlamydomonas* have been studied at the biochemical level, and PI synthesis was found to be highest in the microsomal fraction, suggesting its association with the ER (Blouin et al., 2003).

Extraplastidic PG biosynthesis is known to be associated with both the ER and mitochondria, and three isoforms of phosphatidylglycerolphosphate synthase are encoded in the genome, each with differential targeting prediction probabilities for subcellular localization to the mitochondria, chloroplast or cytosol (Riekhof et al., 2005b; Riekhof and Benning, 2008). However, a detailed analysis of these proteins and their respective genes is not yet available in *Chlamydomonas*. An extraplastidic candidate for CDP-DAG synthase, which provides one of the substrates for phosphatidylglycerolphosphate synthase, has been identified in the genome (Riekhof et al., 2005b). However, as in the case of chloroplast PG biosynthesis, no gene encoding an extraplastidic phosphatidylglycerolphosphate phosphatase is currently known.

IV Fatty Acid Desaturation

Biochemical studies in *Chlamydomonas* have indicated that further desaturation of 16:0 and 18:1 acyl groups occurs after the production of the major glycerolipids, in a manner similar to plants (Giroud et al., 1988). Furthermore, the *Chlamydomonas* fatty acid profile is known to change markedly in response to various environmental conditions, including CO_2 concentration, as well as nitrogen and phosphorous limitation (Tsuzuki et al., 1990; Weers and Gulati, 1997). The elucidation of fatty acid desaturase (*FAD*) genes in *Arabidopsis* is a classic example of the power of genetic and molecular biological approaches in solving biological problems, which prove to be largely intractable through a strictly biochemical approach (Browse and Somerville, 1991; Wallis and Browse, 2002). The identification of many of the *Chlamydomonas* desaturase gene candidates by their similarity to *Arabidopsis* orthologs, combined with a handful of studies of *Chlamydomonas* desaturase mutants and characterization of cloned *FAD* genes, has provided a reasonable picture of the fatty acid desaturation pathways in this alga (Fig. 3).

Putative orthologs for the plastidic desaturases encoded by *Arabidopsis FAD5* (MGDG palmitate-Δ7-desaturase), *FAD6* (ω6-desaturase), and *FAD7* or *FAD8* (encoding ω3-desaturase isozymes) are present in the *Chlamydomonas* genome (Riekhof et al., 2005b; Riekhof and Benning, 2008). Of these, only the *Chlamydomonas FAD6* gene has been studied to date at the molecular/genetic level (Sato et al., 1995b, 1997). The *hf-9* mutant was first isolated by its high chlorophyll fluorescence phenotype, and detailed lipid analysis revealed an apparent defect in ω6-desaturase activity as it showed marked decreases in both 16- and 18-carbon polyunsaturated fatty acyl groups, with concomitant increases in $16{:}1^{\Delta 7}$ and $18{:}1^{\Delta 9}$ (Sato et al., 1995b). The *hf-9* mutant had an increased doubling time and showed reduced photosynthetic O_2 evolution as well as an altered chloroplast ultrastructure (Sato et al., 1995b). The *Chlamydomonas FAD6* gene (first described as *DES6*) was subsequently cloned and found to be highly similar to cyanobacterial Δ12- and seed plant ω6-desaturases, and was also shown to complement the *hf-9* mutant desaturation defects (Sato et al., 1997). However, it did not restore the photosynthetic defects, suggesting that these phenotypes arose from a mutation in another gene (or possibly from multiple loci). As such, the roles of polyunsaturated fatty acids (PUFAs) in the assembly or maintenance of optimally functioning photosynthetic membranes in *Chlamydomonas* cannot be easily deduced from analysis of *hf*-9. The function of PUFAs in this regard have been studied and found to differ in mutants of both plants and cyanobacteria. The *Arabidopsis fad6 fad2* double mutant, which lacks both plastidic and ER ω6-desaturases exhibited severe growth and photosynthetic defects (McConn and Browse, 1998). In contrast,

a mutant of *Synechocystis* sp. PC 6,803 lacking PUFAs had no observable photosynthetic defects under normal growth conditions (Gombos et al., 1992). Thus, it still remains to be determined whether the importance of PUFAs in photosynthetic membrane function in *Chlamydomonas* is more similar to that of seed plants or cyanobacteria. Other plastidic desaturases still have no gene candidates in *Chlamydomonas*. As noted above, the desaturase producing $16{:}1^{\Delta 3trans}$ specifically on plastidic PG is still not identified at the molecular level in plants or algae, although mutants lacking this fatty acid have been obtained in *Arabidopsis* and *Chlamydomonas* (Browse et al., 1985; Maroc et al., 1987). Likewise, a gene encoding Δ4 desaturase activity, which is specific for MGDG-esterified 16-carbon acyl groups based on biochemical studies (Giroud et al., 1988), and presumably produces both the $16{:}3^{\Delta 4,7,10}$ and $16{:}4^{\Delta 4,7,10,13}$ found in *Chlamydomonas* is currently unidentified.

The extraplastidic ω6- and ω3-desaturases, which produce $18{:}2^{\Delta 9,12}$ and $18{:}3^{\Delta 9,12,15}$, are encoded by *FAD2* and *FAD3*, respectively, in *Arabidopsis*, and putative orthologs of these genes have been identified in the *Chlamydomonas* genome (Riekhof et al., 2005b; Riekhof and Benning, 2008). The extraplastidic *Chlamydomonas* lipids DGTS and PE have been shown to contain significant amounts of $18{:}3^{\Delta 5,9,12}$ and $18{:}4^{\Delta 5,9,12,15}$ esterified to the respective *sn*-2 positions of the glycerol back bone (Giroud et al., 1988); these Δ5-unsaturated fatty acids are also found in gymnosperms (Mongrand et al., 2001; Wolff and Christie, 2002). Recently a *Chlamydomonas* gene, *CrDES*, encoding a "front-end" type Δ5-desaturase was identified by a bioinformatics approach through its similarity to a known Δ5-desaturase from the liverwort *Marchantia polymorpha* (Kajikawa et al., 2006). Heterologous expression of *CrDES* in *Pichia pastoris* and analysis of desaturase activity indicated that while the primary substrates were $18{:}2^{\Delta 9,12}$ and $18{:}3^{\Delta 9,12,15}$, low but detectable levels of endogenous $18{:}1^{\Delta 9}$ desaturation were also observed (Kajikawa et al., 2006). Transgenic tobacco plants constitutively expressing the *CrDES* gene exhibited strikingly high levels of $18{:}3^{\Delta 5,9,12}$ and $18{:}4^{\Delta 5,9,12,15}$ (which are normally absent), with the highest combined yield reaching ~45% of leaf total fatty acids, and no apparent morphological phenotypes (Kajikawa et al., 2006). The biological roles of these Δ5-unsaturated fatty acids in the organisms which produce them are largely unknown, and the identification of the *CrDES* gene responsible for $18{:}3^{\Delta 5,9,12}$ and $18{:}4^{\Delta 5,9,12,15}$ production in *Chlamydomonas* will allow for this gene to be targeted for suppression through RNAi technology.

V Neutral Lipid Metabolism

To date, little research has been done on neutral glycerolipid synthesis in *Chlamydomonas*. However, there is an increasing focus on oil production in microalgae due to its potential role as a feedstock for biodiesel or jet fuels (Hu et al., 2008), and as a source of commercial oils and fatty acids (Spolaore et al., 2006). Beginning in the late 1970s, the Department of Energy initiated a two decade-spanning research effort, the Aquatic Species Program, to investigate the possibility of obtaining biodiesel from microalgae (Sheehan et al., 1998). Researchers screened numerous algal strains for oil production, and found many that accumulated oil up to 75% dry weight (Benamotz and Tornabene, 1985; Bigogno et al., 2002; Chisti, 2007). *Chlamydomonas* also accumulates oil in the form of triacylglycerol under certain conditions, as already mentioned above (Weers and Gulati, 1997).

In most algae species, oil production is triggered by environmental stress, suggesting that triacylglycerol plays a role in microalgae beyond energy storage. One of the main stresses investigated is nutrient deprivation, with nitrogen deprivation being the most common condition used. Growing the chlorophyte *Neochloris oleoabundans* in growth media limited for nitrogen resulted in lipids being accumulated up to 56% of the total dry weight, with 80% of that being triacylglycerol (TAG) (Tornabene et al., 1983). The eustigmatophyte *Nannochloropsis* gave similar results (Suen et al., 1987) as did the chlorophyte *Parietochloris incise* (Merzlyak et al., 2007). Growing the chlorophyte *Haematococcus pluvialis* in nitrogen-free medium led not only to an increase in total lipid content, but also to a change in fatty acid composition, with an increase in oleic acid (Zhekisheva et al., 2002). Other nutrient deficiencies can trigger lipid accumulation. For example, silicon deficiency leads to increased lipid content (mainly in the form of TAG) in the diatom

Cyclotella cryptica (Roessler, 1988). Phosphate limitation leads to an increase in TAG and overall lipid levels in some green algae, and to a decrease in lipid content in others (Khozin-Goldberg and Cohen, 2006).

Other factors, such as light, temperature and growth phase also affect oil accumulation in microalgae. The effect of temperature on lipid accumulation varies between strains, with some reporting increases in lipid levels, and others decreases (Richardson et al., 1983; Dempster and Sommerfeld, 1998; de Swaaf et al., 1999). Inhibition of cell cycle in the chlorophyte *Chlorella* by high pH leads to an accumulation of TAG similar to that due to nutrient deprivation, suggesting that environmental stress may indirectly trigger TAG synthesis by inhibiting growth, rather than directly (Guckert and Cooksey, 1990). High light intensity has been shown to increase the ratio of TAG to total lipids, although the total lipid level can remain the same or decrease (Zhekisheva et al., 2002; Khotimchenko and Yakovleva, 2004, 2005). In the chlorophyte *Dunaliella bardawil*, the accumulation of TAG under high-light stress is linked to an accumulation of β-carotene, which suggests that TAG accumulation may help to protect the chloroplasts from photooxidative damage (Benamotz et al., 1989; Rabbani et al., 1998).

The biochemistry of TAGs in microalgae has not been studied, including that in the genetic model *Chlamydomona*s. Previous research has indicated that lipid synthesis in *Chlamydomonas* is homologous to that in plants (Fig. 1), and possibly simpler (Riekhof et al., 2005b; Riekhof and Benning, 2008). Therefore, it is likely that many general aspects of TAG synthesis in *Chlamydomonas* follow that of plants. One common path for TAG synthesis in seed plants is the Kennedy pathway, which involves the stepwise addition of fatty-acyl groups to a glycerol-3-phosphate to form PA, which is converted to DAG by phosphatidic acid phosphatase; DAG is further acylated by diacylglycerol acyltransferase to form TAG, as discussed further below (Kennedy, 1961). Two genes encoding putative extraplastidic phosphatidic acid phosphatases have been identified in the *Chlamydomonas* genome, but have not yet been studied in molecular detail (Riekhof et al., 2005b). Several studies in many different plant species have also indicated that DAG derived from the PC pool also contributes substantially to TAG biosynthesis (Ohlrogge and Browse, 1995). Clearly, PC plays no role in TAG biosynthesis in *Chlamydomonas*, and its role in plants is not universal, as studies of mesocarp microsomes in avocado indicated that only the Kennedy pathway was active (Stobart and Stymne, 1985). It may be possible that the assumed functional analog of PC in *Chlamydomonas*, DGTS, is an intermediate in the biosynthesis of TAG. However, no biochemical studies to determine this have been undertaken to date, and utilization of the DGTS pool to provide DAG precursors would require an as yet unidentified enzyme to remove the ether-linked trimethylhomoserine head group. Regardless of the precursors used in forming DAG, the final step is catalyzed by diacylglycerol acyltransferases, or DGATs, which transfer a fatty acid from acyl-CoA to diacylglycerol. DGATs have been isolated and characterized from several plant species, including *Arabidopsis* (Routaboul et al., 1999; Zou et al., 1999; Hobbs et al., 1999), maize (Zheng et al., 2008) and castor beans (Kroon et al., 2006). The *Chlamydomonas* genome contains a number of putative DGAT isoforms yet to be studied in molecular detail (Riekhof and Benning, 2008; R. Miller and C. Benning, unpublished, 2009).

*Chlamydomona*s may not only utilize the Kennedy pathway for TAG synthesis. Indeed, an alternate pathway for TAG synthesis involves phospholipid: diacylglycerol acyltransferases, or PDATs, to generate TAG using a phospholipid as a fatty acid donor, rather than acyl-CoA. PDATs have also been found in plants (Dahlqvist et al., 2000) and represent a possible second type of enzyme that is also present in *Chlamydomonas* (Riekhof and Benning, 2008). Given the induction of TAG biosynthesis by different stresses, it is likely that the mechanism for the regulation of TAG synthesis differs in *Chlamydomonas* from that in seed plants, which often produce oil during a specific phase of their life cycle and in specialized tissues.

VI Perspectives

The study of lipid metabolism in microalgae is experiencing a renaissance due to their potential for the production of large quantities of biomass

in general, and specifically due to their ability to accumulate neutral lipids following nutrient deprivation. However, during the past decade much of basic research on lipid metabolism in photosynthetic organisms was focused on cyanobacteria and seed plants, in particular, the genetic and genomic model *Arabidopsis*. *Chlamydomonas* has been developed over the years as an excellent genetic model as well, however, not necessarily for the study of lipid metabolism. The availability of the *Chlamydomonas* genomic sequence (Merchant et al., 2007) has made the application of knowledge on well studied lipid metabolism in seed plants to this model alga relatively facile using comparative genomics. The result is a testable hypothesis of lipid metabolism in *Chlamydomonas* (Fig. 1) based on genome annotation (Riekhof et al., 2005b; Riekhof and Benning, 2008), which provides a wealth of opportunities to students of lipid metabolism. Those researchers interested in studying basic lipid metabolism in photosynthetic organisms might wonder what novel concepts research on *Chlamydomonas* could contribute beyond research on *Arabidopsis*. The answer lies in the fact that lipid metabolism in *Chlamydomonas* appears simpler and in some aspects drastically different from that in seed plants as discussed in detail above. The reduced redundancy in *Chlamydomonas* versus *Arabidopsis* permits testing of hypotheses on the function of parallel pathways, e.g., galactoglycerolipid biosynthesis (Härtel et al., 2000), present in *Arabidopsis*. Moreover, the unicellular organization of *Chlamydomonas* and its resulting lifestyle requires completely different input for the regulation of TAG biosynthesis (Hu et al., 2008) than the developmental regulation of storage lipid metabolism in developing seeds of *Arabidopsis* (Santos-Mendoza et al., 2008). The absence of PC in *Chlamydomonas* challenges concepts about the role of this lipid as a central metabolite in lipid trafficking and lipid modification in plants. How widespread the replacement of PC by the betaine lipid DGTS in microalgae is not known at this time, but it would be important to explore, if *Chlamydomonas* is to become the model for the engineering of microalgal biofuel-producing strains.

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Chapter 8

Lipid Biosynthesis and its Regulation in Cyanobacteria

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Summary

According to the endosymbiosis theory, cyanobacteria are assumed to be ancestors of chloroplasts. They are Gram-negative and perform oxygenic photosynthesis via two photosystems, which resemble the photosystems in the chloroplasts of eukaryotic plants. The membranes of cyanobacteria contain four main glycerolipids, three of which are glycolipids, namely, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG), and one of which is the phospholipid phosphatidylglycerol (PG). The lipid composition of cyanobacterial membranes is similar to that of chloroplast membranes, and, in particular, to that of thylakoid membranes, and it is

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different from that of the membranes of most bacteria, which contain phospholipids as the major glycerolipids. The fatty acid composition of the membrane lipids of cyanobacteria differs among strains, and cyanobacteria can be classified into four groups in terms of their fatty acids. Among unsaturated fatty acids, monounsaturated to tetraunsaturated fatty acids have been found in cyanobacteria. In a limited number of cyanobacteria, the fatty acid composition resembles that of chloroplast membranes. The biosynthesis of membrane lipids in cyanobacteria has been studied in vivo by tracer experiments with radio-labeled compounds since the 1980s. However, the characterization and molecular identification of the enzymes involved in the biosynthesis of lipids had to await the cloning of the genes for these enzymes since most of these enzymes are membrane-bound and, thus, their solubilization and subsequent purification are relatively difficult. Genes for the desaturases of cyanobacteria, which introduce double bonds into fatty acids bound to membrane lipids, were cloned in the early 1990s, after identification of the *desA* gene that was able to complement a mutation that resulted in a defect in desaturation at the Δ12 position. Many genes for enzymes involved in the biosynthesis of various lipid classes were identified in the 2000s with the help of newly available databases of cyanobacterial genomic sequences. Many of the proteins encoded by the identified genes are homologous to their respective counterparts in chloroplasts, supporting the endosymbiosis theory, but some of them are structurally distinct. This distinction suggests the possibility that original genes were replaced by unrelated genes during the evolution of chloroplasts from cyanobacteria. The biosynthesis of membrane lipids in cyanobacteria is regulated by environmental conditions, indicating that membrane lipids play an active role in adaptive processes. Studies of cyanobacterial lipids have shed light both on the evolution of chloroplasts and the adaptation of photosynthetic organisms to changes in environmental conditions.

I Introduction

According to the fluid mosaic model of biomembranes, membrane lipids form dynamic bilayers that provide a fluid environment in which membrane proteins can perform their respective functions. Thus, membrane lipids are both structural and functional components of biomembranes (Singer and Nicolson, 1972), playing crucial roles in the biogenesis and structural and functional integrity of individual membrane proteins (see Chapters 11 and 12) and as signaling molecules that respond to physiological stimuli (see Chapters 5 and 18). Association of particular membrane lipids with membrane proteins at specific sites has been demonstrated by x-ray crystallography and suggests direct roles for membrane lipids as cofactors in the activities of membrane proteins (see Chapter 10). Therefore, a full understanding of membrane function requires characterization not only of membrane proteins but also of the membrane lipids that interact with membrane proteins and/or generate signals. Moreover, it is now clear that membrane–lipid metabolism is modulated in response to changes in environmental factors and mediates biochemical responses that are required for adaptation to ambient conditions. Thus, the complete characterization of the enzymes (and their genes) that regulate the biosynthesis of membrane lipids is essential.

Oxygenic photosynthesis in cyanobacterial cells resembles that in the chloroplasts of eukaryotic plants. The endosymbiosis theory posits that cyanobacteria are the ancestors of chloroplasts. Cyanobacteria have three membrane systems, namely, thylakoid membranes, plasma (inner) membranes and outer membranes. The thylakoid membranes are the sites of the photosynthetic apparatus that plays a central role in the conver-

Abbreviations: ACP – Acyl-carrier protein; CDP – Cytidine 5′-diphosphate; CMP – Cytidine 5′-monophosphate; CTP – Cytidine 5′-triphosphate; Cyt b_5 – Cytochrome b_5; DG – Diacylglycerol; DGDG – Digalactosyldiacylglycerol; ER – Endoplasmic reticulum; FAS – Fatty acid synthase; Fd – Ferredoxin; FNR – NADPH: ferredoxin oxidoreductase; GOGAT – Glutamine: 2-oxoglutarate aminotransferase (glutamate synthase); G3P – Glycerol 3-phosphate; GPAT – Glycerol-3-phosphate acyltransferase; LPA – Lysophosphatidic acid; LPAAT – Lysophosphatidic acid acyltransferase; MGDG – Monogalactosyldiacylglycerol; MGlcDG – Monoglucosyldiacylglycerol; ORF – Open reading frame; PA – Phosphatidic acid; PG – Phosphatidylglycerol; PGP – Phosphatidylglycerophosphate; PS – Photosystem; SQDG – Sulfoquinovosyldiacylglycerol; UDP – Uridine 5′-diphosphate; X:Y – A fatty acid with X carbon atoms and Y double bonds. Where indicated, numbers in parenthesis represent the positions of double bonds relative to the carboxyl end of the hydrocarbon chain.

sion of light energy to chemical energy. The plasma membranes and outer membranes contain porins and permeases that facilitate the transport of ions and solutes across these membranes (Jürgen and Benz, 1988; Laudenbach and Grossman, 1991). A full understanding the biochemical and molecular biological aspects of membrane lipids in cyanobacteria is necessary if we are to clarify the ways in which lipids contribute to the general functions of cyanobacterial membrane systems and, also, to verify the endosymbiosis theory as it relates to membrane lipids. Furthermore, clarification of the roles of membrane lipids in cyanobacteria will lead to a clearer understanding of the ways in which chloroplasts utilize membrane lipids to modulate membrane function, and it should also advance discussions of the functional evolution of membrane lipids in photosynthetic organisms.

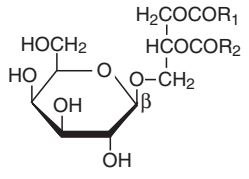
The basic framework for the biosynthesis of membrane lipids was established by tracer experiments with radio-labeled compounds in vivo in cyanobacteria as well as in other organisms. However, many of the enzymes involved in lipid metabolism are integral membrane proteins, which are difficult to solubilize and purify by traditional biochemical techniques. Resultantly, the cloning of the genes for these enzymes presents challenges. Two strategies have been developed to overcome the problems associated with the identification of the enzymes and genes required for lipid biosynthesis in cyanobacteria. First, a procedure has been established for the disruption of any gene of interest by homologous recombination in unicellular cyanobacteria, such as *Synechocystis* sp. PCC 6803 (hereafter called *Synechocystis*), through natural transformation, that is to say, by incubating cells with the DNA to be introduced (Golden, 1988), or by conjugation in the case of filamentous cyanobacteria, such as *Anabaena* sp. PCC 7120 (Cai and Wolk, 1990). Second, databases of genomic sequences have been generated for more than 30 strains of cyanobacteria, including strains that are amenable to transformation (http://bacteria.kazusa.or.jp/cyanobase/index.html). Thus, it is now possible to identify genes for enzymes involved in the biosynthesis of lipids through reverse genetics, namely, by disrupting a putative gene, identified in a database, that encodes a homolog of a known protein involved in lipid biosynthesis, with subsequent characterization of the disruptant. Alternatively, it is possible to take advantage of the small sizes of cyanobacterial genomes (e.g., 3.6 Mb in *Synechocystis*) for cloning of the gene of interest by more standard genetic techniques, namely, by generating mutants, by random mutagenesis, that are defective in the biosynthesis of lipids, with subsequent characterization of fragments of genomic DNA that are able to complement the mutation of interest. The identification of genes involved in the biosynthesis of lipids will enable us to investigate molecular-genetical and biochemical aspects of the encoded enzymes.

Since the publication, a decade ago, of a review of membrane lipids in cyanobacteria by Wada and Murata (1998), remarkable advances have been made in the identification of the genes for enzymes involved in the biosynthesis of lipids and in the elucidation of mechanisms of induction of expression of genes for desaturases in response to low temperature. In this chapter, we summarize recent developments in studies of the membrane lipids of cyanobacteria, including details of the biosynthesis of fatty acids and lipids. We also discuss the enzymes and genes required for the biosynthesis of lipids and their regulation in response to changes in environmental factors. Thus, this chapter can be considered to be a sequel to the above mentioned review.

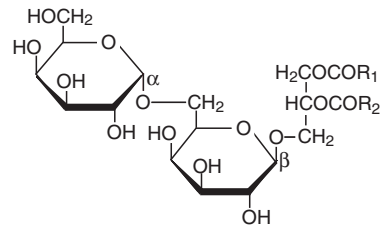
II Characteristics of Cyanobacterial Lipids

A Lipid Classes

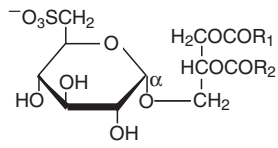
The major membrane lipids of cyanobacteria include four classes of glycerolipid (Wada and Murata, 1998). They have two acyl groups esterified at the *sn*-1 and *sn* -2 positions of the glycerol moiety and a polar head group at the *sn*-3 position, which characterizes each individual class of lipid (Fig. 1). Three classes consist of glycolipids, namely, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG). The other class of lipid is the phospholipid phosphatidylglycerol (PG). All of these cyanobacterial lipids are characteristic lipids in the chloroplast membranes of eukaryotic plants (see Chapter 3). MGDG has a head group of 1β-galactose linked to the diacylglycerol (DG) moiety, whereas DGDG has a head group of digalactose, with the second galactose bound to the first galactose of MGDG by an α1–6 glycosidic link-



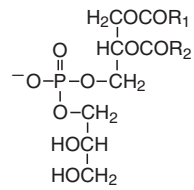
3-β-D-Galactosyl-1,2-diacyl-*sn*-glycerol
(Monogalactosyldiacylglycerol; MGDG)



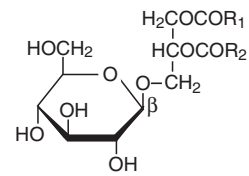
3-[α-D-Galactosyl-(1→6)-β-D-galactosyl]-1,2-diacyl-*sn*-glycerol
(Digalactosyldiacylglycerol; DGDG)



3-[6-Sulfo-6-deoxy-α-D-glucosyl)-1,2-diacyl-*sn*-glycerol
(Sulfoquinovosyldiacylglycerol; SQDG)



3-(3-*sn*-Phosphatidyl)glycerol
(Phosphatidylglycerol; PG)



3-β-D-Glucosyl-1,2-diacyl-*sn*-glycerol
(Monoglucosyldiacylglycerol; MGlcDG)

Fig. 1. Structures of membrane lipids in cyanobacteria. R_1 and R_2 denote hydrocarbon chains of fatty acids.

age (Carter et al., 1956). SQDG contains 6-deoxy-6-sulfo-α1-glucose as the head group, and PG contains *sn*-glycerol 1-phosphate (Benson and Maruo, 1958; Benson, 1963). The latter two lipids are categorized as acidic lipids because of their negative charge at neutral pH. In addition to these four classes of lipids, cyanobacteria contain a minor glycolipid, monoglucosyldiacylglycerol (MGlcDG), which has a 1β-glucose linked to DG (Sato and Murata, 1982a; Wada and Murata, 1998).

In cyanobacteria, MGDG is the most abundant lipid (as it is in chloroplasts), accounting for 40–60% of total lipids, followed by DGDG, SQDG, and PG, each of which represents 10–20% of total lipids (e.g., Murata et al., 1992; Wada and Murata, 1998). Thylakoid membranes are the predominant membrane system in cyanobacteria and, thus, their lipid composition is similar to that of the total cellular membranes (Sakurai et al., 2006). The thylakoid membranes of chloroplasts contain the same lipid classes as those of cyanobacterial cells, with MGDG being the most abundant lipid, and DGDG, SQDG, and PG accounting for the remainder. This similarity provides evidence that suggests that cyanobacteria might be the evolutionary ancestors of chloroplasts (Dorne et al., 1990).

The membranes of *Gloeobacter violaceus* PCC 7421 differ from those described above. They lack SQDG as a result of the absence of the genes required for the synthesis of SQDG (see below; Selstam and Campbell, 1996; Nakamura et al., 2003). *G. violaceus* PCC 7421 is considered to be the most primitive of known extant cyanobacterial strains on the basis of molecular phylogenetic analysis of the small subunit of ribosomal RNA (Douglas and Turner, 1991). In addition, this strain lacks thylakoid membranes and its photosynthetic machinery is localized in the plasma membrane (Rippka et al., 1974; Koyama et al., 2008). These unusual characteristics of *G. violaceus* PCC 7421 might have been inherited from ancient cyanobacteria, namely, from an ancestral form of cyanobacterium that might not have been able to synthesize SQDG. The system for the biosynthesis of SQDG might have been acquired during subsequent evolution that resulted in most of the cyanobacterial strains that have been studied to date.

Recently reported studies of the lipid compositions of marine cyanobacterial species, such as *Prochlorococcus* MED4 and *Synechococcus* sp. WH8103, revealed, by contrast, that SQDG dominates membrane lipids in these species, with levels ranging from 38% to 66% of total lipids, while MGDG and PG account for less than 25% and 10%, respectively, of the total lipids (Van Mooy et al., 2006). The cited authors proposed the evolutional acquisition, by the marine cyanobacteria, of an adaptive mechanism whereby sulfur replaces phosphorus in membrane lipids to reduce the cellular demand for phosphorus in phosphorus-limited regions of the ocean.

B Fatty Acids and their Distribution at the sn-*Positions of the Glycerol Backbone*

Cyanobacteria contain saturated and *cis*-unsaturated fatty acids with chains of 16 and 18 carbon atoms (C16 and C18 acids) as the major fatty acids in their membrane lipids. The extent of unsaturation of these fatty acids varies among strains. Classification of cyanobacteria into four groups in terms of the fatty acid composition of their total lipids was proposed by Kenyon and coworkers (Kenyon, 1972; Kenyon et al., 1972). Subsequently, the classification was reevaluated in terms of the fatty acid composition at each *sn*-position of lipids in the respective lipid classes (Murata et al., 1992; Murata and Wada, 1995; Wada and Murata, 1998).

According to this classification, Group 1 includes strains, such as *Synechococcus* sp. PCC 6301 and *Mastigocladus laminosus*, that have only saturated and monounsaturated fatty acids (Sato et al., 1979; Murata et al., 1992). *M. laminosus* was shown to have the molecular species, 16:1 and 18:1 at the *sn*-1 position/16:0 and 16:1 at the *sn*-2 position (16:1, 18:1/16:0 and 16:1) in MGDG and DGDG and 16:0 and 18:1/16:0 in SQDG and PG (Murata et al., 1992).

In contrast to those in Group 1, strains in Groups 2, 3, and 4 contain polyunsaturated C18 acids, namely, α-18:3 [18:3(9,12,15)], γ-18:3 [18:3(6,9,12)], and 18:4(6,9,12,15), respectively. Group 2 includes strains such as *Anabaena variabilis* and *Synechococcus* sp. PCC 7002. The former strain contains the molecular species, 18:1, 18:2, α-18:3/16:0, 16:1 and 16:2 in MGDG and DGDG and 18:1, 18:2 and α-18:3/16:0 in SQDG and PG (Sato et al., 1979). Because of the presence of 18:1, 18:2, and α-18:3, the fatty acid composition of strains in Group 2 is similar to that of chloroplasts. By contrast, Group 3 includes strains, such as *Spirulina platensis* and *Synechocystis* sp. PCC 6714, that contain the molecular species 18:1, 18:2 and γ-18:3/16:0 in MGDG and DGDG, and 18:1 and 18:2/16:0 in SQDG and PG (Murata et al., 1992). Group 4 includes strains such as *Synechocystis* and *Tolypothrix tenuis*. The former strain contains the molecular species 18:1, 18:2, γ-18:3, and 18:4/16:0 in MGDG and DGDG, and 18:1, 18:2 and α-18:3/16:0 in SQDG and PG (Wada and Murata, 1990; Murata et al., 1992). This classification of cyanobacteria also reflects the specific desaturases that are present in each group of cyanobacteria (see below; Murata and Wada, 1995; Nishida and Murata, 1996; Wada and Murata, 1998; Chintalapati et al., 2006). Moreover, profiles of the fatty acids of total lipids are reflected to some extent in the taxonomic and phylogenetic classification of the genera *Anabaena* and *Nostoc* that belong to Group 2 (Li and Watanabe, 2001; Liu et al., 2003).

Strong specificity at the *sn*-2 position for C16 acids is evident in the respective lipid classes of strains in all four groups. The molecular species of membrane lipids of eukaryotic plants are of only two types: the prokaryotic type with C16 acids at the *sn*-2 position; and the eukaryotic type with C18 acids at this position. The relative levels of the prokaryotic and the eukaryotic types in chloroplast lipids, excluding PG, depend on the plant species and the ratio seems to have decreased during plant evolution. Chloroplast lipids are predominantly of the prokaryotic type in some green algae. Both the prokaryotic and eukaryotic types are found in other green algae and in primitive angiosperms, while the eukaryotic type is found exclusively in other angiosperms (Giroud et al., 1988; Browse and Somerville, 1991; Sato et al., 2003a). In this regard, *G. violaceus* PCC 7421 in Group 2 is an exception. In this strain, C18 fatty acids in MGDG account for as much as 68% of the total, indicating that at least 18% of C18 acids are located at the *sn*-2 position. Thus, more than 36% of MGDG can be accounted for by molecular species of the eukaryotic types with C18 fatty acids at the *sn*-2 position (Selstam and Campbell, 1996).

III Biosynthesis of Lipids

A Saturated Fatty Acids

Fatty acids are synthesized via sequential reactions that are catalyzed by acetyl-CoA carboxylase and fatty acid synthase (FAS) (see Chapter 2). Acetyl-CoA is used as a substrate by acetyl-CoA carboxylase and as a primer for fatty acid synthesis. Acetyl-CoA carboxylase catalyzes the first reaction in the synthesis of fatty acids, namely, the formation of malonyl-CoA from acetyl-CoA and CO_2. All the enzymatic reactions for the biosynthesis of fatty acids, with the exception of the

conversion of acetyl-CoA to malonyl-CoA, are catalyzed by FAS. The FAS of fungi and animals, designated type I FAS, is a multi-functional enzyme that has all the functional domains necessary for fatty acid synthesis and for its role as an acyl-carrier protein (ACP; Wakil et al., 1983). By contrast, the FASs of cyanobacteria and plants consist of several subunits that catalyze individual steps in fatty acid synthesis, and they are designated type II FASs (see Chapter 2; Ohlrogge and Browse, 1995). The malonyl-CoA generated by acetyl-CoA carboxylase is converted to malonyl-ACP by malonyl-CoA: ACP transacylase, a component of FAS. Then malonyl-ACP is converted to butyryl-ACP (C_4) via sequential reactions that are catalyzed by other components of FAS, namely, 3-ketoacyl-ACP synthase, 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase. The resultant butyryl-ACP is further elongated with an acetyl unit from malonyl-ACP via the same reaction cycle, which is repeated until the chain length of the fatty acid reaches C_{16} or C_{18}. Finally, palmitoyl-ACP (16:0-ACP) and stearoyl-ACP (18:0-ACP) are synthesized and used for the biosynthesis of lipids (Lem and Stumpf, 1984a; Stapleton and Jaworski, 1984a).

Early biochemical characterization of cyanobacterial FASs was limited to purification of malonyl-CoA:ACP transacylase from *A. variabilis* (Stapleton and Jaworski, 1984b) and of ACP from *A. variabilis* and *Synechocystis* (Froehlich et al., 1990). However, genes that encode proteins homologous to the components of type II FASs in other organisms can be found in the database of cyanobacterial genomes (Table 1). Moche et al. (2001) identified the gene for β-ketoacyl-ACP synthase II in the genome of *Synechocystis* and overexpressed the gene in *Escherichia coli.* Then they determined the structure of the protein

Table 1. Cyanobacterial enzymes for lipid biosynthesis and their evolution, as suggested by their amino acid sequences.

Enzyme in cyanobacteria in chloroplasts	Cloning	Comparison of amino acid sequence with a counterpart	Reference
FAS	Yes[a]	Related[b]	Moche et al. 2001
GPAT	No	Unrelated[b]	
LPAAT	Yes	Related[b]	Weier et al. 2005 Okazaki et al. 2006
PA phosphatase	Yes	Related	Nakamura et al. 2007
MGlcDG synthase	Yes	Unique[c]	Awai et al. 2006
MGlcDG epimerase	No	Unique[c]	
DGDG synthase	Yes	Unrelated	Awai et al. 2007 Sakurai et al. 2007
UDP-sulfoquinovose synthase	Yes	Related	Güler et al. 1996 Aoki et al. 2004
SQDG synthase	Yes	Related	Güler et al. 2000
CDP-DG synthase	Yes	Related[b]	Sato et al. 2000
PGP synthase	Yes	Related	Hagio et al. 2000
PGP phosphatase	Yes[d]	Unknown	Wu et al. 2006
Δ9 desaturase	Yes	Unrelated[e]	Sakamoto et al. 1994b Chintalapati et al. 2006
Δ12 desaturase	Yes	Related	Wada et al. 1990
Δ15 (ω3) desaturase	Yes	Related	Sakamoto et al. 1994a
Δ6 desaturase	Yes	Unique[f]	Reddy et al. 1993

[a]The gene for β-ketoacyl-ACP synthase II, one of the enzymes for fatty acid synthesis, has been cloned.

[b]Evaluated by comparison of genomic sequence data between genomes of cyanobacteria and *A. thaliana*.

[c]The enzyme is absent in higher plants.

[d]The gene for one isozyme has been cloned.

[e]Higher plants utilize soluble Δ9 acyl-ACP desaturase and/or membrane-bound Δ7 acyl-lipid desaturase for production of monounsaturated fatty acids in chloroplasts. *A. thaliana* has Δ9 acyl-lipid desaturases in the endoplasmic reticulum, but not in chloroplasts (Heilmann et al., 2004).

[f]It has been proposed that, in some plant species, such as *Borage officinalis*, the enzyme is present in the endoplasmic reticulum, but not in chloroplasts (Sayanova et al., 1997).

that was purified from the *E. coli* cells by x-ray crystallographic analysis, and comparison of its three-dimensional structure with those of other condensing enzymes from different pathways led to findings of the structure-function relationships in the condensing enzymes.

B Lipid Classes

1 Phosphatidic Acid

The glycerol backbone for the synthesis of phosphatidic acid (PA) is provided by glycerol 3-phosphate (G3P), and PA is the common intermediate in the synthesis of all classes of lipids (Fig. 2; Nishida and Murata, 1996; Wada and Murata, 1998). G3P is acylated first at the *sn*-1 position by G3P acyltransferase (GPAT) to yield lysophosphatidic acid (LPA) and then at the *sn*-2 position by LPA acyltransferase (LPAAT) to yield PA. It has been assumed for many years that cyanobacterial GPAT and LPAAT preferentially utilize 18:0-ACP and 16:0-ACP, respectively, as donors of the acyl moiety (Lem and Stumpf, 1984b).

Screening of open reading frames (ORFs) that encode the acyltransferase motif in the genomic sequence database of *Synechocystis* resulted in the identification of two genes (*sll1752* and *sll1848*) for membrane-bound LPAATs (Weier et al., 2005; Okazaki et al., 2006), but no gene(s)

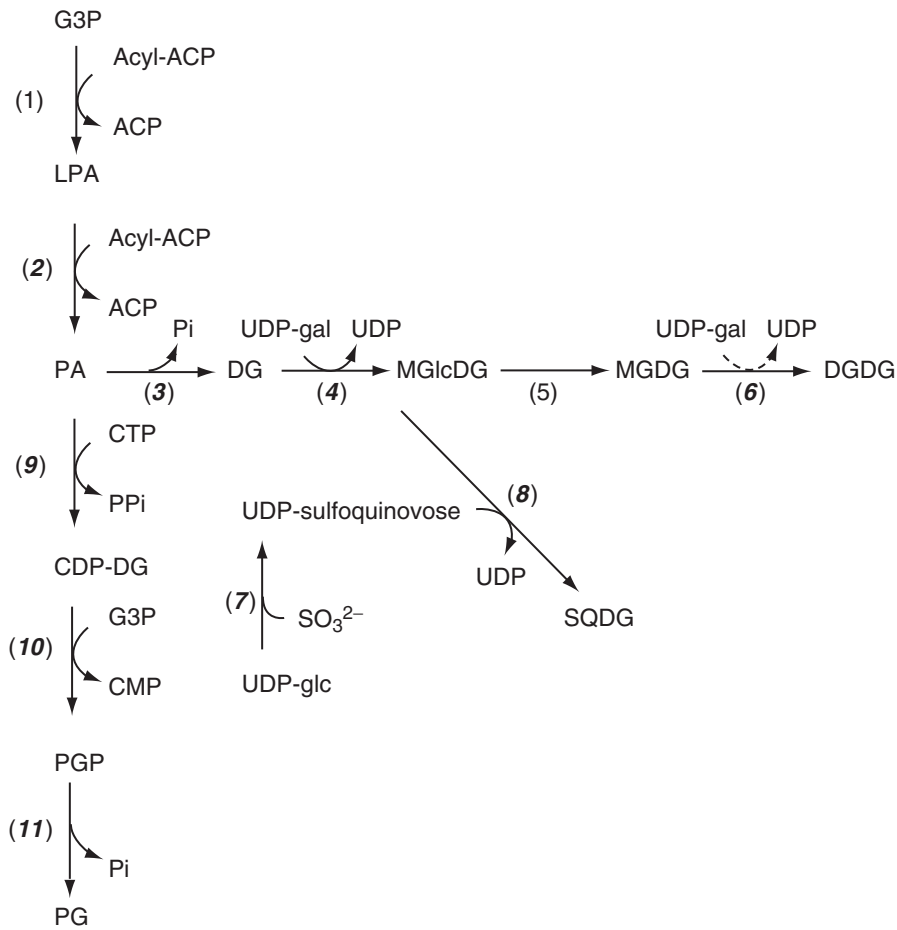


Fig. 2. Biosynthetic pathways to lipids in cyanobacteria. Enzymes that characterize the indicated reactions are as follows: (1) G3P acyltransferase; (2) LPA acyltransferase; (3) PA phosphatase; (4) MGlcDG synthase; (5) MGlcDG epimerase; (6) DGDG synthase; (7) UDP-sulfoquinovose synthase; (8) SQDG synthase; (9) CDP-DG synthase; (10) PGP synthase; and (11) PGP phosphatase. The donor of galactose in reaction (6) is postulated to be UDP-gal. The enzymes whose genes have been identified are indicated in bold italics. Abbreviations: Pi, Inorganic phosphate; PPi, pyrophosphate; UDP-gal, UDP-galactose; and UDP-glc, UDP-glucose.

for GPAT has yet been identified (Table 1). Disruption of *sll1848* (Δ*sll1848*) dramatically decreased the relative levels of C16 acids at the *sn*-2 position in the lipids of the resultant mutant cells, with concomitant increases in levels of C18 acids. Moreover, the product of *sll1848* that was overexpressed in *E. coli* had 130-fold higher specific activity, as LPAAT, for 16:0-CoA than for 18:0-CoA when examined with acyl-CoAs as substrates instead of acyl-ACPs. These results indicated that *sll1848* encodes the major LPAAT, which has strong specificity for 16:0-ACP, while another LPAAT (Sll1752), which prefers 18:0-ACP is dominant in Δ*sll1848* cells. The Sll1752 protein, purified from *E. coli* cells that overexpressed *sll1752* of *Synechocystis*, had LPAAT activity with a preference for 18:0-CoA rather than 16:0-CoA, and the expression of *sll1752* was induced in Δ*sll1848* cells of *Synechocystis* (Okazaki et al., 2006). These findings suggested that *sll1752* intrinsically encodes a minor LPAAT with a preference for 18:0-ACP but the expression of this gene is enhanced so that its product makes a much greater contribution to the cell's LPAAT activity when the expression of *sll1848* is repressed. The chloroplast LPAAT that is encoded by the *ATS2* gene of a higher plant *Arabidopsis thaliana* (Yu et al., 2004) is structurally similar to Sll1848 (Table 1).

2 Galactolipids

After the synthesis of PA, the biosynthetic pathway to lipids in cyanobacteria divides into two branches (Wada and Murata, 1998), as is also the case in chloroplasts (Browse and Somerville, 1991). PA is dephosphorylated by PA phosphatase to yield DG for the synthesis of glycolipid on one branch of the pathway, whereas PA is converted to cytidine 5′-diphosphate-DG (CDP-DG) by CDP-DG synthase for the synthesis of PG on the other branch of the pathway.

Recently, a homolog of the gene for the lipid phosphate phosphatase of the green sulfur bacterium *Chlorobium tepidum* was found in the genome of *Synechocystis*. Nakamura et al. (2007) verified that the gene encodes a PA phosphatase by an assay in vitro of the activity of the gene product, after its expression in *E. coli*, and by complementation, by heterologous expression of the gene, of a lethal mutation in a yeast mutant that lacked PA phosphatase activity. They also used this cyanobacterial gene to identify genes that encode isoforms of plastid PA phosphatase in *A. thaliana* (Table 1).

The DG produced by PA phosphatase is utilized as the substrate for the synthesis of MGlcDG and SQDG. The reaction catalyzed by MGlcDG synthase that converts DG to MGlcDG was shown, in vitro, to proceed via the transfer of glucose from uridine 5′-diphosphate-1α-glucose (UDP-1α-glucose) to the *sn*-3 position of DG in *Synechocystis* and *Anabaena* sp. PCC 7120 (Sato and Murata, 1982c; Awai et al., 2006). This reaction is conserved in cyanobacteria but has not been found in plants. Awai et al. (2006) recently identified the gene for MGlcDG synthase in *Synechocystis* and *Anabaena* sp. PCC 7120 by comparative genomic analysis (Table 1). They first identified candidate genes for MGlcDG synthase in the genome of *Synechocystis* by assuming that (i) the gene product would include a glycosyltransferase motif but would have been categorized, originally, as a protein of unknown function and (ii) the gene would be conserved between the genomes of *Synechocystis* and *Anabaena* sp. PCC 7120. Among the candidate genes, *sll1377* in *Synechocystis* and its homolog, *all3944*, in *Anabaena* sp. PCC 7120 were confirmed as genes for MGlcDG synthase by measurements of MGlcDG synthase activity when each gene was expressed in *E. coli*.

Pulse-chase experiment with $NaH^{14}CO_3$ in *A. variabilis* cells indicated that the radioactivity in the glucose unit of MGlcDG decreased during the chase period, concomitant with an increase in the radioactivity of the galactose unit of MGDG (Sato and Murata, 1982a), while the sum of the radioactivity associated with glucose and galactose units remained almost unchanged. This observation suggested that MGDG might be synthesized by epimerization of the glucose moiety to galactose in MGlcDG. However, the gene responsible for the epimerization remains to be identified (Table 1). The biosynthesis of MGDG from DG via these two reactions in cyanobacteria is quite distinct from that of MGDG in chloroplasts. In chloroplasts, MGDG is synthesized via the direct transfer of galactose from UDP-1α-galactose to DG in a reaction catalyzed by MGDG synthase (see Chapter 3; Table 1; Shimojima et al., 1997).

The net incorporation of radioactivity into DGDG in *A. variabilis* cells during incubation

with $NaH^{14}CO_3$ decreased by only 52% in the presence of cerulenin, an inhibitor of FAS, despite the dramatically reduced incorporation of radioactivity into MGlcDG and MGDG (Sato and Murata, 1982a). The incorporation of radioactivity into DGDG in the presence of cerulenin suggested that DGDG might be synthesized via the transfer of galactose to preexisting MGDG. Although the molecule that donates galactose to DGDG synthase has not been identified in cyanobacteria, it is likely to be UDP-1α-galactose, as it is in chloroplasts (see Chapter 3; Kelly and Dörmann, 2002). No homologs of the gene for DGDG synthase of the "green lineage" (see Table 1; Dörmann et al., 1999; Nishiyama et al., 2003; Riekhof et al., 2005) have been found in cyanobacterial genomes. However, a candidate gene (*dgdA*) for a cyanobacterial DGDG synthase was identified in *Synechocystis* by comparative genomic analysis (Awai et al., 2007; Sakurai et al., 2007). Disruption of the *dgdA* gene in *Synechocystis* resulted in a mutant that was defective in DGDG synthesis, and when the *dgdA* gene was expressed in *E. coli* together with a cucumber gene for MGDG synthase, DGDG was produced, as anticipated. These findings indicated that the *dgdA* gene of *Synechocystis* encodes a DGDG synthase (Table 1). Among eukaryotic photosynthetic organisms, a homolog of the *dgdA* gene has been found only in the plastid genome of the unicellular red alga *Cyanidioschyzon merolae*. The glycosyl residues of both the MGlcDG and MGDG that are synthesized in cyanobacteria are in the β-anomeric configuration, and the glycosidic linkage between two galactose moieties of DGDG is in the α-anomeric configuration (Fig. 1). In view of the anomeric configuration of the hexose donor, we can classify both the MGlcDG synthase and the MGDG synthase of cyanobacteria as members of the inverting glycosyltransferase family, whereas cyanobacterial DGDG synthase seems to be a retaining glycosyltransferase, as is the DGDG synthase found in chloroplasts.

3 Sulfoquinovosyldiacylglycerol

In higher plants, SQDG is synthesized from DG by a two-step reaction in chloroplasts (see Chapter 3). First, UDP-6-sulfo-6-deoxy-1α-glucose (UDP-sulfoquinovose), which is the donor of sulfoquinovose for the synthesis of SQDG, is synthesized from UDP-glucose and sulfite by UDP-sulfoquinovose synthase in the stroma (Sanda et al., 2001). Then, SQDG is synthesized via the transfer of sulfoquinovose from UDP-sulfoquinovose to DG in a reaction that is catalyzed by SQDG synthase in envelope membranes (Heinz et al., 1989). In *A. thaliana*, UDP-sulfoquinovose synthase and SQDG synthase are encoded by the *SQD1* and *SQD2* genes, respectively (Essigmann et al., 1998; Yu et al., 2002). Homologs of *SQD1* and *SQD2* have been found in cyanobacterial genomes and participate in the synthesis of SQDG in cyanobacteria (see below; Table 1). Therefore, the two sequential reactions mentioned above are also assumed to lead to the synthesis of SQDG in cyanobacteria.

A set of the genes for the synthesis of SQDG was initially identified in the anoxygenic photosynthetic bacterium *Rhodobacter sphaeroides* as a result of cloning of genes that complemented mutations that interfered with the synthesis of SQDG. These genes were designated *sqdA*, *sqdB*, *sqdC*, and *sqdD* (Benning, 1998). After identification of these genes in *R. sphaeroides*, a homolog of *sqdB* was cloned from *Synechococcus* sp. PCC 7942 (hereafter called *Synechococcus*) by heterologous hybridization with a probe that included part of the sequence of *sqdB* from *R. sphaeroides*. Güler et al. (1996) confirmed that the cloned gene encoded an enzyme involved in the biosynthesis of SQDG by showing that disruption of the gene in *Synechococcus* resulted in a mutant that was defective in the synthesis of SQDG. A gene for a protein with a glycosyltransferase motif was then found just downstream of *sqdB* in the genome of *Synechococcus* (Güler et al., 2000). Since disruption of this latter gene also interfered with the synthesis of SQDG in *Synechococcus*, this gene, which presumably encodes an SQDG synthase, was designated *sqdX*. SQDG synthase is classified as a retention-type glycosyltransferase, as is DGDG synthase, while MGlcDG synthase and MGDG synthase are both of inversion-type enzymes. In contrast to the location of the genes in *Synechococcus*, homologs of the *sqdB* and *sqdX* genes in *Synechocystis* are located 1.8 Mb apart on the genome. Homologs of *sqdB* and *sqdX* have been found not only in cyanobacteria but also in the higher plant *A. thaliana*, the red alga *C. merolae*, and the diatom *Thalassiosira pseudonana*, which is presumed to be the secondary

symbiont of a red alga (Armbrust et al., 2004; Matsuzaki et al., 2004). Thus, it seems that the system for the synthesis of SQDG in cyanobacteria, unlike those for the synthesis of MGDG and of DGDG, has been conserved through the putative evolution of cyanobacteria into chloroplasts. Among the four *sqd* genes for the synthesis of SQDG in *R. sphaeroides*, three genes, namely, *sqdA*, *sqdC* and *sqdD* are specific to this species (Benning, 1998). We can conclude, then, that the system for the synthesis of SQDG in oxygenic photosynthetic organisms is not linked evolutionarily to that in *R. sphaeroides*, except with respect to *sqdB*, which is also found in nonphotosynthetic bacteria, such as *Sinorhizobium meliloti* (Weissenmayer et al., 2000) and *Sulfolobus tokodaii* (Kawarabayasi et al., 2001).

The UDP-sulfoquinovose synthase that is encoded by *sqdB* in cyanobacteria and by *SQD1* in eukaryotic photosynthetic organisms is particularly relevant to the putative evolution of cyanobacteria into chloroplasts. A molecular phylogenetic analysis of UDP-sulfoquinovose synthase allows the division of cyanobacteria into two groups: one that includes cyanobacteria, such as *Synechocystis* and *Anabaena* sp. PCC 7120, and one that includes the genera *Prochlorococcus* and *Synechococcus*. The green alga *Chlamydomonas reinhardtii*, and the higher plant *A. thaliana* are positioned close to the former group of cyanobacteria, whereas the red alga *C. merolae* and the diatom *T. pseudonana* are positioned near the latter group on the phylogenetic tree (Sato et al., 2003b; Van Mooy et al., 2006). Although the number of sequences available for the "red" lineage is too limited to allow meaningful conclusions, the close relationship among the former group of cyanobacteria, *C. reinhardtii* and *A. thaliana* suggests the selection of cyanobacteria that harbor the *sqdB* gene of the former type in the evolution to chloroplasts (Sato, 2004). However, the enzymatic function of UDP-sulfoquinovose synthase seems to be conserved between the two phylogenetic groups because the SQDG-deficient phenotype of *Synechococcus* Δ*sqdB* cells was partially rescued by the expression of a cDNA derived from the *SQD1* gene of *C. reinhardtii* (Sato et al., 2003b). Shimojima et al. (2005) purified SQD1 from *Spinacia oleracea* as a protein complex and found that the complex contained both SQD1 and ferredoxin-dependent glutamate synthase (Fd-GOGAT), which exploited a flavin mononucleotide as its cofactor for the binding of sulfite. The protein complex had stronger affinity for sulfite than that of the free SQD1 protein and, thus, the cited authors inferred that Fd-GOGAT might play a role in channeling sulfite to SQD1. In this regard, it is noteworthy that a yeast two-hybrid assay of *Synechocystis* proteins revealed that both SqdB and Fd-GOGAT were able to associate with Hik21, which is a sensory histidine kinase that is a member of a family of two-component sensory systems (Sato et al., 2007).

Physiological characterization of SQDG-deficient mutants revealed that SQDG is essential for growth and for the normal functioning of photosystem (PS) II in *Synechocystis*. However, SQDG is dispensable in *Synechococcus* (Güler et al., 1996; Aoki et al., 2004). A requirement for SQDG was also demonstrated in the case of PSII of *C. reinhardtii* as in *Synechocystis* (Sato et al., 1995a; Minoda et al., 2002, 2003). However, the absence of a requirement for SQDG for growth, as observed in *Synechococcus*, was also evident in *R. sphaeroides*, *C. reinhardtii*, and *A. thaliana* during studies of the respective SQDG-deficient mutants (Benning et al., 1993; Sato et al., 1995b; Yu et al., 2002).

4 Phosphatidylglycerol

The synthesis of PG from PA involves three reactions in *E. coli*, namely, transfer of cytidine 5′-monophosphate (CMP) from cytidine 5′-triphosphate (CTP) to PA to yield CDP-DG in a reaction catalyzed by CDP-DG synthase, conversion of CDP-DG to phosphatidylglycerophosphate (PGP) as a result of the transfer of G3P to the PA moiety of CDP-DG by PGP synthase, and dephosphorylation of PGP by PGP phosphatase (Dowhan, 1997). PG is probably synthesized via the same sequence of reactions in cyanobacteria (see Chapter 11; Wada and Murata, 1998). In 2000, analysis of the genomic sequence of *Synechocystis* revealed the presence of two genes homologous to the *cdsA* and *pgsA* genes of *E. coli*, which encode CDP-DG synthase and PGP synthase, respectively (Hagio et al., 2000; Sato et al., 2000). These genes were identified as genes for the corresponding enzymes by cited authors, who showed that mutations in the respective genes resulted in *Synechocystis* cells with a defect

in the synthesis of PG and that the synthesis of PG in a *pgsA* mutant of *E. coli* was rescued by heterologous expression of the *pgsA* gene from *Synechocystis* (Table 1; Hagio et al., 2000; Sato et al., 2000). Both mutants of *Synechocystis* that had defects in the synthesis of PG required exogenous PG for growth, as well as for construction of the photosynthetic apparatus, indicating that PG is physiologically essential in this cyanobacterium. This observation reflects similar findings with a *pgp1* mutant of *A. thaliana* (see Chapter 11). After identification of the *pgsA* gene in *Synechocystis*, the *PGP1* gene for a PGP synthase, which is homologous to the *pgsA* gene of *Synechocystis* and whose product is localized both in chloroplasts and mitochondria, was identified in *A. thaliana* (Table 1; Hagio et al., 2002; Xu et al., 2002; Babiychuk et al., 2003).

Even though two genes, *pgpA* and *pgpB*, which encode independent PGP phosphatases, are present in *E. coli*, only two homologs of *pgpB*, but no homologs of *pgpA*, have been found in the genome of *Anabaena* sp. PCC 7120. One of the two homologs (*alr1715*) was characterized as the gene responsible for some of the PGP phosphatase activity in *Anabaena* cells: the level of PG in Δ*alr1715* mutant cells was 30% lower than in wild-type cells (Wu et al., 2006). It is likely that another uncharacterized gene(s), such as the other homolog of the *pgpB* gene of *E. coli* (*all7623*), might also encode a PGP phosphatase. Homologs of *alr1715* are found in a limited group of cyanobacteria that includes strains, such as *Anabaena variabilis* ATCC 29413 and *Nostoc punctiforme* ATCC 29133. No other homologs of *pgpA* and *pgpB* have been found in currently available cyanobacterial genomes, indicating that cyanobacterial PGP phosphatases are structurally unrelated to PgpA and PgpB of *E. coli*. By contrast, homologs of the *cdsA* and *pgsA* genes are present in all cyanobacterial genomes that have been sequenced to date.

C Desaturation of Fatty Acids

1 The Fatty Acid Desaturation Reaction

The desaturation of fatty acids involves O_2-dependent dehydrogenation reactions that are catalyzed by desaturases via an electron-donating system (Fig. 3). Each reaction proceeds in a highly stereoselective and regioselective manner. Thus, a given desaturase is able to catalyze insertion of only one *cis*- or one *trans*-double bond at one specific position of fatty acids. Desaturases commonly contain non-heme di-iron as catalytic centers, which probably form a high-valency di-iron oxo species for the dehydrogenation reaction (Behrouzian and Buist, 2003). They can be divided into two structurally distinct classes. One class consists of a large number of integral membrane proteins with a di-iron moiety that is probably coordinated to three conserved histidine motifs (Los and Murata, 1998). The other class consists of a small number of soluble desaturases with a carboxylate-bridged di-iron moiety (Lindqvist et al., 1996). Moreover, the former class can be further divided into two subclasses on the basis of acyl substrates, namely, acyl-CoA and acyl-lipid desaturases. The latter enzymes are known as acyl-ACP desaturases (Murata and Wada, 1995; Wada and Murata, 1998).

Pulse-chase experiment with $NaH^{14}CO_3$ in *A. variabilis* demonstrated the stepwise desaturation of 18:0/16:0 species of MGlcDG, MGDG, SQDG, and PG at the Δ9, Δ12, and Δ15 positions of C18 acids and of MGlcDG and MGDG at the Δ9 and Δ12 positions of C16 acids (Sato and Murata, 1982b). Unexpectedly, no positive evidence was obtained with respect to desaturation on DGDG. It was assumed, therefore, that the desaturation of fatty acids occurs exclusively when fatty acids are bound to lipids other than DGDG, and cyanobacterial desaturases were classified as acyl-lipid desaturases (Murata and Wada, 1995; Wada and Murata, 1998). Accordingly, Murata and colleagues proposed the following group-dependent modes of fatty acid desaturation in cyanobacteria that they based on the fatty acids at the *sn*-1 and -2 positions of the respective membrane lipids (Fig. 3b; Murata et al., 1992; Murata and Wada, 1995; Wada and Murata, 1998):

Group 1: Desaturation at the Δ9 position of 18:0 at the *sn*-1 position of MGDG, of 16:0 at the *sn*-1 and *sn*-2 positions of MGDG, and of 18:0 at the *sn*-1 positions of SQDG and PG.

Group 2: Desaturation at the Δ9, Δ12, and Δ15 (or ω3, see below) positions of C18 acids at the *sn*-1 positions of MGDG, SQDG and PG, and desaturation at the Δ9 and Δ12 positions of C16 acids at the *sn*-2 position of MGDG.

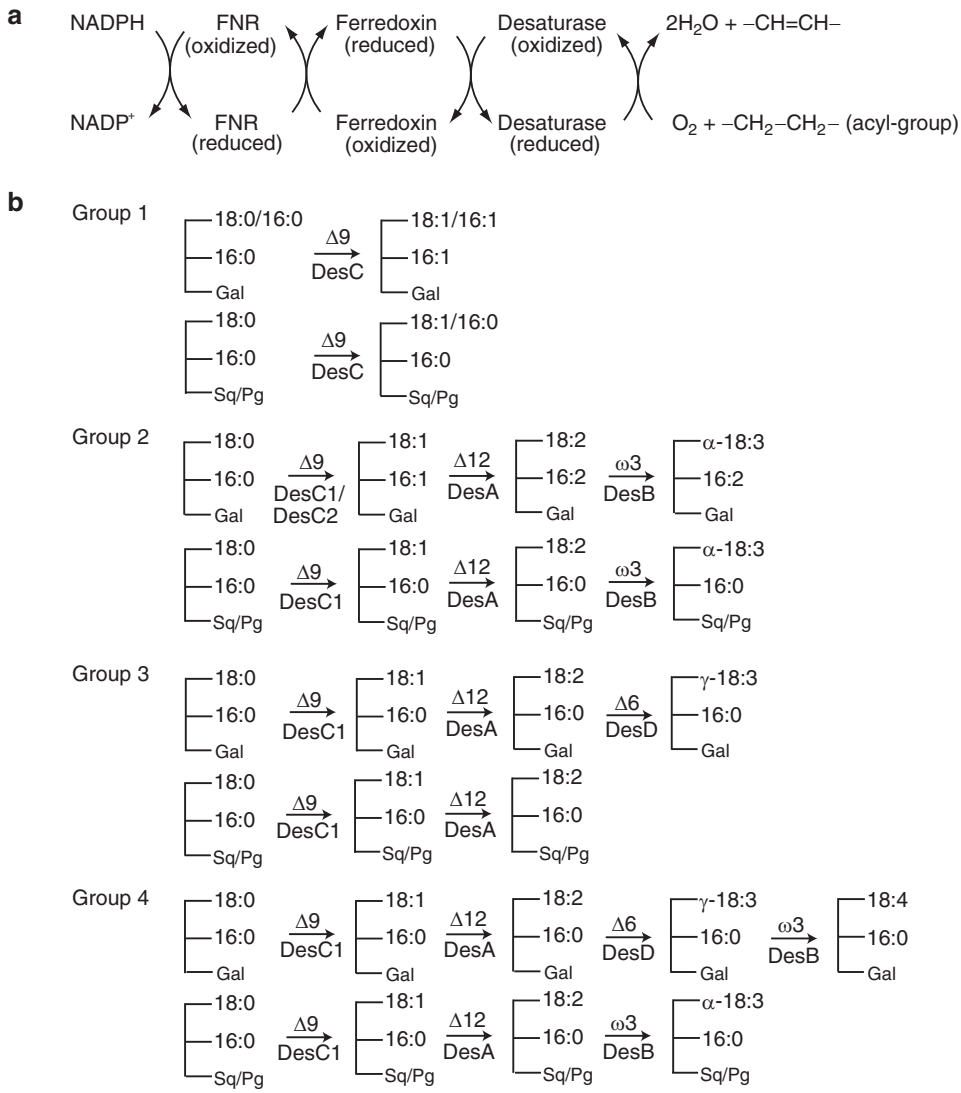


Fig. 3. Desaturation of fatty acids in cyanobacteria. (**a**) Components involved in desaturation reactions. FNR, Ferredoxin oxidoreductase. (**b**) Main pathways for fatty acid desaturation in the indicated groups of cyanobacteria. The desaturation of fatty acids on MGDG and the desaturation of fatty acids on SQDG and PG are indicated independently. Desaturation of fatty acids at the Δ9, Δ12, ω3, and Δ6 positions is catalyzed by desaturases DesC, DesA, DesB, and DesD, respectively. DesC can be classified as DesC1, DesC2, and additional DesC clades on the basis of molecular phylogenetic analysis, with group-dependent distribution (Chintalapati et al., 2006). The Δ9 and Δ12 desaturation reactions at the *sn*-1 and *sn*-2 positions on MGDG in Groups 1 and 2 proceed independently but are shown coupled here for simplicity's sake. Note that the desaturation of 18:0 and 16:0 at Δ9 is catalyzed by DesC1 and DesC2, respectively, in strains that belong to Group 2. Gal, Galactose; Pg, phosphoglycerol; and Sq, sulfoquinovose.

Group 3: Desaturation at the Δ9, Δ12, and Δ6 positions of C18 acids at the *sn*-1 position of MGDG, and desaturation at the Δ9 and Δ12 positions of C18 acids at the *sn*-1 positions of SQDG and PG.

Group 4: Desaturation at the Δ9, Δ12, Δ6, and Δ15 (ω3) positions of C18 acids at the *sn*-1 position of MGDG, and desaturation at the Δ9, Δ12, and Δ15 (ω3) positions of C18 acids at the *sn*-1 positions of SQDG and PG.

Since a given desaturase is responsible for introduction of a double bond at one specific site in fatty acids, the group-dependent modes of fatty acid desaturation can be explained in terms of the types of desaturase, namely, the Δ6, Δ9, Δ12, and Δ15 (ω3) desaturases, that are present

in each group of cyanobacteria (see below). Furthermore, recent classification of the Δ9 desaturases into DesC1, DesC2, and additional clades on a molecular phylogenetic tree accords with the categorization of cyanobacteria on the basis of fatty acid composition (Chintalapati et al., 2006): strains in Groups 3 and 4 have a *desC1* gene for a Δ9 desaturase that acts on 18:0 at the *sn-1* position of lipids. By contrast, strains in Group 2 have both a *desC1* and a *desC2* gene that encode Δ9 desaturases. The DesC2 desaturase introduces a double bond at the Δ9 position of fatty acids bound to the *sn-2* position of MGDG. However, it seems that each strain in Group 1 has only one *desC* gene (Kiseleva et al., 2000; see databases of genomic sequences, for example, the database of *Synechococcus* at http://bacteria.kazusa.or.jp/cyanobase/index.html), and that the gene, therefore, encodes a Δ9 desaturase that acts both at the *sn*-1 and *sn*-2 positions. It is noteworthy that the DesC desaturases of strains in Group 1 are phylogenetically diverse and cannot be categorized as one particular clade.

The mechanisms by which cyanobacterial desaturases determine the site of insertion of a double bond into a fatty acid was investigated by determining positions of double bonds in fatty acids bound to lipids from *Synechocystis* cells that had been cultured in the presence of a heptanoic acid (7:0) (Higashi and Murata, 1993). In addition to C16 and C18 acids, the cells synthesized C15 acids with a double bond at the Δ9 position and C17 and C19 acids with double bonds at the Δ6, Δ9, Δ12, and ω3 positions. This distribution of double bonds in the unusual fatty acids indicated that the desaturases responsible for the desaturation at the Δ9 and ω3 positions of fatty acids (hereafter called Δ9 and ω3 desaturases, respectively) determine the position at which they will insert a double bond by "measuring" the distance from the carboxyl and methyl termini, respectively, and that the desaturases responsible for desaturation at the Δ6 and Δ12 positions (hereafter called Δ6 and Δ12 desaturases, respectively) determine the position not from the methyl terminus but rather from either the carboxyl terminus or from a preexisting double bond at the Δ9 position.

When supplied with radio-labeled fatty acids with an odd number of carbon atoms, the green alga *Chlorella vulgaris* synthesized methyl group-interrupted diunsaturated fatty acids (C15, C17, and C19 acids) with a double bond at either the Δ9 or the ω9 position (Gurr, 1971). This result suggested that the chloroplast desaturase responsible for conversion of 18:1(9) to 18:2(9,12) might determine the position for insertion of a double bond by reference to the position of a preexisting double bond. Subsequently, it was demonstrated that one of the chloroplast desaturases in *C. reinhardtii* and in *A. thaliana* catalyzes conversion not only of 18:1(9) to 18:2(9,12) but also of 16:1(7) to 16:2(7,10). This desaturase (hereafter called ω6 desaturase) determines the position for insertion of a double bond not from either the carboxyl terminus or the methyl terminus but, rather, by reference to a preexisting double bond. The cloning of the gene and/or the cDNA for the ω6 desaturase from *C. reinhardtii* and *A. thaliana* revealed the structural similarity between the chloroplast ω6 desaturase and the cyanobacterial Δ12 desaturase (Falcone et al., 1994; Sato et al., 1997; see below), suggesting that the cyanobacterial Δ12 desaturase as well as the chloroplast ω6 desaturase determines the position for insertion of a double bond in terms of the distance from the preexisting double bond.

2 Protein Components for Fatty Acid Desaturation

Murata and colleagues published several reviews in the 1990s of the cloning of cyanobacterial genes for fatty acid desaturases (Murata and Wada, 1995; Nishida and Murata, 1996; Wada and Murata, 1998). The entire set of genes for the membrane-bound acyl-lipid desaturases in *Synechocystis* was first cloned more than a decade ago (Table 1), and it includes the *desA* gene for Δ12 desaturase (Wada et al., 1990), the *desB* gene for ω3 desaturase (Sakamoto et al., 1994a), the *desC* gene for Δ9 desaturase (Sakamoto et al., 1994b), and the *desD* gene for Δ6 desaturase (Reddy et al., 1993). The *desA* gene was cloned first as a gene for an acyl-lipid desaturase and it was identified as a genomic clone that complemented the low temperature-sensitive phenotype of mutant cells that were deficient in Δ12 desaturation. The *desD* gene was cloned by a gain-of-function method with *Anabaena* sp. PCC 7120, which is normally unable to desaturate fatty acids at the Δ6 position. The *desC* gene was found in the 5′-upstream region of the *desA* gene in *A. variabilis* as a

homolog of genes for acyl-CoA desaturases in yeast and mammals. Then this gene was used to clone the *desC* gene of *Synechocystis* by heterologous hybridization. The *desB* gene was found in *Synechocystis* by heterologous hybridization, with the *desA* gene as the probe. The discovery of these genes for desaturases in *Synechocystis*, together with the more recent identification of two homologs of *desC* in *Nostoc* sp. (SO-36), designated *desC1* and *desC2* (Chintalapati et al., 2007), has contributed to the classification of cyanobacteria in terms of fatty acid synthesis at the genomic level.

With respect to the positions of the hydrocarbon chains of fatty acids that are desaturated, the cyanobacterial desaturases DesC, DesA, and DesB correspond to the Δ9 acyl-ACP desaturase in chloroplasts, to the ω6 acyl-lipid desaturases in chloroplasts and the endoplasmic reticulum (ER), and to the ω3 acyl-lipid desaturases in chloroplasts and the ER, respectively. Molecular phylogenetic analysis suggests that DesA and DesB are structurally similar to the ω6 and ω3 desaturases in chloroplasts, an observation that is consistent with the endosymbiosis theory (Table 1; Sperling et al., 2003). However, while DesC (the membrane-bound Δ9 acyl-lipid desaturase) is structurally unrelated to the soluble Δ9 acyl-ACP desaturase in chloroplasts, it is similar to the Δ9 acyl-CoA desaturase in the ER of mammals and yeast (Sakamoto et al., 1994b). In general, the desaturases of photosynthetic organisms are distinct from the desaturases of mammals and yeast, with the latter belonging to the family of acyl-CoA desaturases (Sperling et al., 2003).

The electron-donation system for fatty acid desaturation depends on the intracellular localization of each desaturase. With an exogenous supply of Fd, NADPH: Fd oxidoreductase (FNR), and NADPH, thylakoid membranes isolated from *Synechocystis* were capable of desaturating 18:0 to 18:1(9) and 18:1(9) to 18:2(9,12) when the fatty acids were bound to MGDG (Wada et al., 1993a). The dependence of cyanobacterial Δ12 desaturase activity on this electron-donation system was confirmed with extracts of *E. coli* cells, in which the *desA* gene had been overexpressed (Wada et al., 1993b). It had been assumed that cyanobacterial desaturases accept electrons from Fd, which is reduced by electrons from NADPH via the action of FNR, as demonstrated in the case of Δ9, ω6, and ω3 desaturases of chloroplasts (Fig. 3a; McKeon and Stumpf, 1982; Schmidt and Heinz, 1990a, b). By contrast, ω6 and ω3 acyl-lipid desaturases in the ER of higher plants, as well as the Δ9 acyl-CoA desaturase in the ER of mammals, utilize cytochrome b_5 (Cyt b_5) as the electron donor, with Cyt b_5 being reduced by electrons from NADH via the action of NADH: Cyt b_5 oxidoreductase (Kearns et al., 1991). Napier and colleagues showed, moreover, that a cytochrome b_5-like domain, which functions as an electron donor, is fused to the carboxyl terminus of the Δ9 acyl-CoA desaturase in ER of yeast and to the amino terminus or middle regions of the Δ6 and Δ5 desaturases in ER of plants and mammals (Napier et al., 1999). However, none of the cyanobacterial desaturases identified to date includes a Cyt b_5-like domain. It is likely, therefore, that all the cyanobacterial desaturases use Fd as the electron donor. These observations suggest that desaturases have evolved to utilize electron donors that are readily available, namely, Fd in the case of desaturases in cyanobacteria and chloroplasts, and Cyt b_5 in the case of desaturases in the ER.

IV Regulation of Lipid Biosynthesis

A Increases in the Extent of Unsaturation of Membrane Lipids in Response to Low Temperature

Membrane fluidity decreases upon the exposure of membranes to low temperatures and such decreases result in membrane dysfunction (for an early report of this phenomenon, see Sinensky et al., 1974). In several strains of cyanobacteria, the extent of unsaturation of fatty acids in individual lipid classes increases in response to low temperatures, thereby maintaining appropriate membrane fluidity (Sato et al., 1979; Wada and Murata, 1990, 1998; Murata and Wada, 1995; Nishida and Murata, 1996; Quoc and Dubacq, 1997; Chintalapati et al., 2007). Details of the molecular mechanism responsible for the activation of desaturation at low temperatures were clarified in *Synechocystis* (see Chapter 15; Los et al., 1997). As described in Chapter 15, levels of transcripts of the *desA*, *desB*, and *desD* genes rose upon transfer of cell cultures from 34°C to 22°C

as a consequence of enhanced transcription and increases in the stability of the respective transcripts, which, in turn, resulted in elevated levels of the DesA, DesB, and DesD proteins. Extensive analysis indicates that the *hik33* gene encodes the sensory histidine kinase of a two-component system (see below). This kinase perceives the low-temperature signal and promotes signal transduction that induces expression of the *desB* gene (Suzuki et al., 2000; Murata and Los, 2006; Murata and Suzuki, 2006). By contrast, in psychrotolerant *Nostoc* sp. (SO-36) from the Antarctica, which optimally grows at 25°C and can grow even at 10°C, levels of transcripts of the genes for desaturases are unaltered upon transfer of cell cultures from 25°C to 10°C. This observation suggests that increases in the extent of unsaturation of membrane lipids in this strain are induced by the posttranscriptional control of the expression of these genes (Chintalapati et al., 2007).

B Variability in Levels of Phosphatidylglycerol and Sulfoquinovosyldiacylglycerol under Phosphorus-Limiting Conditions

Carbon, phosphorus (P), and sulfur (S) are the primary components of cyanobacterial membrane lipids. Thus, we might reasonably expect that the biosynthesis of these lipids should be regulated by pathways that control the metabolism of these elements. In *Synechococcus* cells when exposed to P-limiting conditions, the relative level of MGDG fell from 60% to 42% and that of PG fell from 16% to 7%, with concomitant increases in relative levels of DGDG from 12% to 28% and of SQDG from 10% to 22% (Güler et al., 1996). In particular, levels of the two acidic lipids, SQDG and PG, rose and fell, respectively, such that the sum of their relative levels changed only a little (26–29%). This phenomenon has also been observed in the eukaryotic photosynthetic organisms *C. reinhardtii* and *A. thaliana* (Essigmann et al., 1998; Riekhof et al., 2003) and in the anoxygenic photosynthetic bacterium *R. sphaeroides* (Benning et al., 1993). The decrease in the level of PG can be interpreted in terms of a metabolic response that shifts the flow of P from the synthesis of PG to the synthesis of compounds that are more important physiologically, such as nucleotides and nucleic acids. The increase in the level of SQDG can be explained in terms of a compensatory mechanism that operates to mitigate the decrease in the level of PG to maintain an appropriate charge balance on, for example, the thylakoid membranes. The *sqdB* mutant of *Synechococcus* is not only incapable of synthesizing SQDG but is unable to limit the synthesis of PG under P-limiting conditions, and it has a much lower ability to adapt to such conditions than the wild type (Güler et al., 1996). SQDG-deficient mutants of *C. reinhardtii*, *A. thaliana*, and *R. sphaeroides* exhibit a similar phenotype (Benning et al., 1993; Yu et al., 2002; Riekhof et al., 2003). These observations suggest that the balanced regulation of the biosynthesis of PG and SQDG and changes in this balance under P-limiting conditions are physiologically relevant to photosynthetic organisms.

Prokaryotes, yeast cells and plant cells have a variety of two-component regulatory systems, each of which exploits a sensory histidine kinase and a response regulator for induction of appropriate gene expression in response to changes in environmental conditions. The histidine kinase senses a physical or chemical stimulus and then it autophosphorylates its transmitter domain and transfers the phosphate group to the receiver domain of the cognate response regulator, which acts as a transcription factor for the target gene(s). Putative genes for 43 sensory histidine kinases and 38 response regulators have been found in the genome of *Synechocystis* (Mizuno et al., 1996). One possible scenario under P-limiting conditions is that a sensory histidine kinase perceives a shortage of P and transduces a signal that suppresses the biosynthesis of PG, and then another sensory kinase, which monitors membrane charge, contributes to the signal transduction that enhances the biosynthesis of SQDG. DNA microarray analysis revealed that SphS and SphR are components of a two-component system that is involved exclusively in the induction of gene expression in response to P limitation in *Synechocystis* (Suzuki et al., 2004).

The opposing changes in levels of MGDG and DGDG in *Synechococcus* under P-limiting conditions might reflect stimulation of the galactosylation of MGDG to DGDG to meet an increased demand for DGDG by an as yet uncharacterized mechanism. Thus, for example, a mutant of *Synechocystis* lacking DGDG with a mutation in the *dgdA* gene for DGDG synthase exhibited

substantially retarded growth under P-limiting conditions, as compared to the wild type, while the mutation had little effect on growth when there was an adequate supply of P (Awai et al., 2007). Furthermore, it is relevant that *R. sphaeroides* accumulates a dihexosyl lipid, glucosylgalactosyl diacylglycerol, under P-limiting conditions (Benning et al., 1995), and relative levels of DGDG also increase in response to P limitation in *A. thaliana* (Härtel et al., 2000). However, the latter increase in DGDG content occurs not only in chloroplast membranes but also in non-chloroplast membranes, such as plasma membranes and mitochondrial membranes, to compensate for decreased levels of phospholipids under P-limiting conditions.

C Compensation for a Genetic Defect in Lipid Biosynthesis

Genetic manipulation of metabolic enzymes often results in changes in metabolic flow that cannot be explained simply by a substrate–product relationship. Such changes suggest that a compensatory system might operate to mitigate the metabolic changes caused by the artificially induced decrease or increase in the activity of the enzyme of interest. Such observations might provide clues to novel mechanisms that regulate metabolic pathways. For example, disruption of the gene for the major LPAAT in *Synechocystis*, which is strongly specific for 16:0-ACP, induced the minor LPAAT, which prefers 18:0-ACP to 16:0-ACP as its substrate (Okazaki et al., 2006). It is possible that *Synechocystis* has a mechanism that regulates the ratio of C16 to C18 fatty acids at the *sn*-2 position of membrane lipids by modulating the levels of these two LPAATs.

Loss of PG by disruption of the *pgsA* gene for PGP synthase in *Synechocystis* and of the *pgpB* gene for PGP phosphatase in *Anabaena* sp. PCC 7120 resulted in elevated levels of the other acidic lipid, SQDG (Hagio et al., 2000; Wu et al., 2006), which are reminiscent of the changes in levels of these two acidic lipids in photosynthetic organisms under P-limiting conditions. In addition, loss of SQDG by disruption of the *sqdB* and/or *sqdX* gene in *Synechococcus* and *Synechocystis* raised levels of PG, apparently compensating for the loss of SQDG (Güler et al., 1996, 2000; Aoki et al., 2004). Moreover, mutants of *C. reinhardtii*, *A. thaliana*, and *R. sphaeroides* that are defective in the synthesis of one acidic lipid, either SQDG or PG, raise levels of the other one (Benning et al., 1993; Sato et al., 1995b; Yu et al., 2002; Yu and Benning, 2003). It seems reasonable, therefore, to conclude that a regulatory mechanism that balances levels of PG and SQDG might exist in a broad range of photosynthetic organisms, acting to maintain an appropriate charge balance on membranes under both normal and stressed conditions.

V Conclusions and Future Perspectives

The lipid components of cyanobacterial membranes resemble those of chloroplast membranes and, in particular, those of thylakoid membranes. The metabolic map of lipid biosynthesis in cyanobacteria is close to completion as a result of investigations of the metabolic flow of ^{14}C-labeled compounds, such as $NaH^{14}CO_3$, the biochemical characterization of the enzymes involved, and the identification of the genes for the various enzymes. Further studies should lead to the molecular identification of GPAT, MGlcDG epimerase, and PGP phosphatase. For explorations of the as yet uncharacterized genes for these enzymes, researchers will be able to exploit comparative genomics, using sequences of cyanobacterial genomes, and DNA microarray analysis of the genome-wide expression of genes under conditions that are expected to induce or repress the expression of target genes. Table 1 summarizes the information that is currently available about cyanobacterial enzymes involved in lipid biosynthesis and compares these enzymes with those in chloroplasts. Similarities between cyanobacterial and chloroplast enzymes can be explained by the endosymbiosis theory, which postulates that cyanobacteria are the ancestors of chloroplasts. However, some cyanobacterial genes are not related to those in chloroplasts and appear to have been replaced by phylogenetically unrelated genes during evolution. Why is this the case and what are the origins of the chloroplast-specific genes for lipid–biosynthetic enzymes? Accumulation of genomic sequences from larger numbers of plant species might lead to the discovery of the ancestors of these chloroplast-specific genes, which

might, in turn, provide clues to the evolutional and physiological aspects of the discontinuity in lipid biosynthesis between cyanobacteria and chloroplasts.

Increases in the extent of fatty acid unsaturation in membrane lipids, which maintain membrane fluidity in response to low temperatures, are regarded as evidence of acclimation, and the mechanism responsible has been characterized at the molecular level in *Synechocystis* (see Chapter 15). Hik33 has been identified as the signaling component for enhancement of the expression the *desB* gene, and it plays an important role in the regulation of the expression of cold-inducible genes for control of membrane fluidity, of the transcription and translation of many other genes, and of the cell's energy status (Suzuki et al., 2001; Inaba et al., 2003). Future, identification of signaling pathways that lead to changes in lipid metabolism in response to other environmental stimuli will contribute to the elucidation of mechanisms of acclimation that involve not only membrane function but also a broad spectrum of physiological processes.

In both oxygenic and anoxygenic photosynthetic organisms, a decrease in the relative level of PG and a concomitant increase in that of SQDG have been observed under P-limiting conditions. The observed changes are essential for the adaptive growth of these organisms and can be interpreted as being crucial to maintenance of the charge balance on membranes. Furthermore, photosynthetic organisms, in general, seem to be able to raise the relative level of one acidic lipid, PG or SQDG, if the level of the other falls. The molecular mechanisms of such regulation of the biosynthesis of these two acidic lipids remain to be elucidated. Studies of signaling pathways involved in the control of the levels of these two acidic lipids upon P limitation or upon the loss of the gene responsible for the synthesis of one of these acidic lipids can be modeled on the earlier studies of Hik33 in *Synechocystis*. The information obtained by such studies will lead to a better understanding of the ways in which cyanobacteria and other photosynthetic organisms cope with P limitation, thereby providing a basis for genetic manipulation that might enhance the ability of plants to tolerate limited availability of P.

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Chapter 9

Heterocyst Envelope Glycolipids

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Summary

Heterocyst-forming cyanobacteria simultaneously photosynthesize, producing oxygen (O_2), and fix dinitrogen (N_2), initially into ammonia, using nitrogenase enzymes that are rapidly inactivated by O_2. These cyanobacteria enable nitrogenases to function in an oxic environment by segregating them within specialized cells, called heterocysts, in which O_2 is not produced, respiration is highly active, and an envelope barrier of glycolipids greatly slows the rate of entry of O_2. We will describe the chemical structure of the heterocyst-specific glycolipids (Hgls), their physiological role, and what is known of their deposition. We will then discuss the clustered genes that encode the proteins required for their biosynthesis, how

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the glycolipids are believed to be synthesized, and what is known of the regulation of their biosynthesis. Finally, we will examine the relationship between their biosynthetic enzymes and other polyketide synthases, with an emphasis on those from cyanobacteria.

I Introduction

Cyanobacteria have been grouped into five sections (Rippka et al., 1979). Section I comprises unicellular strains, for example, *Synechocystis*, *Synechococcus*, *Prochlorococcus*, *Thermosynechococcus*, and *Microcystis*, whose cells divide by binary fission. Cells of strains in section II divide internally, producing small daughter cells, and may also undergo binary fission. Cyanobacteria of strains of sections III, IV, and V are filamentous. The cells of filaments of cyanobacteria of section V can divide in more than one plane, resulting in branching, whereas those of sections III (e.g., *Lyngbya* and *Trichodesmium*) and IV (e.g., *Nostoc* and *Anabaena*) divide in only one plane. Only cells of sections IV and V can differentiate morphologically and biochemically into dinitrogen(N_2)-fixing cells called heterocysts. Cyanobacteria, whose photosystems are very similar to those of chloroplasts, are widely accepted as the evolutionary progenitors of chloroplasts (Douglas, 1994; Deusch et al., 2008). As discussed in Chapters 3 and 8, the lipid compositions of thylakoids from cyanobacterial cells, including heterocysts (Winkenbach et al., 1972), and chloroplasts are also very similar, supporting the hypothesis that chloroplasts and contemporary cyanobacteria are related.

Nitrogenase comprises dinitrogenase and dinitrogenase reductase, both of which are labile to oxygen. Heterocysts protect these proteins from oxygen in three ways (Wolk et al., 1994): (i) in those cells, PS II loses the ability to evolve oxygen; (ii) electron flow to oxygen (e.g., respiration) increases, thereby consuming oxygen; and (iii) a thick envelope that comprises an inner layer of glycolipid (HGL, Fig. 1; for a transmission electron micrograph, see for example, Winkenbach et al., 1972; Fan et al., 2005; Moslavac et al., 2007) and an outer layer of polysaccharide (HEP, Fig. 1; Cardemil and Wolk, 1979, 1981a) is synthesized, and decreases the rate of diffusion of oxygen into the cells.

Heterocyst envelope glycolipids (Hgls) were discovered four decades ago as lipids specific to heterocyst-forming cyanobacteria (Nichols and Wood, 1968) and were then found to be specific to the heterocysts (Walsby and Nichols, 1969; Wolk and Simon, 1969). The inner, laminated layers of the heterocyst envelope, isolated by sucrose density gradient centrifugation, were found to consist of Hgls, accompanied by only a very small amount of non-lipid, organic material (Winkenbach et al., 1972).

II Chemical Structure of the Heterocyst Envelope Glycolipids

Hgls are long-chain polyhydroxy alcohols with a hexose head group (Bryce et al., 1972). Detailed structural analysis showed that the aglycones have 26–32 carbons with three or four oxygenated groups, at most one of which is a ketone. There are hydroxyls at the 1 and ω-2 (penultimate) positions, either a hydroxyl or a ketone at the 3-position, and sometimes a hydroxyl or a ketone at the ω-4 position. Most Hgls analyzed so far are from section IV cyanobacteria, with only two strains of section V cyanobacteria analyzed. With few exceptions, the polar head group is α-linked glucose, but α-linked galactose (in *Anabaena* sp. strain WSAF), β-linked glucose (in *Tolypothrix tenuis* and *Anabaena* sp. strain WSAF), and α-linked mannose (in *Calothrix desertica* and – in section V – *Chlorogloeopsis fritschii*) have also been reported (Lambein and Wolk, 1973, revised by Soriente et al., 1995; Soriente et al., 1992, 1993; Gambacorta et al., 1995, 1996, 1998). The structures of the

Abbreviations: ACP– Acyl carrier protein; AT – Acyl transferase; KS – β-ketoacyl synthase; CLF – Chain length factor; cAMP – Cyclic adenosine monophosphate; DH – Dehydrase; ER – Enoyl reductase; FAS – Fatty acid synthase; Hgl – Heterocyst envelope glycolipid; HGL or HGL layer – Laminated layer of Hgls; HEP – Layer of heterocyst envelope polysaccharide; KR – Ketoacyl reductase; PKS – Polyketide synthase; PUFA – Polyunsaturated fatty acid; TER – Thioester reductase

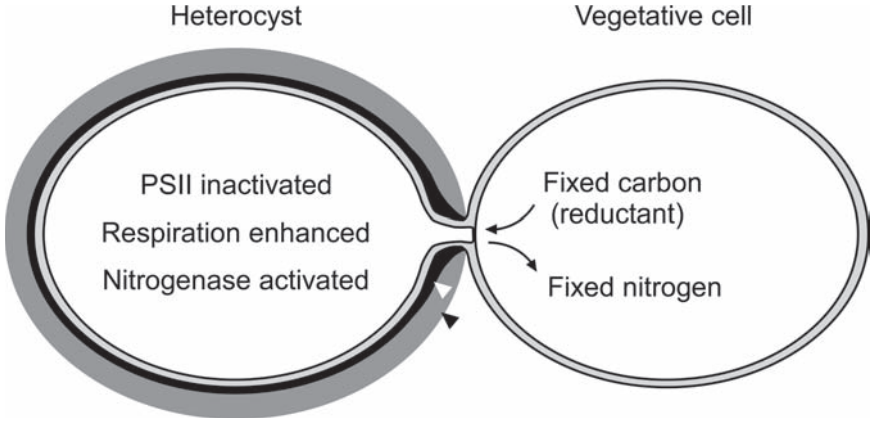

Fig. 1. Schematic representation of a heterocyst and a vegetative cell of a filamentous cyanobacterium. Heterocysts fix nitrogen and the fixed nitrogen moves into vegetative cells; similarly, vegetative cells fix carbon dioxide, and products of its fixation move into heterocysts, where they are oxidized to produce reductants and are used as substrate for biosynthesis (Wolk et al., 1994). In mature heterocysts, the homogeneous layer of heterocyst envelope polysaccharide (HEP, *dark gray area*, *black arrowhead*) and the laminated layer (HGL, *black area*, *white arrowhead*) of heterocyst envelope glycolipids (Hgls) surround the peptidoglycan-containing wall with its periplasmic space (*light gray area*). The wall is bounded on the inside by the cytoplasmic membrane (*solid line*) and on the outside by the outer membrane (seen as a *solid line* at the outside of the vegetative cell). The outer membrane is discernible in heterocysts of some mutants that lack a HGL layer, but has not been clearly seen in heterocysts of a wild-type strain. The periplasmic space is thought to be continuous along the filament, providing a conduit between vegetative cells and heterocysts (Flores et al., 2006; Mariscal et al., 2007).

Hgls from *Anabaena* sp. strain PCC 7120 (sometimes referred to as *Nostoc* PCC 7120, but hereinafter called *Anabaena* PCC 7120) are presented in Fig. 2 as examples. The Hgls of this strain are 1-α-glucosyl-3,25-hexacosanediol (at the bottom of the main vertical sequence) and 1-α-glucosyl-3-keto-25-hexacosanol (at the bottom of the right-hand branch) in a ratio of approximately 5:2 (Gambacorta et al., 1996).

The detailed ultrastructure of HGL layers has been investigated, but remains uncertain. The thickness of an individual lamina has been reported to be 3–4 nm (Winkenbach et al., 1972; Granhall, 1976) and 7–8 nm (Golecki and Drews, 1974; Giddings and Staehelin, 1978); the thickness of the laminated layer of the envelope may vary with the O_2 content of the environment (Kangatharalingam et al., 1992) and with the position along the cell. From electron micrographs, five or more layers of glycolipid seem to surround heterocysts that have differentiated under ambient O_2 (Winkenbach et al., 1972).

The layer of glycolipid is perforated at each polar pore of heterocysts, so that gases, as well as other chemicals, may more easily enter (or leave) there. At the outer end of the pore, the heterocyst is connected to an adjacent vegetative cell (Lang and Fay, 1971). However, many more layers of glycolipid surround the pores at the ends of the heterocysts than are present midway along the cells, suggesting that gases may enter not indiscriminately at the pore, but in a regulated manner, as proposed by Walsby (2007). The region of cytoplasm near the inner end of the pore contains "honeycomb" respiratory membranes that presumably help to protect nitrogenase from oxygen that enters through the pore (Wolk et al., 1994; Valladares et al., 2007).

III Physiological Role of the Heterocyst Envelope Glycolipids

Heterocysts were first proposed to be involved in, or the site of, nitrogen fixation by Fay and coworkers (Fay and Walsby, 1966; Fay et al., 1968). Later, heterocysts were isolated gently by sequential treatment of filaments first with lysozyme and then with bath cavitation. Lysozyme weakens the walls of vegetative cells, whereas the walls of heterocysts, protected by their envelope layers, are much less affected. Bath

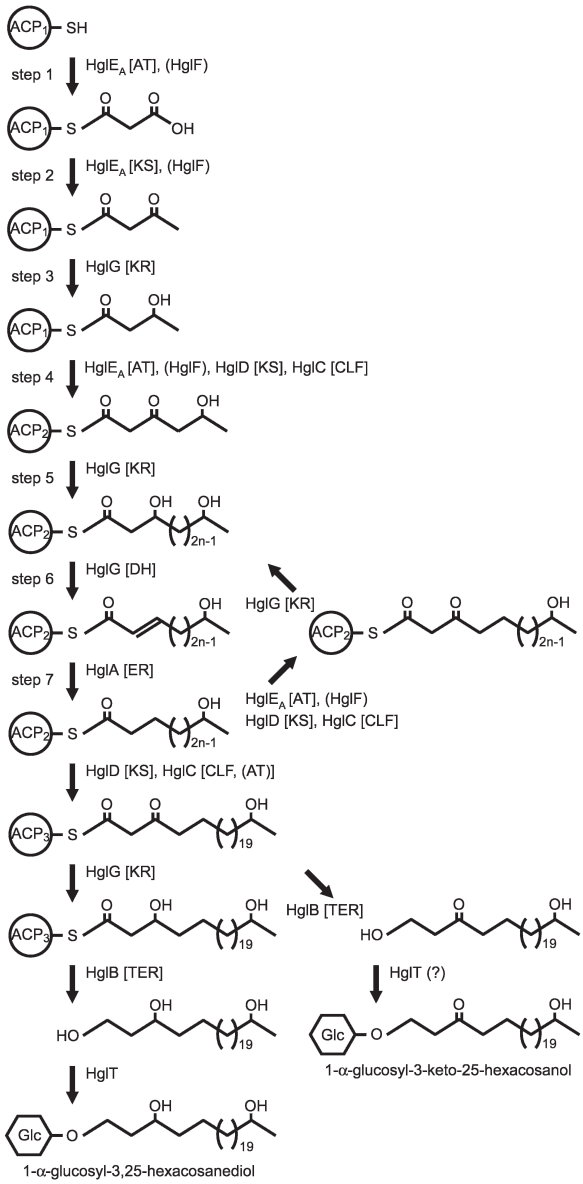

Fig. 2. Proposed pathway for biosynthesis of Hgls of *Anabaena* PCC 7120. ACP_1, ACP_2, and ACP_3 refer, respectively, to the first- and second-used ACP domains in $HglE_A$ and to the ACP domain in HglB. The sulfur moiety (S) to the right of ACP indicates that the ACP has already been phosphopantetheinylated. Because HglF contains no obvious domain and its function could not be predicted, we have placed it in parentheses. It is placed adjacent to $HglE_A$ because, for reasons discussed in the text, we suggest that it is associated with $HglE_A$. Large parentheses with a number 2n − 1 indicate an odd number of repetitions of the methylene residue enclosed within the parentheses; n increases from 1 to 10 (2n − 1 increases from 1 to 19) during repetitions of the cycle shown midway down at the *right*. The main vertical sequence ends in the principal Hgl of *Anabaena* PCC 7120, 1-α-glucosyl-3,25-hexacosanediol, and the bottom right-hand branch ends in the minor Hgl of that strain, 1-α-glucosyl-3-keto-25-hexacosanol. Steps 1–7 are discussed in the text.

cavitation, which exerts a low energy density compared with probe cavitation ("sonication"), suffices to rupture the lysozyme-weakened vegetative cells while wreaking much less damage on the heterocysts. Heterocysts thereupon recovered by low-speed centrifugation accounted for a majority of the nitrogenase activity of the original filaments, and a very large fraction of the dinitrogenase (the MoFe protein) and the dinitrogenase reductase (the Fe-protein), labeled with ^{99}Mo and ^{55}Fe, of the original filaments (Peterson and Wolk, 1978). Further results that confirm the conclusion that heterocysts are the sites of nitrogen fixation in aerobically grown *Anabaena* have been summarized (Wolk et al., 1994). However, it has been found that under anoxic conditions, a different Mo-based nitrogenase is expressed also in vegetative cells of *Anabaena variabilis* strain ATCC 29413 (Thiel et al., 1995), and that some unicellular and non-differentiating cyanobacteria can, in the presence of oxygen, cycle between an N_2-fixing state in the dark and an O_2-producing state in the light (Fay, 1992; Gallon, 1992). Moreover, cultured *Trichodesmium* can fix N_2 in the light under aerobic conditions, with nitrogenase expressed in all of the cells (Ohki, 2008) or only in particular, morphologically undifferentiated cells (El-Shehawy et al., 2003).

The glycolipid layer is thought to be the principal barrier to entry of oxygen (Walsby, 1985, 2007). This premise derives in part from the observation that mutants whose HGL layer is missing or disrupted can grow in air only in the presence of fixed nitrogen and can maintain nitrogenase activity only under micro-oxic or anoxic conditions (Currier et al., 1977; Haury and Wolk, 1978; Murry et al., 1984; Murry and Wolk, 1989; Ernst et al., 1992; Fan et al., 2005).

The polysaccharide layer is thought to protect the more fragile layer of glycolipid. The latter interpretation comes from the observation that mutations – of presumptively sugar transferase-encoding genes and others – that block formation, apparently specifically, of the polysaccharide layer frequently leave the glycolipid layer fragmented. One such mutation was initially misinterpreted as resulting in the production of abundant, non-compacted polysaccharide (Wolk et al., 1988; Murry and Wolk, 1989; Holland and Wolk, 1990) until examined by high-resolution electron microscopy (Wolk, 2000).

Anabaena sp. strain PCC 7119, deprived of boron, shows extensive disruption of heterocyst envelopes and loss of Hgl (Garcia-Gonzalez et al., 1988, 1991), and *A. variabilis* also shows a boron requirement for growth specifically on N_2 (Malek, 2002). Boron forms di-ester bridges between *cis*-hydroxyl-containing molecules (Bolaños et al., 2004), but extraction of glycolipids does not extract boron (Malek, 2002). Moreover, the resolution of the relatively low-magnification transmission electron micrographs presented by Garcia-Gonzalez et al. (1991) is inadequate to be sure, but the images are suggestive of loss of HEP and initial retention of HGL. Therefore, envelope-localized boron may be bound to HEP rather than to HGL, and the loss of Hgls from boron-deprived cells of *Anabaena* sp. strain PCC 7119 (Garcia-Gonzalez et al., 1991) may be a secondary effect of loss of HEP.

It is difficult to imagine how the polysaccharide layer could be deposited through the glycolipid layer, and electron micrographs suggest that although deposition of glycolipid is not initiated simultaneously everywhere around the heterocyst, it is – in the wild-type strain – deposited locally after formation of the polysaccharide layer. Nitrogen deprivation elicits heterocyst differentiation. Although few time points were reported, the data of Ehira and coworkers (Ehira et al., 2003; Ehira and Ohmori, 2006a; see also Xu et al., 2008) suggest that after the start of nitrogen deprivation, activation of genes required for formation of the polysaccharide layer (Huang et al., 2005; Wang et al., 2007) tends to precede activation of genes involved in synthesis of the glycolipid layer (Fan et al., 2005).

IV Deposition of the Heterocyst Envelope Glycolipids

A gene involved in the deposition of Hgls was first identified from a library of transposon mutants unable to grow aerobically on N_2 (Ernst et al., 1992). Mutant strain M7 formed less Hgl than did the wild-type strain. The transposon in M7 was found to be present in a gene whose predicted product is an ATP-binding cassette protein of a bacterial multi-subunit ABC transporter. The gene was designated *devA* (Maldener et al., 1994). The *devA* gene was shown to be expressed principally, perhaps specifically, in developing heterocysts (Maldener et al., 1994), suggesting that it has a role in the process of cellular differentiation. Genes *devB* and *devC* neighbor *devA* in *Anabaena* PCC 7120 and in *A. variabilis* (Fiedler et al., 1998a, b). Knock-out mutants of any one of these genes or of *alr2887*, aka *hgdD* (for *h*eterocyst *g*lycolipid *d*eposition; see also Wolk et al., 2007), exhibit the same phenotype as a *devA* mutant, namely, they accumulate Hgls when deprived of fixed nitrogen but lack a HGL layer in the heterocyst envelope. The *devB* gene encodes a protein similar to a membrane fusion protein of a bacterial ABC transporter that may traverse the periplasmic space. The *devC* gene encodes an integral membrane protein (Fig. 3). Finally, HgdD may trimerize to form a TolC-like tunnel (Maldener et al., 2003; Moslavac et al., 2007) through which Hgls might – in interaction with DevBCA – traverse the periplasm from inside of the plasma membrane to outside of the outer membrane (Fig. 3). HgdB shows similarity to DevB, HgdC shows similarity to DevC, and *hgdB* and *hgdC* mutations result, respectively, in spatial and temporal defects of Hgl deposition (Fan et al., 2005). These results cumulatively suggest that ABC transporter proteins DevA, DevB, DevC, HgdB, HgdC, and TolC-like protein HgdD participate in transferring Hgls from within the cell to a position between the outer membrane and the polysaccharide layer of the heterocyst envelope.

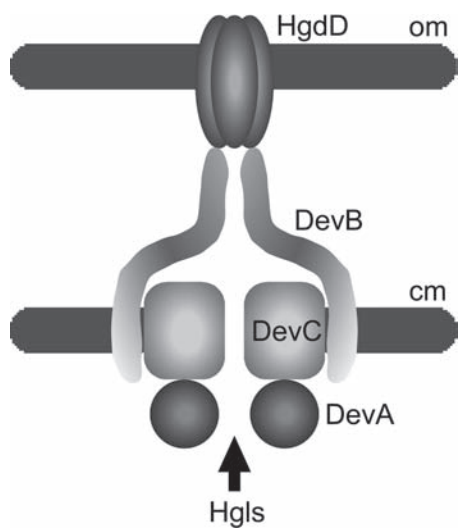

Fig. 3. Machinery for deposition of Hgls, based on the model of Moslavac et al. (2007). Abbreviations: DevA, ATP-binding cassette protein; DevB, membrane fusion protein; DevC, permease protein; HgdD, homotrimeric TolC-like protein; cm, cytoplasmic membrane; and om, outer membrane. The *arrow* indicates the direction of Hgl deposition.

HglK is a protein that contains four membrane-spanning domains followed by 36 repeats of a pentapeptide sequence (consensus, AxLxx). The insertion of a transposon near the 5′ end of the *hglK* gene (mutant M8 of Ernst et al., 1992; R. Kong and C.P. Wolk, unpublished) results in a phenotype similar to that of a mutation that creates a stop codon immediately preceding the pentapeptide repeats of HglK (All0813) (Black et al., 1995). Both mutants synthesize Hgl, but electron microscopy fails to show a laminated layer (Black et al., 1995). Thus, the pentapeptide repeat appears to be required for HglK to enable proper localization of Hgls. Although such a pentapeptide repeat is conserved in cyanobacterial species (Bateman et al., 1998), its function – possibly simply to serve as a multi-level scaffold – remains unknown. Mutations in *hglK* accounted for 3% of the mutations in a large library of Fox$^-$ transposon mutants (mutants unable to *f*ix N_2 in the presence of *ox*ygen: Fan et al., 2006).

Mutation of the glycosyltransferase gene whose product catalyzes the final step of Hgl biosynthesis (Awai and Wolk, 2007) resulted in a strain that accumulates the principal Hgl aglycone and forms no laminated layer, indicating that the Hgl deposition machinery distinguishes Hgls from their precursors. Proper synthesis of outer membrane lipopolysaccharide is also required for a Fox$^+$ phenotype (Xu et al., 1997), as are certain penicillin-binding proteins that are thought to function in peptidoglycan synthesis (Lázaro et al., 2001; Leganés et al., 2005). The latter results were interpreted as suggesting "that re-fashioning of the cell wall of the differentiating vegetative cell by the products of those genes may be a prerequisite for the correct assembly of the heterocyst envelope" (Leganés et al., 2005).

V Biosynthetic *hgl* Genes; Predicted Heterocyst Envelope Glycolipid Biosynthetic Pathway

A Hgl biosynthetic gene, denoted *hetM* and later renamed *hglB*, was found by Black and Wolk (1994), who recognized it as bearing an acyl carrier protein (ACP) domain and another domain that resembled portions of multifunctional polyketide synthases and fatty acid synthases. Black and Wolk emphasized study of its downstream genes, which they denoted *hetN* and *hetI*. *hetN*, although it possesses a ketoreductase (KR) domain, is an important regulator of heterocyst differentiation and proved not to be required for synthesis of Hgls (Callahan and Buikema, 2001; Orozco et al., 2006); and Black and Wolk (1994) were unable to segregate a mutation in *hetI* (see below). Bauer and co-workers (Bauer, 1994; Bauer et al., 1997), recognizing the requirement of *hetM* for Hgl biosynthesis, renamed it *hglB*. They also showed that nearby genes are required for Hgl biosynthesis, and named them *hglC* and *hglD* (Bauer, 1994; Bauer et al., 1997).

Campbell et al. (1997) isolated Fox$^-$ transposon mutants of *Nostoc punctiforme* strain ATCC 29133. In one such mutant, heterocysts differentiated under conditions of nitrogen deprivation, but the mutant was unable to synthesize Hgls. The mutated gene, designated *hglE*, encoded a protein similar to polyketide synthases. HglE was found to have a ketosynthase domain, an acyltransferase domain and two ACP domains, indicative of a type I polyketide synthase in which a single polypeptide bears a series of functional domains (Fan et al., 2005; Jenke-Kodama et al., 2005). PfaA, a polyketide synthase for C-20 and C-22 polyunsaturated fatty acids (PUFA; Yazawa, 1996; Metz et al., 2001), shows ca. 45% amino acid identity to HglE through a length of over 1,500 amino acids. Two homologs of HglE were found in each of the four available genomic sequences of heterocyst-forming cyanobacteria (see below). In *Anabaena* PCC 7120, the homologs were designated $HglE_A$ and HglE2 (Fan et al., 2005). A knockout mutant of $hglE_A$, which is clustered with *hglB*, *hglC* and *hglD*, lacks Hgls and a HGL layer. We know of no attempt to determine what the phenotype of a *hglE2* mutant might be.

A chromosomal region (Fig. 4) that contains *hglB*, *hglC*, *hglD*, and $hglE_A$ was denoted an expression island by Ehira et al. (2003) because at least some of its genes are up-regulated in response to nitrogen deprivation. Fan et al. (2005) identified a cluster of transposon insertions in that region that led to a Fox$^-$ phenotype. Complementation and other analyses led to the interpretation that open reading frames (orfs) *alr5351* through *alr5357* (denoted, in order, $hglE_A$, *hglF*, *hglG*, *hglD*, *hglC*, *hglA*, and *hglB*) encode the enzymes that biosynthesize the Hgl aglycones; the product of orf *all5341* (denoted *hglT*) glycosylates at least the major aglycone; *orfs all5345* (*hgdA*) and

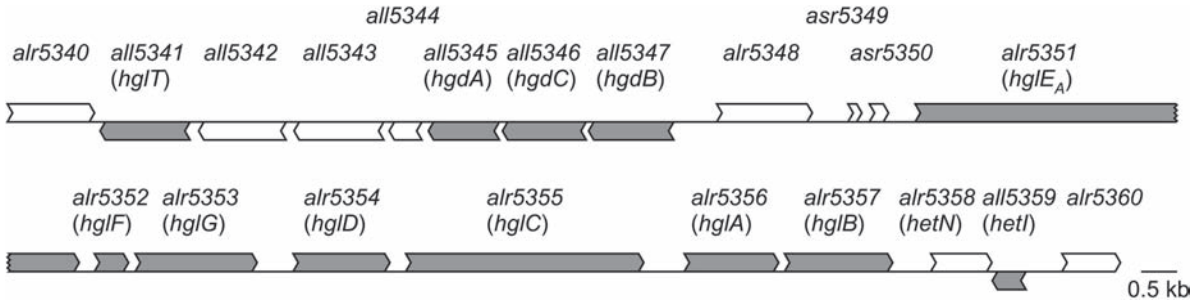

Fig. 4. Map of the Hgl island in the chromosome of *Anabaena* PCC 7120.

all5346 (*hgdC*) may be involved in Hgl transport near a pore and farther from a pore, respectively; and orf *all5347* (*hgdB*) may influence the relative timing of deposition of Hgls and of envelope polysaccharide (Fan et al., 2005). Fan and co-authors referred to the region as the Hgl island.

Co-expression of *Anabaena* PCC 7120 HetI with *Schizochytrium* PUFA synthase in *E. coli* resulted in synthesis of docosahexaenoic acid (Hauvermale et al., 2006), and the HetI homolog encoded by the *hetMNI* region of *Nodularia spumigena* pantotheinylated several proteins in vitro (Copp et al., 2007). It was concluded that *hetI* encodes a phosphopantetheinyl transferase. In addition, it is apparently the only gene of *Anabaena* PCC 7120 that encodes a phosphopantetheinyl transferase, accounting for the lack of segregation of a *hetI* mutation (Black and Wolk, 1994; Fan et al., 2005).

The steps of synthesis described below suggest that the machinery for Hgl biosynthesis, and also for some PUFA synthesis, involves unusual examples of an iterative Type I PKS (Shen, 2003). Figure 2 summarizes a proposed biosynthetic pathway (Fan et al., 2005) of Hgl in *Anabaena* PCC 7120. In that figure, HetI has already transferred a phosphopantetheinyl moiety from coenzyme A to one of the two ACP domains of HglE$_A$, thus activating the domain (Lambalot et al., 1996; Copp and Neilan, 2006); bacterial PUFA synthases are known to have up to six ACP domains (Okuyama et al., 2007). Acyl transferase (AT) domains catalyze the transfer of precursor subunits to ACP prior to condensation by a KS domain (Serre et al., 1995; Rangan and Smith, 1996). HglE$_A$ would use its acyl transferase (AT) domain to malonylate the ACP domain of HglE$_A$ (Fig. 2, step 1) and its β-ketoacyl synthase (KS) domain to transfer an acetyl residue to the activated CH_2 of the malonyl group, providing the ω-1 (final) and ω-2 carbons of the Hgl, with decarboxylation of C-3 of the malonyl group (Fig. 2, step 2). HglF, encoded downstream from HglE$_A$, is predicted to function with HglE$_A$ because their homologs in both *A. variabilis* and *N. punctiforme* are fused (see below). We have suggested (Fan et al., 2005) that at the initially used ACP domain, steric hindrance may, following keto-reduction (Fig. 2, step 3), block dehydration and enoyl reduction, thus accounting for the ω-2 hydroxyl of the aglycone, and that the chain may then be moved to the second ACP domain of HglE$_A$ for a series of subsequent chain elongation (e.g., Fig. 2, step 4), ketoreduction (e.g., Fig. 2, step 5), dehydration (e.g., Fig. 2, step 6), and enoyl reduction steps (e.g., Fig. 2, step 7).

The chain lengths of Hgls may be determined primarily by the chain length factor (CLF) domain of HglC (Tang et al., 2003), a domain that is closely related to a ketoacyl synthase domain. As discussed by Fan et al. (2005), the condensations in Fig. 2 step 4 and later are probably catalyzed by the AT domain of *hglE$_A$* together with a KS domain, and would involve attachment of a malonyl-CoA unit to the ACP, and transfer of the previously elongated chain to the activated C-2 of the malonyl unit with release of its C-3. Additional KS and CLF domains are found in HglD and HglC, respectively; as discussed below, homologs of these two genes are fused in other cyanobacteria. Which domain(s) would be involved in condensation is unclear, but we suggest in Fig. 2 that it would be the KS of HglD together with the CLF of HglC (Keatinge-Clay

et al., 2004). However, no evidence now excludes the possibility that the HglDC dimer is responsible for initiation and $HglE_A$ is involved in elongation. Ketoreduction and dehydration are attributable to HglG, and enoyl reduction to HglA (Fan et al., 2005). An alternative derivation of the Hgl aglycones has been considered: that they normally are, or can be, synthesized by elongation of fatty acids such as palmitoleic acid, linoleic acid or linolenic acid, followed by hydroxylation. This possibility was not experimentally supported (Gambacorta et al., 1995).

HglB contains an N-terminal ACP domain, and as the only plausible means of chain termination that we identified, a C-terminal thioester reductase (TER) domain. We found, for example, by comparison of HglB with the biochemically characterized Lys2 protein of yeast (GenBank AAA34747; Ehmann et al., 1999), 52% similarity with an Expect value of 4exp-38 in the TER-domain-containing region of amino acids 137–503. HglB also shows substantially lesser similarity (42% similarity, Expect = 8exp-5) to the N-terminal region, in which we find no TER domain, of a fatty acid reductase from jojoba, an enzyme that can reduce a fatty acyl-coenzyme A to a primary alcohol (Metz et al., 2000). How the Hgl biosynthetic system leaves a hydroxyl or ketone at the C-3 position is unclear, but may possibly be related to steric hindrance around the ACP domain in HglB or that may occur as the chain approaches its final length. Finally, HglT transfers a glucose moiety (probably from UDP-glucose; Cardemil and Wolk, 1981b) to the 1-hydroxyl residue of one or both aglycones to synthesize the Hgls of *Anabaena* PCC 7120 (Awai and Wolk, 2007).

VI Regulation of Heterocyst Envelope Glycolipid Biosynthesis

It might be anticipated on the basis of the apparent role of the HGL layer that the concentration of oxygen in the environment would regulate HGL formation. Indeed, working with *Anabaena spiroides* strain PCC 6310 in the presence of DCMU (to block production of O_2) under an atmosphere of argon, Rippka and Stanier (1978) observed a fibrous layer but no laminated Hgl layer or homogeneous Hep layer by low-magnification transmission electron microscopy of heterocysts. Also, Kangatharalingam and coworkers (1992), working with *Anabaena flos-aquae* under atmospheres that included a range of O_2 concentrations from 5 to 40 kPa O_2 (1 atm = 101.325 kPa), 0.04 kPa CO_2, balance N_2, obtained light micrographs that suggest that between 10 and 40 kPa O_2, the heterocyst envelope increases in thickness. Their low-magnification transmission electron micrographs using "a method of fixation" that "does not stain the laminated glycolipid layer of the heterocyst" and may "cause shrinkage in the various layers of the heterocyst envelope" hinted at a similar conclusion. Because Kulasooriya et al. (1972), who gassed *Anabaena cylindrica* with 5% CO_2 in N_2, failed to prevent O_2 production by the experimental material, their conclusion, based on electron microscopy, that all layers of the heterocyst envelope form in the "absence of exogenous oxygen" cannot be accepted at face value. Further evidence showing whether pO_2 regulates HGL formation would be welcome. The current model of Hgl biosynthesis (Fan et al., 2005) invokes no role of free O_2 as a precursor of any of the oxygenated groups in Hgls. Therefore, if that model is correct, the absence of Hgls under anoxic conditions cannot be attributed to the biosynthetic pathway.

Hgls are generated as part of the normal process by which, in response to nitrogen deprivation, a regulatory cascade leads to heterocyst differentiation. When filaments of heterocyst-forming cyanobacteria are deprived of fixed nitrogen, 2-oxoglutarate accumulates, activating NtcA, a transcription factor that globally regulates nitrogen metabolism (Li et al., 2003; Laurent et al., 2005). In turn, NtcA enhances expression of many genes, including *nrrA*, which encodes a response regulator (Ehira and Ohmori, 2006a; Muro-Pastor et al., 2006), and genes that encode the Hgl-transporter, DevBCA (Fiedler et al., 2001). NrrA binds directly to the promoter region of *hetR* (Ehira and Ohmori, 2006b), which encodes the master regulator of heterocyst differentiation. Further details of this regulatory cascade have been reviewed (Zhang et al., 2006; Xu et al., 2008; Zhao and Wolk, 2008). Microarray analyses (data of Ehira and Ohmori, 2006a, re-evaluated by Xu et al., 2008) have shown subsequent up-regulation of the following genes that are involved in Hgl biosynthesis or deposition (Table 1): *hglA*, *hglB*, *hglC*, *hglD*, $hglE_A$, *hglF*, *hglG*, *hglT*, *devA*, *devB*,

Table 1. Genes involved in Hgl biosynthesis and deposition in *Anabaena* PCC 7120.

Gene name	Orf no. in *Anabaena* PCC 7120	Length, amino acids	Domain(s) or annotation	References
			Biosynthesis	
hglA	alr5356	442	Enoyl reductase	Fan et al., 2005
hglB	alr5357	506	Acyl carrier protein; thioester reductase	Bauer, 1994; Black and Wolk, 1994; Bauer et al., 1997
hglC	alr5355	1,109	Chain length factor; acyl transferase-like domain	Fan et al., 2005
hglD	alr5354	453	Ketoacyl synthase	Fan et al., 2005
$hglE_A$	alr5351	1,541	Ketoacyl synthase and acyl transferase and two acyl carrier protein domains	Campbell et al., 1997; Fan et al., 2005
hglF	alr5352	162	Probably interacts with $HglE_A$	Fan et al., 2005
hglG	alr5353	570	Ketoreductase and dehydrase	Fan et al., 2005
hetI	all5359	160	Phosphopantetheinyltransferase	Black and Wolk, 1994
hglT	alr5341	421	Glucosyltransferase	Awai and Wolk, 2007
			Deposition	
devA	alr3712	244	ATPase subunit of ABC transporter	Maldener et al., 1994; Fiedler et al., 1998a, b
devB	alr3710	474	Membrane fusion protein subunit of ABC transporter	Fiedler et al., 1998a, b
devC	alr3711	385	Permease subunit of ABC transporter	Fiedler et al., 1998a, b
hgdA	all5345	333	Oxidoreductase	Fan et al., 2005
hgdB	all5347	399	Membrane fusion protein subunit of ABC transporter	Fan et al., 2005
hgdC	all5346	392	Permease subunit of ABC transporter	Fan et al., 2005
hgdD	alr2887	742	TolC-like protein	Moslavac et al., 2007
hglK	all0813	727	Function unknown; has 36 pentapeptide repeats	Black et al., 1995

devC, *hgdB*, *hgdC*, *hgdD*, and *hglK*. In addition, the up-regulation of individual genes in *Anabaena* PCC 7120 has been reported for *hglB* (Maldener et al., 2003), *hglC* and $hglE_A$ (Jang et al., 2007), *hglK* (Black et al., 1995), *devA* (Maldener et al., 1994), and *hgdD* (Maldener et al., 2003; Moslavac et al., 2007), as was induction of a gene encoding biotin carboxyl carrier protein, a subunit of acetyl-coA carboxylase (Gornicki et al., 1993). In work with *N. punctiforme*, up-regulation of *hglE* was reported (Campbell et al., 1997).

DevH, a transcription factor similar to NtcA, is required for Hgl synthesis and is also induced by nitrogen deprivation (Hebbar and Curtis, 2000; Curtis and Hebbar, 2001; Ramírez et al., 2005). In a *devH* mutant, expression of $hglE_A$ is diminished, indicative of involvement of the transcription factor in regulation of Hgl biosynthesis, and so is expression of *hglE2* (Ramírez et al., 2005).

Mutants defective in *henR* (*alr1086*) and *hepS* (*all2760*), which presumptively encode a response regulator and a serine/threonine kinase, respectively, both showed down-regulation of Hgl island genes (Lechno-Yossef et al., 2006); *henR* mutant FQ621 lacked, whereas *hepS* mutant FQ1641 formed, a HGL layer (Fan et al., 2006). Two other presumptive serine/threonine kinases, Pkn44 (All1625) and Pkn30 (All3691), each sufficed for biosynthesis of the less abundant Hgl (Fig. 2, bottom right) in the presence, but not in the absence, of the other (Shi et al., 2007). A mutant with both genes inactivated showed retarded expression of Hgl island genes, lacked the HGL layer as well as the minor Hgl, possessed a diminished or delayed HEP layer, and – under micro-oxic conditions – showed nitrogenase activity. Contrariwise, presumptive protein phosphatase PrpJ (All1731) appeared to be required specifically for biosynthesis of the principal

Hgl (Fig. 2, bottom left) (Jang et al., 2007). The implication that beyond the step(s) regulated by DevH, syntheses of different Hgls in an organism are independently regulated was foreshadowed by the observation that during differentiation of heterocysts of *Anabaena cylindrica*, production of radio-labeled alkanetriol aglycones decreases and production of radio-labeled alkanediol aglycones increases between 15 and 56 h of nitrogen deprivation (Krepski and Walton, 1983). Mutation of *prpJ* also changed the temporal pattern of expression of $hglE_A$, but of neither *hglC* nor *hglK*. Lipid analysis of the mutant showed that only the major Hgl was missing, with the polysaccharide layer but not the HGL layer formed. These results, taken together, suggest that deposition of a HGL layer requires the presence of both Hgls (Jang et al., 2007).

Locally enhanced expression of *hetR* in cells differentiating into heterocysts can be visualized by 3.5 h of nitrogen deprivation (Black et al., 1993); at unknown times, the autofluorescence of differentiating cells decreases and those cells stop dividing. A *hetC* mutation blocks differentiation at a very early stage, but localized expression of *hetR* is observed in cells with low autofluorescence that can continue to divide, forming small progeny (Khudyakov and Wolk, 1997; Xu and Wolk, 2001). Genes *hepA* and *hepC* are required for synthesis of heterocyst envelope polysaccharide (Holland and Wolk, 1990; Zhu et al., 1998; Wolk, 2000). Proteins Abp2 (All1939) and Abp3 (Alr3608) bind chromosomal DNA of *Anabaena* PCC 7120 upstream from *hepC*. Insertional inactivation of *abp2* and *abp3* greatly reduced the transcriptional response of *hepC* and *hepA* to nitrogen stepdown, but prevented formation of a HGL layer with, surprisingly, much less evident effect on the HEP layer (Koksharova and Wolk, 2002). By use of transcriptional fusions to *gfp*, encoding green fluorescent protein, Wang and Xu (2005) observed restriction of expression of *hglD* and $hglE_A$ to heterocysts, but lack of expression of those two genes in the small cells corresponding to developmentally aborted heterocysts of a *hetC* mutant.

Ohmori and associates determined that addition of NaCl to the culture medium of *Anabaena* PCC 7120 to 200 mM induced a rapid increase of cyclic AMP (cAMP), which stimulated expression of *hglC*, $hglE_A$, *hglG*, and *hdgB*; those authors identified start sites for transcription of *hgdB* and $hglE_A$ (Imashimizu et al., 2005). They also observed strong binding of recombinant cAMP-binding protein AnCrpA to regions upstream from *hgdB*, $hglE_A$, and *hglG*, presumably regulating the expression of those genes (Suzuki et al., 2007). Whether cAMP normally mediates the accumulation of Hgls is unknown.

VII The Organization of *hgl* Genes Resembles that of Polyunsaturated Fatty Acid-Biosynthetic Genes and Has Been Found in Only One Cyanobacterium that Does Not Form Heterocysts

We identified clusters of PKS genes similar to the cluster in the Hgl island of *Anabaena* PCC 7120 (Fan et al., 2005) by searching for orthologs of the described proteins in other cyanobacteria. Searches were conducted using functions and databases, found on the BioBike server (http://biobike.csbc.vcu.edu:8003/biologin; Massar et al., 2005), that include all of the available cyanobacterial genome sequencing projects. A high degree of homology throughout the cluster was shared only with the other heterocyst-forming strains, namely, *A. variabilis* ATCC 29413, *N. punctiforme* ATCC 29133, and *N. spumigena* CCY9414. Additionally, as mentioned by Fan et al. (2005), *Gloeobacter violaceus* PCC 7421 contains orthologs of most of the *hgl* biosynthetic genes, but these orthologs are not physically clustered in the genome. The DNA sequence identity of the entire biosynthetic coding region (from *hglE* to *hetI*; *alr5351* to *all5359*) is a very high 89% between *Anabaena* PCC 7120 and *A. variabilis*, 72% between *N. punctiforme* and *N. spumigena*, and 64% between the two *Anabaena* species and either of the other strains. Although the structures of Hgls of heterocyst-forming cyanobacteria in section V have been described and found to differ slightly from those of cyanobacteria in section IV (Gambacorta et al., 1998), no sequence data are available for those organisms of section V. Therefore, no direct correlation can be made between the structures of the Hgl products and the sequences of the biosynthetic proteins.

Functional groups in all of the *hgl*-encoded biosynthetic proteins were described by Fan et al. (2005).

The functional groups in the *hgl*-like region were identified using the conserved domain function at NCBI (Marchler-Bauer et al., 2007). We used the region of the aligned sequences with highest similarities to generate a position-specific scoring matrix with which to search cyanobacterial genome sequences for the presence of the specific domain. In each of the genomes of the four heterocyst-forming cyanobacteria analyzed there is an additional, *hgl*-like region – in *Anabaena* PCC 7120, *all1642* through *all1646* (*hglE2*; see above) – that bears a second sequence of genes that is similar to the sequence from $hglE_A$ through *hglA*. The DNA sequence identity in this second region ranges from 92% between the *Anabaena* species to 47% between them and those of *Nostoc* or *Nodularia*. Figure 5a and b presents the similarly organized functional domains in the *hgl* and *hgl*-like regions, respectively, of the four heterocyst-forming strains. Significantly, HglE and HglF are fused in *A. variabilis*, *N. punctiforme*, and *N. spumigena*, and HglD and HglC are fused in *N. punctiforme* and *N. spumigena*.

The functional domains and their organization in the *hgl* and *hgl*-like regions do, however, differ significantly. Neither a KS domain homologous to that of HglD nor a domain for chain termination was found in the *hgl*-like cluster (Fig. 5b). A cluster very similar to the *hgl*-like region from *Anabaena* PCC 7120 and *A. variabilis* is present in *Microcystis aeruginosa* NIES-843 (Fig. 5b), a toxin-forming, non-differentiating, unicellular cyanobacterium. That cluster and the *hgl*-like regions in the four heterocyst-forming cyanobacteria are all found downstream of other genes that are PKS-related (data not shown). The joint role of the genes encoded by the *hgl*-like gene cluster is unclear. In our near-saturation mutational analysis (Huang et al., 2005), no mutation in the *hgl*-like gene cluster was identified that caused a Fox$^-$ phenotype (83 such mutations were identified in the region *alr5351-alr5357*; Fan et al., 2005). Additionally, with the exception of *all1645*, no increase in expression of any of the genes in this region in *Anabaena* 7120 was detected by microarray following nitrogen deprivation (Ehira and Ohmori, 2006a). These observations and the finding of a highly similar region in *Microcystis aeruginosa* suggest that the proteins encoded by this cluster are not directly involved in Hgl synthesis during normal heterocyst development. Additionally, the absence of a chain termination

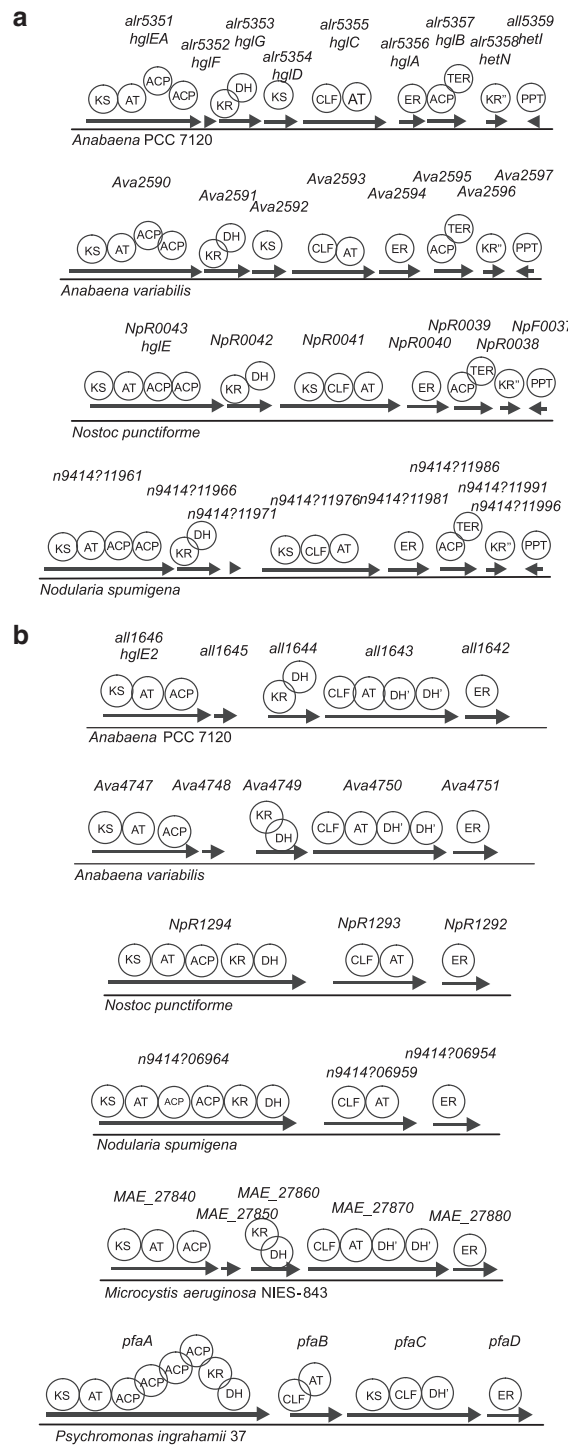

Fig. 5. Modular organization of Hgl proteins encoded in (**a**) the *hgl* region and (**b**) the *hgl*-like region, of *Anabaena* PCC 7120, *Anabaena variabilis* ATCC 29413, *Nostoc punctiforme* ATCC 29133, and *Nodularia spumigena* CCY9414. Part (**b**) includes a comparison with genomic regions from strains of *Microcystis*, a unicellular cyanobacterium that does not form heterocysts, and of the non-cyanobacterium, *Psychromonas* (PfaA: YP_943075, PfaB: YP_943076, PfaC: YP_943077, PfaD: YP_943078).

domain suggests that the protein complex that is encoded by the *hgl*-like region works together with a protein or proteins encoded elsewhere in the genome.

PUFA is a product that is similar in length and structure to the Hgl aglycones. Figure 5a and b illustrates a striking similarity to the organization of domains in the Hgl islands, the *hgl*-like clusters, the aforementioned region from *M. aeruginosa* NIES 843, and clusters that are involved in PUFA synthesis, for example, in *Psychromonas ingrahamii* 37 (Fig. 5b) and *Photobacterium profundum* (Allen and Bartlett, 2002). The following discussion shows a more detailed similarity: corresponding functional domains of the *hgl*-like and corresponding *M. aeruginosa* and *G. violaceus* genes predict amino acid sequences that cluster with those of the PUFA genes of *P. ingrahamii*. We are thereby led to suggest that the *hgl*-like region of the heterocyst-forming strains, the similar cluster in *M. auruginosa* NIES843, and the homologous genes of *G. violaceus* PCC 7421 may be involved in PUFA synthesis.

VIII Are Heterocyst Envelope Glycolipid Biosynthetic Domains Distinguishable from those of Other Polyketide Synthases?

We illustrate the clustering of products of *hgl*-like genes of heterocyst-forming strains and of orthologous genes from *M. aeruginosa* and *G. violaceus*. Most of the sequence conservation of acyl carrier proteins (ACPs) is found in helix II of those proteins, where the phosphopantetheinyl prosthetic group is attached and interactions with other domains and proteins take place (Zhang et al., 2003). The ACP domains in orthologs of HglE and HglE2 cluster with ACP domains of proteins related to PUFA biosynthetic proteins with 90% bootstrap support (Fig. 6).

With 98% bootstrap support, the AT domains of HglE-related proteins cluster with the AT domains of proteins related to PUFA synthesis (Fig.7). The AT domain of a protein (MAE_27840; see Fig. 5b) from *Microcystis aeruginosa* NIES843 is also very closely related to the AT domains of HglE proteins. The AT domains of HglC and its orthologs form a monophyletic cluster (albeit without statistical support), but do not cluster with the AT domains in the HglC-related proteins in the *hgl*-like regions analyzed (Fig. 7). This distinctive phylogeny concords with the fact that the sequence of this domain in HglC and its orthologs contains GYSxG in the catalytic domain, rather than the AT consensus signature GHSxG (Fan et al., 2005, and data not shown).

The presence of a KS and a CLF domain in one protein, as in the HglD-HglC fusions in *N. punctiforme* and *N. spumigena*, is found in some PfaC proteins involved in PUFA synthesis, for example, one from *Psychromonas ingrahamii* 37 (NCBI GenPept YP_943077, Fig. 5b). The sequences of KS domains in *N. punctiforme* HglE, HglE2, and their orthologs form a monophyletic cluster that is closely related to those in PfaA proteins involved in PUFA synthesis (96% bootstrap support, Fig. 8). KS domains in HglC and HglD relatives cluster in two separate clusters, along with domains of proteins related to PfaC. In All1643 and its orthologs in the *hgl*-like cluster are CLF domains related to that in HglC (Fig. 8). KS domains from *Gloeobacter* cluster with domains in HglE (p-gll1959, p-gll4225, p-gll4226, and p-glr2848) and HglD (p-gll1957), and a KS domain and a CLF domain from *Microcystis* cluster with domains in HglE (p-MAE_27840) and HglC (p-MAE_27870), respectively.

Fig. 6. Phylogenetic tree of 88 ACP domains in proteins found in representative cyanobacteria and some reference organisms constructed using the neighbor-joining method. Bootstrap values higher than 50% are shown above the branches. A bootstrap test was performed with 1,000 replications. Sequences were aligned using ClustalX2 (Larkin et al., 2007). Evolutionary distances, computed using the Dayhoff matrix-based method, are presented in units of the number of amino acid substitutions per site. All positions containing gaps were excluded from the dataset. The final data set included 62 positions based on $HglE_A$ positions 1,310–1,383 and 1,450–1,522, corresponding to the two ACP domains of that protein. Phylogenetic analysis was conducted in MEGA4 (Tamura et al., 2007). *Circles* and *small triangles* refer to proteins from, respectively, the *hgl* and *hgl*-like regions of the four heterocyst-forming strains listed in the legend of Fig. 5. Gene products that resulted from BioBike searches have been given prefixes that are abbreviations of the names of the organisms: *Anabaena* PCC 7120 (A7120); *A. variabilis* (A29413B); *N. punctiforme* (Npun); *N. spumigena* (nod); *Trichodesmium erythraeum* IMS101 (ter); *Lyngbya* PCC 8106 (lyn); *Crocosphaera watsonii* WH8501 (Cwat); *Cyanothece* CCY0110 (ctc); *Synechococcus* OS types A′ and B′ (cya and cyb); *Prochlorococcus marinus* AS9601 (pmb); *Synechococcus* WH8102 (S8102); *G. violaceus* PCC 7421 (Gvi); *Synechococcus elongatus* PCC 6301 (S6301);

Fig. 6. (continued) *Microcystis auruginosa* NIES843 (Mae); and *Thermosynechococcus elongatus* BP1 (TeBP1). A7120, A29413B, Npun, and nod form heterocysts; ter and lyn are also filamentous, but lack heterocysts, and ter fixes N_2 aerobically in the light whereas lyn fixes N_2 only under anoxic conditions (http://www.pasteur.fr/recherche/banques/PCC/docs/pcc8106.htm). Unicellular Cwat and ctc can fix N_2 aerobically in the dark. The remaining strains are unicellular and do not fix N_2.

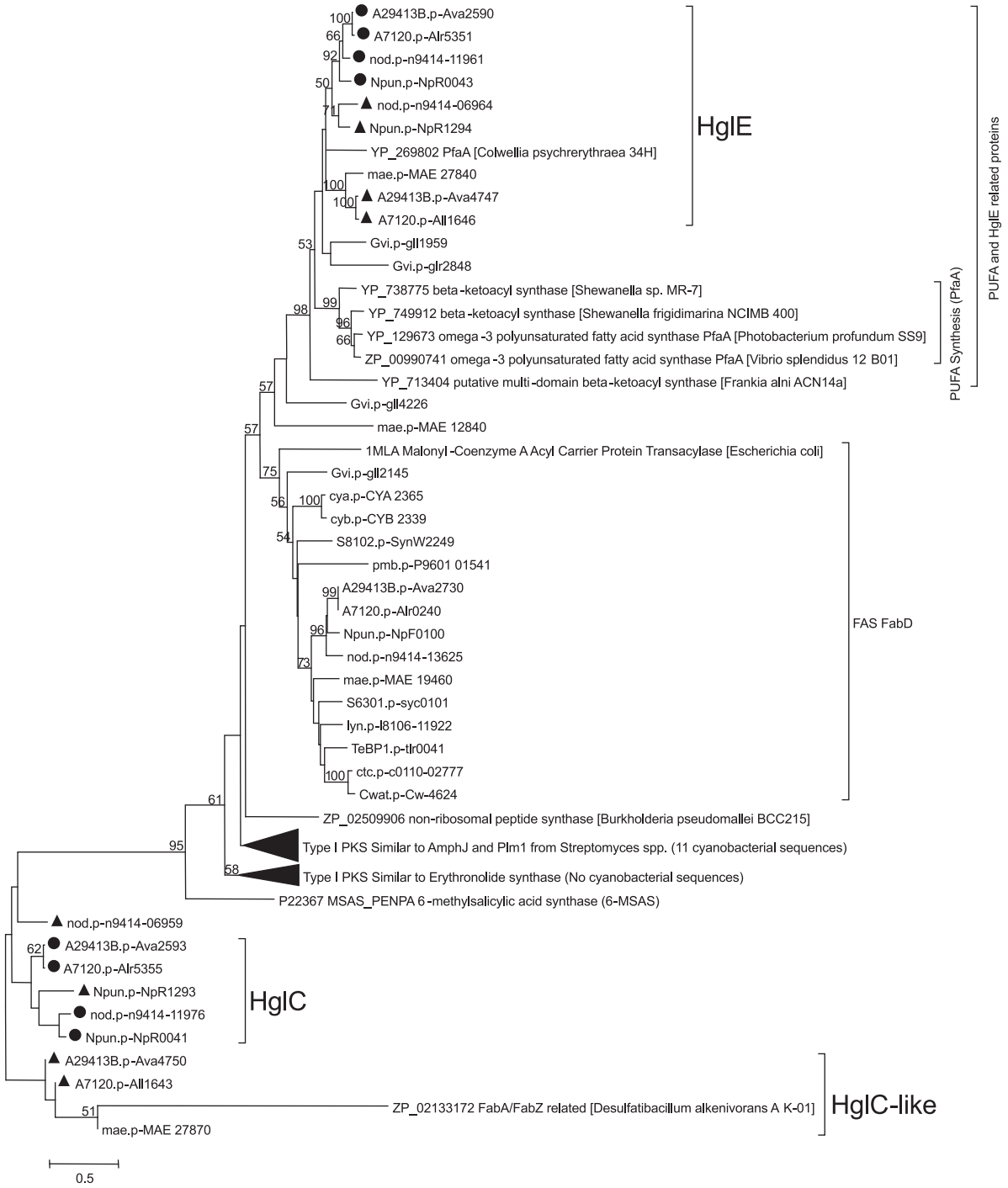

Fig. 7. Phylogenetic tree (see Fig. 6) of 75 acyl transferase domains in proteins found in cyanobacteria and some reference organisms, with only 100 bootstrap repetitions. The final data set, based on $HglE_A$ positions 685–857, included 121 positions.

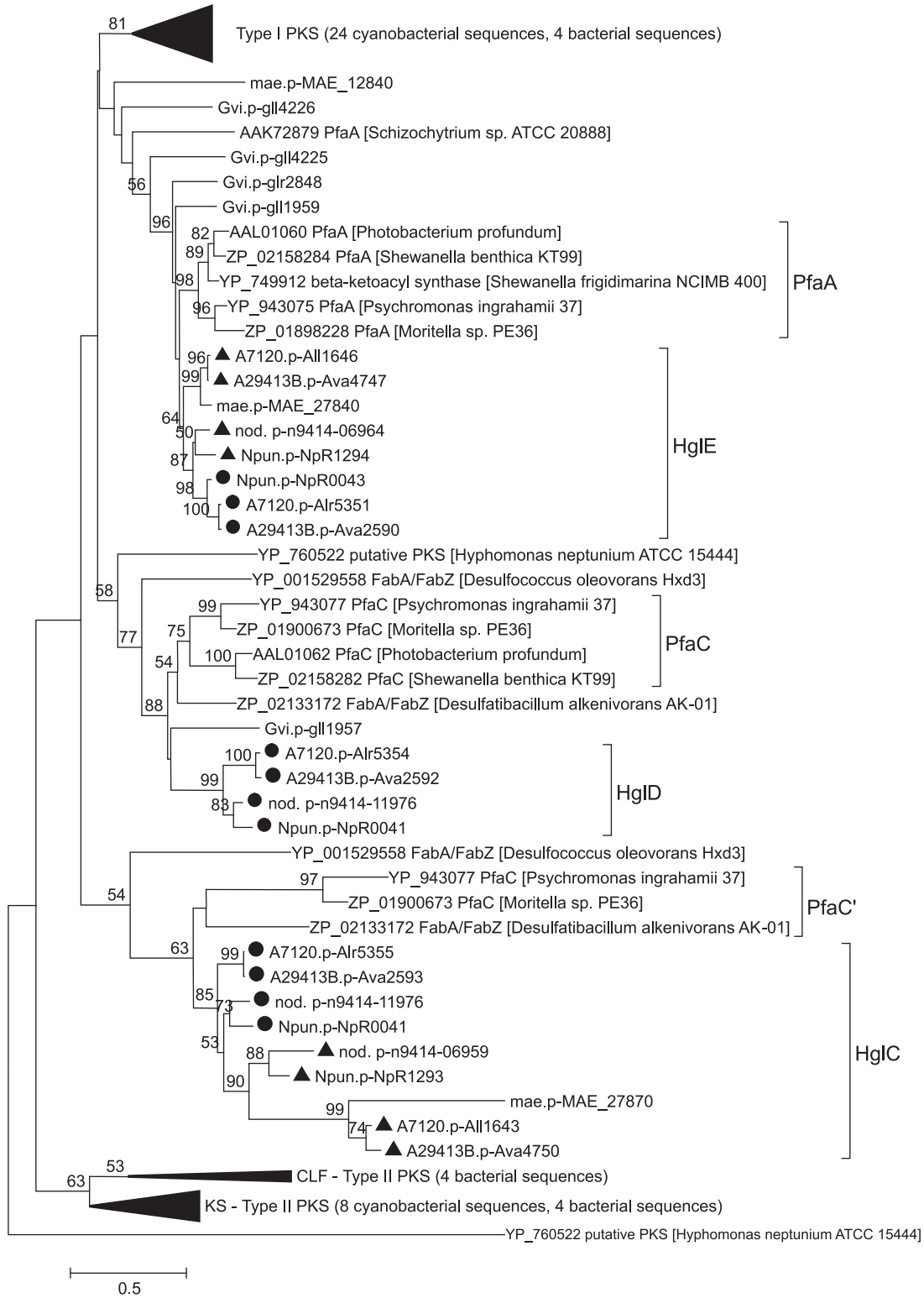

Fig. 8. Phylogenetic tree (see Fig. 6) of 89 KS and CLF domains in proteins found in cyanobacteria and some reference organisms. The final data set, based on $HglE_A$ positions 225–483, included 169 positions.

The KR domain in HglG and its related proteins (including All1644 in the *hgl*-like region, and the orthologs of All1644) is similar to the KR domain found in PUFA biosynthetic proteins (Fig. 9), and clusters separately from the domain found in FabG-related proteins that are involved in fatty acid synthesis. Associated with the KR in HglG and its homologs is a DH domain that is similar to the one found in PUFA synthases and in some proteins annotated as Type I PKSs (Fig. 10). This domain differs substantially in sequence from those found in FabA and FabZ that are involved in fatty acid synthesis. DH domains like those found in FabA and FabZ are found in the *hgl*-like region of *Anabaena* PCC 7120 and of *A. variabilis*, as well as in some PUFA-synthetic gene clusters, but are not found in *N. punctiforme* or *N. spumigena* (Fig. 11). Because FabA functions as a dimer, it is not surprising to find two copies of this domain in All1643 and in Ava4750. Interestingly, the two domains differ; each is related to domains in a different group of proteins. The organization KS-DH′-DH′ is present in a few other proteins, including MAE_27870, and a few PUFA, but normally the FabA/Z domain (conserved domain CD00493) appears only once in combination with a KS domain. Because KS also functions as a dimer, it seems likely that proteins that contain a KS-DH′ organization function as a homodimer.

The similarity of the enoyl reductase in HglA to the one in PfaD (involved in PUFA synthesis), noted earlier (Fan et al., 2005), is represented in Fig. 12. This domain differs significantly from those found in FabI and FabK that are involved in FAS in various bacteria (data not shown).

The TER domain in HglB is the only domain in the *hgl* region that may be involved in chain termination (Fan et al., 2005), and is similar to the TER domain found in Lys2, the yeast protein involved in release of newly synthesized lysine from its synthesizing protein (Ehmann et al., 1999; Hijarrubia et al., 2001). This domain is found in some PKS and non-ribosomal peptide synthases (NRPSs) (Fig. 13). In the cyanobacteria analyzed, only 15 proteins with that domain were found, nine of them in strains of section IV. We found no TER domain in PUFA synthases or in the *hgl*-like regions. Among the cyanobacteria analyzed, the combination of ACP and TER domains in one protein is unique to HglB-related proteins (data not shown).

Finally, we sought to determine whether Hgl proteins bear one or more sequences that can distinguish them from non-Hgl sequences and might help identify Hgl regions in metagenomic analyses. For example, ERWSDEQLTIPDLYLKAKR is a sequence (amino acids 445–463 of HglB of *Anabaena* PCC 7120), that finds TER sequences by tBLASTn (with default settings) in the four heterocyst-forming cyanobacteria whose genomes have been sequenced, and as of this writing identifies no other protein with a similar sequence.

IX Perspectives

Although we have presented a rational interpretation of the pathway of Hgl biosynthesis, that interpretation remains to be tested, either in vitro with isolated enzymes or by heterologous expression of the genes in a different host. Even if the general idea is correct, it remains to be determined with certainty which ACP, which AT, and which KS is active at particular steps of the biosynthesis. Surely it would be helpful if there were *hgl*-region sequence data for the organisms whose various Hgls have been structurally analyzed by the Sodano laboratory.

Humans have a fascination with patterns, be they patterns of notes in music; recurring temporal patterns of day and night, the fullness of the moon, or seasons; or motifs on pottery, on fabric, or in art. As a result, there has been an understandable emphasis on understanding how the pattern of spaced heterocysts is established (Haselkorn, 1998) and maintained (Wolk, 1967). How synthesis of the Hgls is regulated is part of the larger question: what is the network of interactions by which heterocysts mature? Transcription factors HetR dimer (Huang et al., 2004), NtcA (Herrero et al., 2004), and – in the case of Hgls – DevH (Ramírez et al., 2005) are bound to be important players, but we anticipate that there remain numerous other macromolecules whose roles must be identified and conceptually integrated with the roles of those already identified.

Nitrogen fixation and the production of hydrogen gas are biological processes that developed when the world was anoxic, and remain exquisitely sensitive to O_2. They are, respectively, of great and potentially great importance to mankind. Even

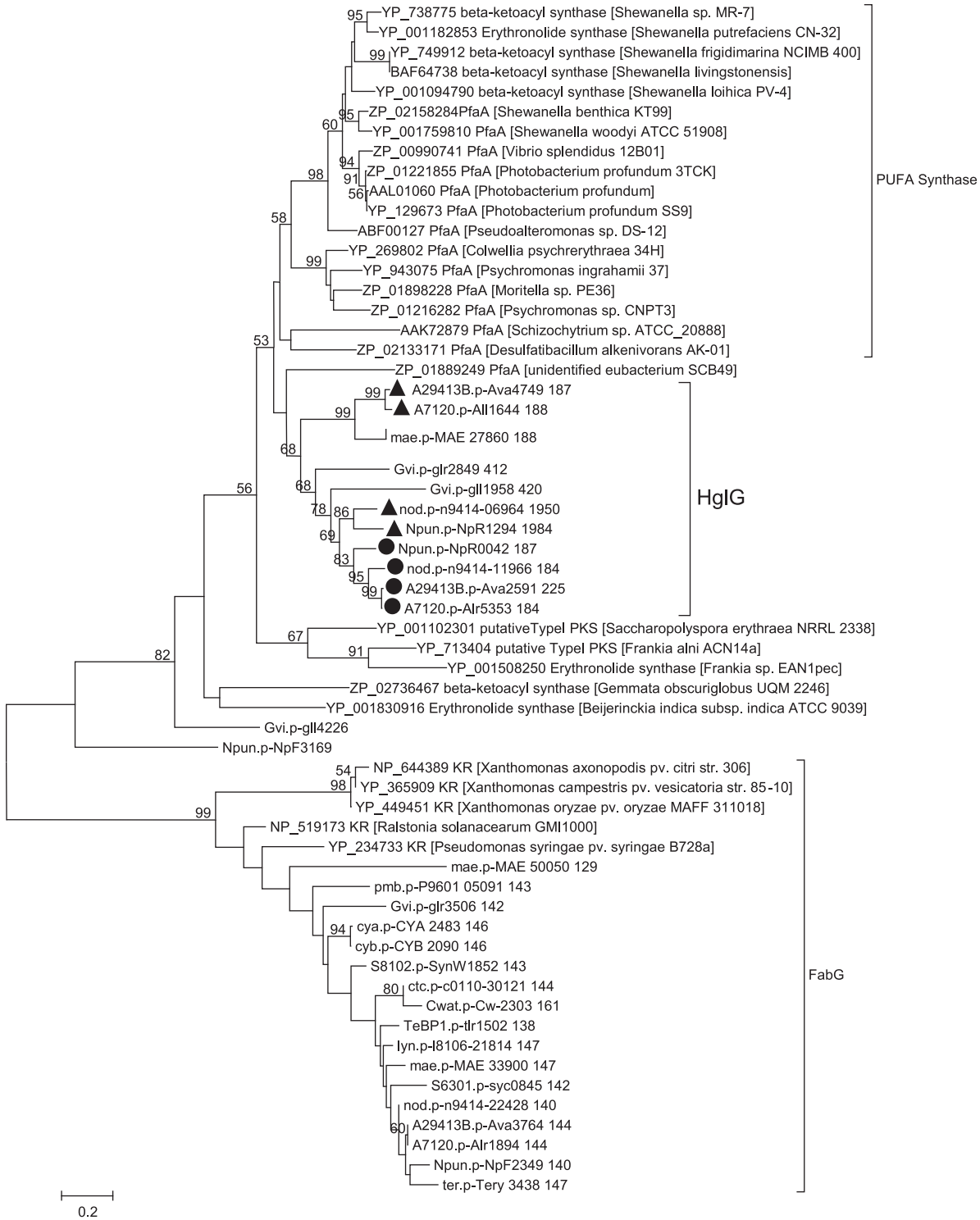

Fig. 9. Phylogenetic tree (see Fig. 6) of 59 ketoacyl reductase domains in proteins found in cyanobacteria and some reference organisms. The final data set included 116 positions based on HglG positions 134–332.

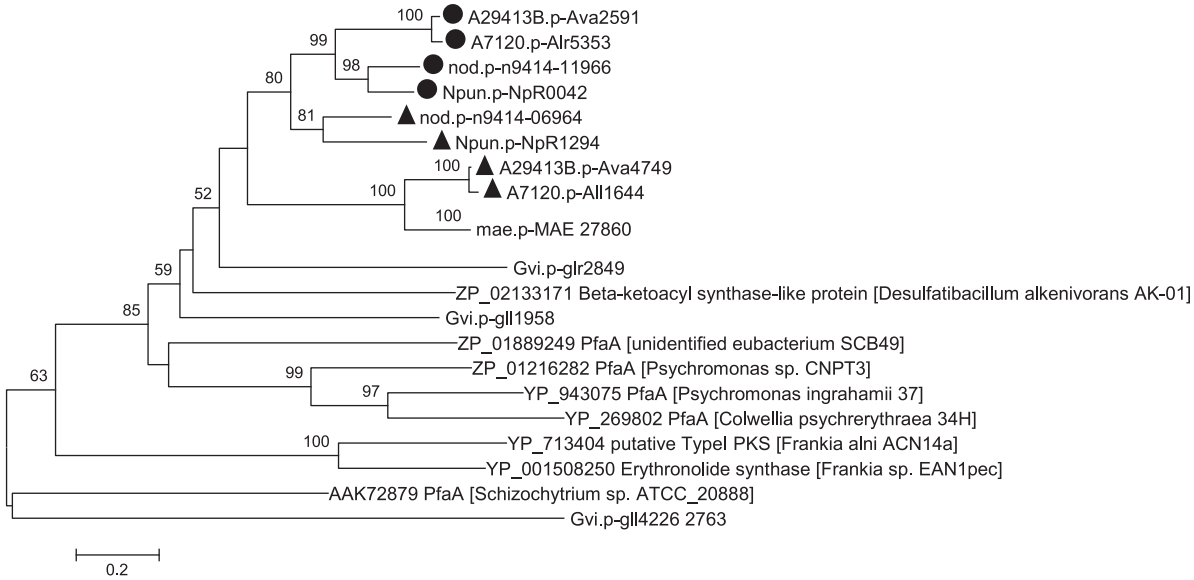

Fig. 10. Phylogenetic tree (see Fig. 6) of 20 dehydrase domains common to HglG-related proteins and to PUFA-synthesis gene clusters. The final data set included 158 positions based on HglG positions 257–455.

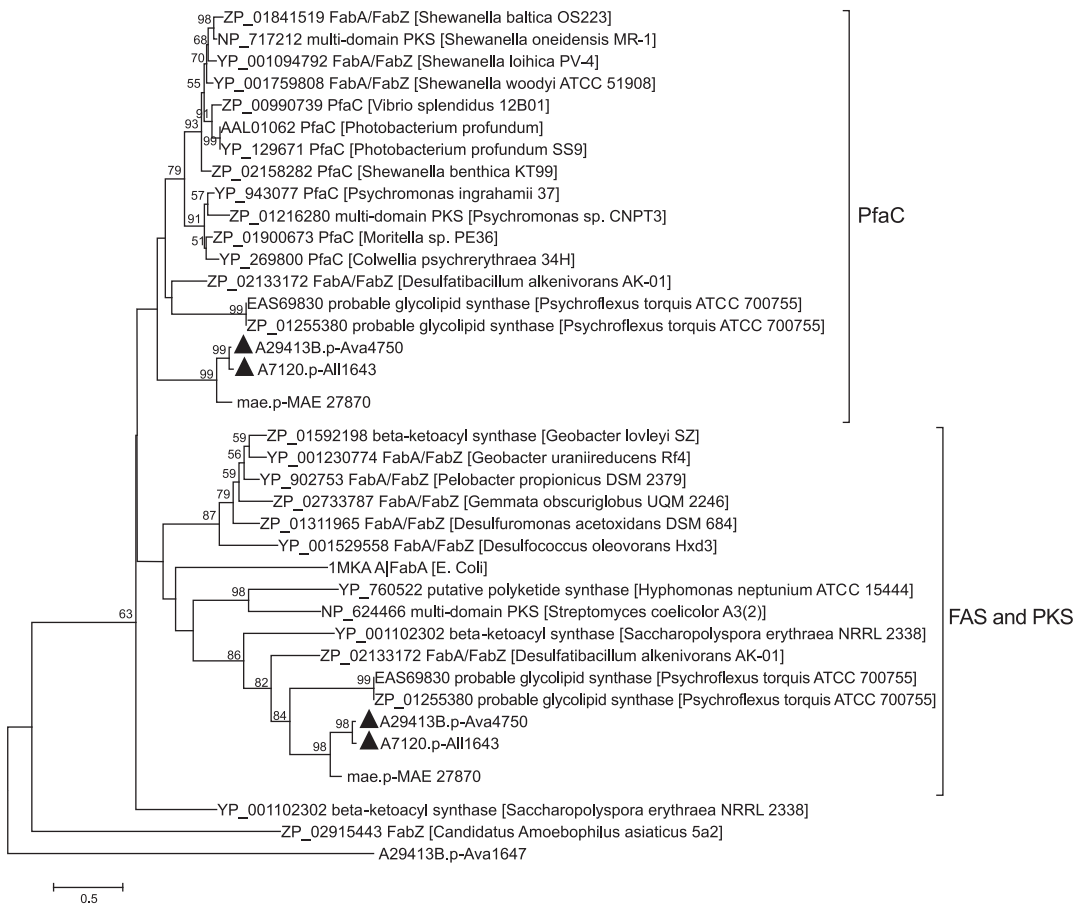

Fig. 11. Phylogenetic tree (see Fig. 6) of 37 dehydrase domains related to FabA and FabZ, found in cyanobacteria and other bacteria. The final data set included 150 positions based on All1643 positions 1,032–1,230 and 1,426–1,601, corresponding to the two DH′ domains in that protein.

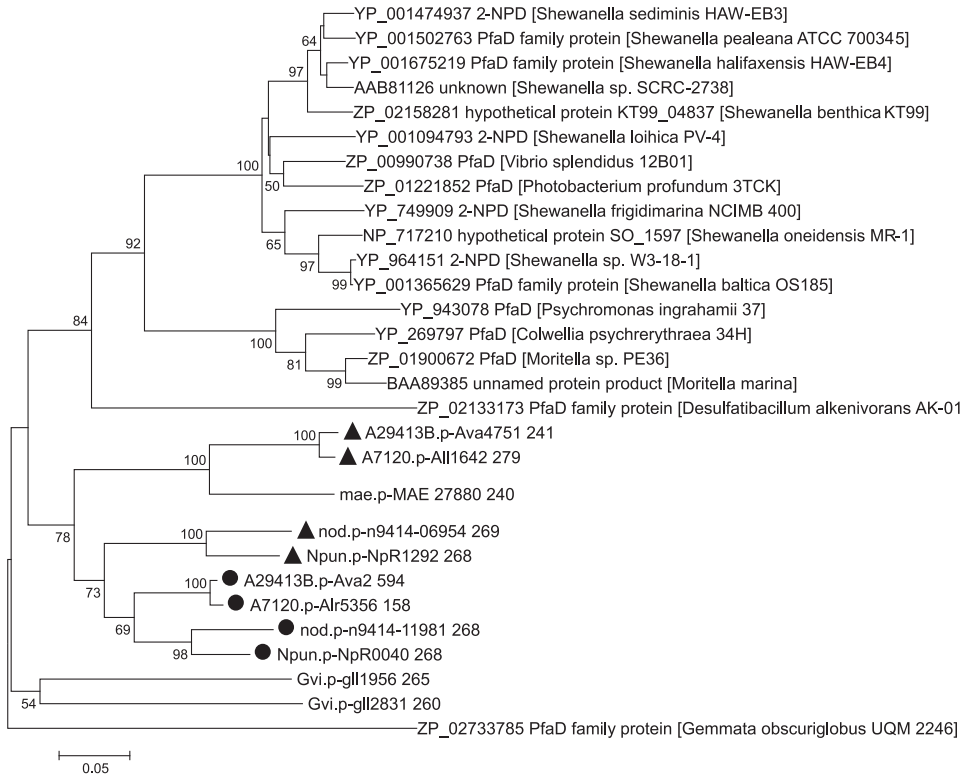

Fig. 12. Phylogenetic tree (see Fig. 6) of 29 enoyl reductase domains related to PfaD. The final data set included 199 positions based on HglA positions 108–306.

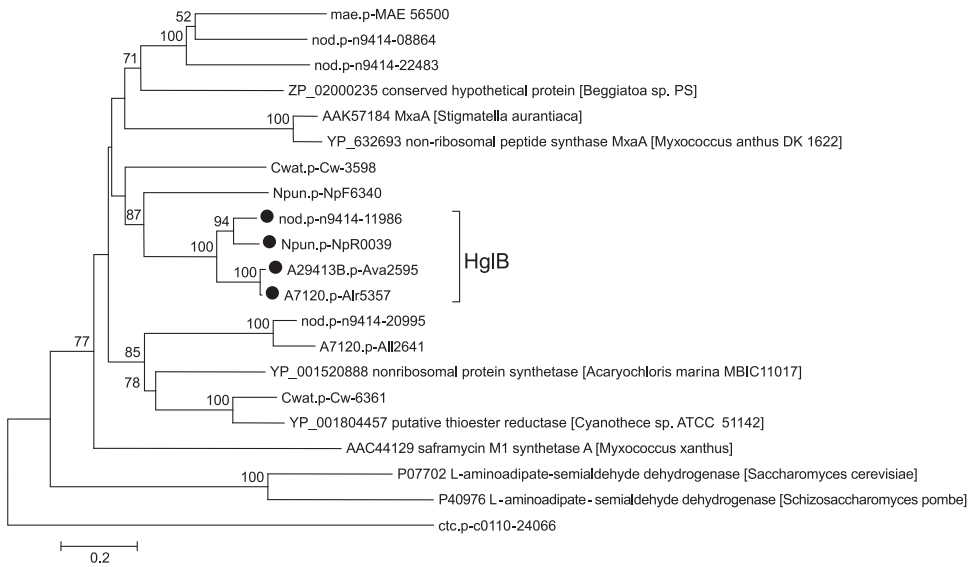

Fig. 13. Phylogenetic tree (see Fig. 6) of 21 thioester reductase domains related to Lys2. After exclusion of positions with gaps, the final data set included 334 positions based on HglB positions 130–503.

though our atmosphere is now replete with oxygen, these processes can be carried out in heterocysts. It is up to those who have an interest in cyanobacteria to find out how to manage that ability of heterocysts in such a way that it can enrich the world. The HGL layer is an important part of the system for maintaining a micro-oxic environment in heterocysts. Even though genes have been identified that are required for the synthesis of Hgls and for their ordered deposition, we still have very little idea what internal or external signals determine how much HGL will be deposited, and even less idea of how those signals are transduced so as to regulate Hgl deposition. These are worthy goals for the near future.

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Chapter 10

Lipids in the Structure of Photosystem I, Photosystem II and the Cytochrome b_6f Complex

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Summary

This chapter describes the data accumulated in the last decade regarding the specific function of lipids in oxygenic photosynthesis, based on crystal structures of at least 3.0 Å resolution of the main photosynthetic membrane protein–pigment complexes, photosystem I, photosystem II and cytochrome b_6f. Comparisons with other structures of membrane protein complexes like the bacterial reaction center and the external antenna system from the plant light harvesting complexes II reveal the functional versatility of integral lipids. A detailed structural description of the membrane protein complexes pinpoints the various interactions of integral lipids between protein and pigments (e.g., chlorophylls, carotenoids, quinones) and gives a deep insight into their functional roles. A particular focus in this chapter is on the lipid-filled plastoquinone exchange cavities in photosystem II and cytochrome b_6f. The differences in extent and lipophilic character of these cavities will be discussed in the light of the resulting plastoquinone/plastoquinol exchange mechanism. An exceptional feature of PS II is the water splitting reaction enabled by the Mn_4Ca cluster. This results in the release of protons to the lumenal aqueous phase, release of electrons to a chain of acceptors, which provides metabolically available reduction equivalents, and release of dioxygen to the atmosphere. The high content of lipids in the interior of photosystem II will be correlated with possible diffusion pathways of the dioxygen and the turnover of the D1 protein, necessary to counteract the photodamage occurring within photosystem II. More structural details of integral lipids derived from higher resolution data from these remarkable membrane protein complexes in combination with data from mutant and/or spectroscopic studies will lead to extended functional insights in the future.

Abbreviations: Car – β-carotene; CL – Cardiolipin; Chl – Chlorophyll; DGDG – Digalactosyldiacylglycerol; DOPC – Dioleylphosphatidylcholin; GGDG – Glucosylgalactosyldiacylglycerol; ISP – Rieske iron-sulfur protein; LHCII – Light harvesting complex II; MGDG – Monogalactosyldiacylglcyerol; β-DM – *n*-dodecyl-β-D-maltoside; BNG – *n*-nonyl-β-D-glucoside; UMQ – *n*-undecyl-β-D-maltopyranoside; Pheo – Pheophytin; PC – Phosphatidylcholine; PE – Phosphatidylethanolamine; PG – Phosphatidylglycerol; PS I – Photosystem I; PS II – Photosystem II; Q_K – Phylloquinone; PQH_2 – Plastoquinol 9; PQ – Plastoquinone 9; PBRC – Purple bacterial reaction center; RC – Reaction center; SQDG – Sulfoquinovosyldiacylglycerol; TMH – Transmembrane α-helix; TDS – Tridecylstigmatelin

I Introduction

A Lipids in Protein Structures

Interactions between lipids and the photosynthetic machinery have been studied for many years. These studies have covered a wide range: for example, general effects of changes in lipid composition of the thylakoid membrane on photosynthetic activity, effects of specific exchange or depletion of individual lipids on different components of the photosynthetic machinery or experiments trying to pinpoint specific interactions between individual

lipids and the photosynthetic protein–pigment complexes. The role of lipids in photosynthesis is discussed in detail in Chapters 11 through 13, and 17 and in various reviews (Pali et al., 2003; Jones, 2007; Wada and Murata, 2007).

Questions regarding specific lipid–protein interactions can only be answered in detail in the light of detailed structural information on the protein–pigment complex involved. Although early biochemical information allowed assumptions to be made regarding lipid locations within protein–pigment complexes (Voß et al., 1992; Kruse and Schmid, 1995; Makewicz et al., 1996; Kruse et al., 2000) the real "break through" has come within the last decade with x-ray crystallographic structural information on detergent solubilized lipid–protein complexes, which has enhanced understanding of the role of integral lipid molecules in protein complexes related to oxygenic photosynthesis. Complete structures of the three main complexes photosystem I (PS I) (Jordan et al., 2001; Amunts et al., 2007), photosystem II (PS II) (Loll et al., 2005; Guskov et al., 2009) and the cytochrome b_6f complex (cyt b_6f) (Kurisu et. al., 2003; Stroebel et al., 2003), were resolved from different organisms with sufficient resolution (around 3.0 Å and better) to localize the positions of lipids within these complexes. In addition, two structures with high resolution of the light harvesting complex LHCII from green plants have been obtained in recent years (Liu et al., 2004; Standfuss et al., 2005), which have unambiguously revealed the location of lipids within these complexes (see Table 1).

In this chapter we will summarize the structural information on lipids in the complexes of oxygenic photosynthesis available to date. By describing the various interactions found between protein, lipids and other cofactors, as well as the possible functional roles of lipids within these complexes, we show that lipids are employed as a novel class of cofactors in membrane proteins of oxygenic photosynthesis, providing specific functionality to them.

Lipids interacting with proteins embedded in a membrane can be classified depending on their structural properties (Palsdottir and Hunte, 2004; Hunte, 2005). The first class is the bulk lipids, which show only non-specific interactions with the protein. The second class is the annular lipids that form a belt of lipids around the protein and are in direct contact with the membrane-exposed part of the protein. The third class is integral lipids, which are bound to the interior of the protein, often at the interface between two subunits or between transmembrane α-helices (TMH). Due to their well-defined positions only annular and integral lipids can be visualized in protein crystallographic studies. The latter often have specific functions for example in protein assembly or cofactor binding.

Table 1. Structures of membrane protein complexes involved in oxygenic photosynthesis.

Protein	Organism	Resolution (Å)	No of lipids/monomer	Lipids/TMH[a]	Reference	pdb code
PS I	*T. elongatus*	2.5	4 (3 PG, 1 MGDG)	0.125	Jordan et al., 2001	1jb0
PS I	*P. sativum*	3.4	–	–	Amunts et al., 2007	2o01
PS II	*T. elongatus*	2.9	25 (11 MGDG, 7 DGDG, 5 SQDG, 2 PG) (+7 β-DM)	0.69 (0.89)	Guskov et al., 2009	3bz1, 3bz2
PS II	*T. elongatus*	3.0	14 (6 MGDG, 4 DGDG, 3 SQDG, 1 PG) (+3 β-DM)	0.39 (0.47)	Loll et al., 2005	2axt
PS II	*T. vulcanus*	3.7	–		Kamiya and Shen, 2003	1izl
cyt b_6f	*M. laminosus*	3.0	2 (2 DOPC)[b]	0.15	Kurisu et al., 2003	1vf5
cyt b_6f	*M. laminosus*	3.0	3 (2 DOPC, 1 SQDG) (+4 UMQ)[b]	0.23 (0.54)	Yamashita et al., 2007	2e74 2e75 2e76
cyt b_6f	*C. reinhardtii*	3.1	3 (2 MGDG, 1 SQDG)	0.23	Stroebel et al., 2003	1q90
LHCII	*P. sativum*	2.5	2 (1 PG, 1 DGDG)	0.67	Standfuss et al., 2005	2bhw
LHCII	*S. oleracea*	2.7	2 (1 PG, 1 DGDG) (+1 BNG)[b]	0.67 (1.0)	Liu et al., 2004	1rwt

[a]Values in parentheses count lipids + detergent together.
[b]With addition of non-endogenous lipids.

B Properties and Composition of the Thylakoid Membrane

A general property of the thylakoid membrane of cyanobacteria, algae and plants is to provide the matrix for photosynthetic protein–pigment complexes (PS II, cyt b_6f, PS I and ATP synthetase) catalyzing the reactions of oxygenic photosynthesis (Nelson and Ben-Shem, 2004; Iverson, 2006; Fromme, 2008) and to hinder the free diffusion of ions, a prerequisite for the generation of an electrochemical potential difference across the membrane that drives the ATP synthetase. The lipid composition of thylakoid membranes is highly conserved among oxygenic photosynthetic organisms. It is composed of uncharged monogalactosyldiacylglycerol (MGDG; ~50%) and digalactosyldiacylglycerol (DGDG; ~30%), as well as anionic sulfoquinovosyldiacylglycerol (SQDG; ~5–12%) and phosphatidylglycerol (PG; ~5–12%) (see Chapters 3 and 8). Cyanobacteria also contain a minor glycerolipid monoglucosyldiacylglycerol, which is absent from chloroplasts (Sato and Murata, 1982). It should be noted that the lipid composition is dependent on the growth conditions, see for example for cyanobacteria (Kiseleva et al., 1999) and Chapter 8.

Because of its high concentration in the thylakoid membranes, MGDG is the most abundant polar lipid species in nature. Compared to animal, bacterial and non-chloroplastic cellular membranes, which are dominated by the presence of phospholipids, thylakoid membranes contain smaller amounts of phospholipids. The lipid molecules and proteins located in the membrane (membrane-intrinsic) each contribute about 50% of the total thylakoid mass. This high protein-to-lipid ratio is typical for energy converting membranes. Specific roles of PG and the glycerolipids are discussed in Chapters 11 and 12, respectively.

C Oxygenic Photosynthesis

The thylakoid membrane of all organisms capable of oxygenic photosynthesis contains the two large protein–cofactor complexes PS I and PS II, conducting light-driven charge separation across the membrane, as well as the cyt b_6f complex, coupling the two photosystems via the plastoquinone pool and the soluble electron transport proteins plastocyanin/cytochrome c_6 (Fig. 1a).

In oxygenic photosynthesis, the photochemical events generate a strongly oxidizing potential at the lumenal side of PS II (Fig. 1b and c) and a strongly reducing potential at the cytoplasmic/stromal side of PS I (Fig. 1d and e), resulting in the oxidation of water and release of molecular oxygen by PS II and the production of reduced electron carriers, which finally reduce $NADP^+$ in a reaction catalyzed by ferredoxin-$NADP^+$ reductase, by PS I. By oxidizing PQH_2 from the plastoquinone pool, which is the final product of transmembrane charge separation in PS II, and by reducing plastocyanin or cytochrome c_6, which serve as reductants for the oxidized side of PS I, cyt b_6f (Fig. 1f) is essential for the completion of the reaction cycles in PS II and PS I. The cyt b_6f complex completes a linear flow of electrons powered by light, which facilitates the endergonic reduction of $NADP^+$ to NADPH by water. The reactions of the Q-cycle catalyzed by cyt b_6f contribute together with the other reactions of the linear electron transfer chain (proton release from water oxidation and proton uptake during PQ reduction at PS II and $NADP^+$ reduction) to the build-up of a proton concentration gradient across the thylakoid membrane (Fig. 1a), which in turn powers the synthesis of ATP from ADP and inorganic phosphate catalyzed by the H^+-ATP synthase. A proton gradient can also be generated by another mechanism involving

Fig. 1. (continued) shown. (**d**) One monomer of cyanobacterial PS I (pdb: 1jb0), view is along the membrane plane. The three cytoplasmic subunits PsaC (magenta), PsaD (blue) and PsaE (green) are shown at the top, other subunits are indicated in panel e, Fe_4S_4 clusters are shown as red and yellow spheres, other cofactors are colored as in panel b. (**e**) Trimeric PS I, view is from the cytoplasmic side onto the membrane, membrane extrinsic parts are omitted. In monomer I all cofactors are shown, in monomer II only Chls are given and in monomer III subunit assignments and positions of Car, lipids and phylloquinones (light blue) are given and TMH of PsaA and PsaB are labeled a–k. (**f**) Protein and cofactor assignment in the cyt b_6f dimer from *M. laminosus* (pdb: 2e74). Cofactors are colored as in previous panels; subunits are given in yellow (ISP), cyan (cyt b_6), red (cyt *f*), green (subunit 8), and magenta (subunit 4). (**g**) Top view (from the cytoplasmic side) of the trimeric LHCII complex from pea (pdb: 2bhw). Cofactors are colored as in other panels, protein in grey, TMH a-c are labeled in one monomer. (See Color Fig. 7 on Color Plate 5).

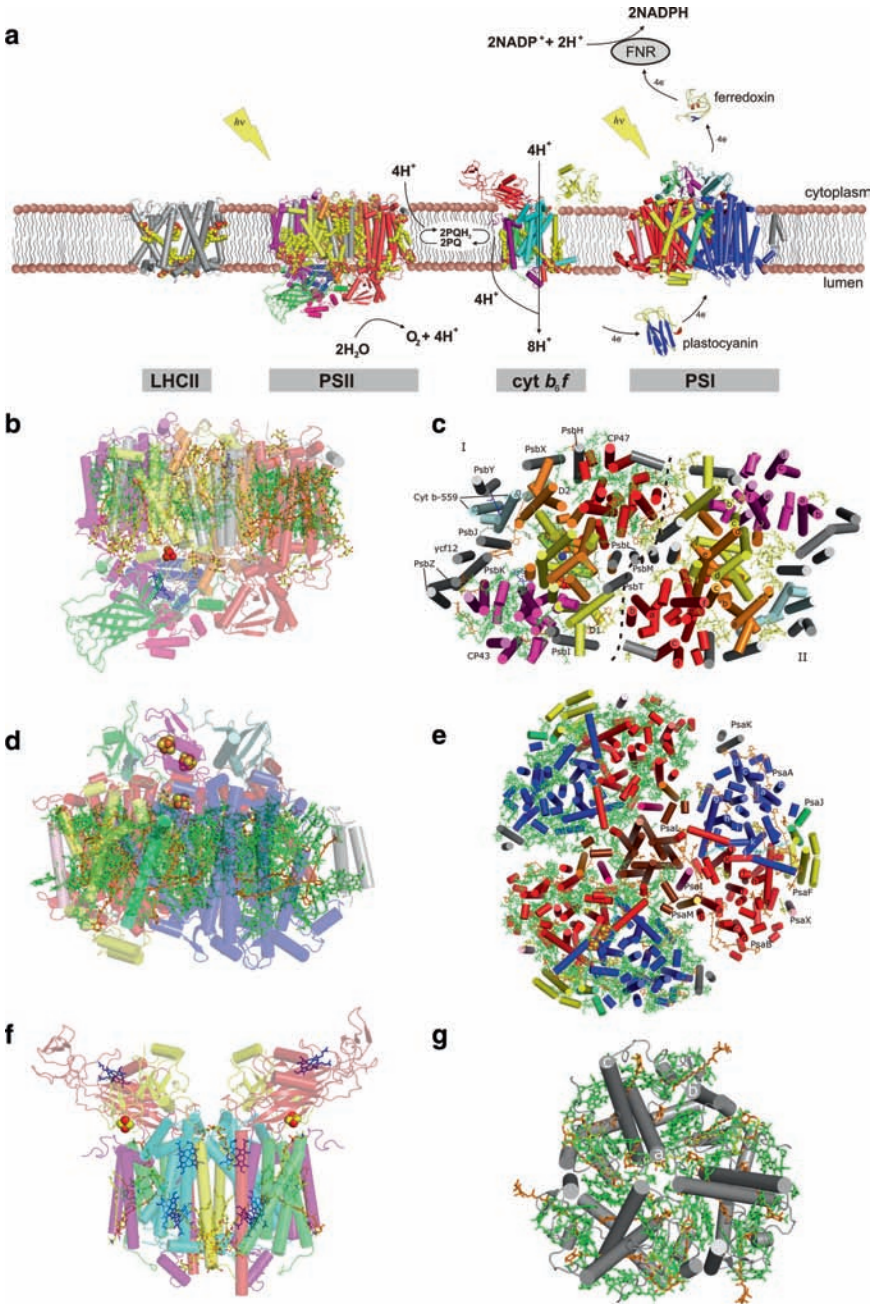


Fig. 1. Lipids in protein complexes of oxygenic photosynthesis. (**a**) Schematic view of the thylakoid membrane with PS I, PS II, LHCII and cyt b_6f embedded. For simplicity only a monomer of each complex is shown, TMHs are shown as cylinders, all cofactors except lipids are omitted. The lipids intrinsic to the structures are shown in space filling representation with yellow spheres for carbon and red spheres for oxygen. Proton as well as electron fluxes are indicated by *arrows*. (**b**) One monomer of cyanobacterial PS II (pdb: 3bz1), view is along the membrane plane. Chls are shown in green, Car in orange, lipids in yellow, heme in blue. Mn (red) and Ca (orange) of the Mn_4Ca cluster and the non-heme Fe^{2+} (blue) are shown as spheres. Membrane intrinsic subunits are assigned in panel c; the three lumenal membrane extrinsic subunits PsbO (green), PsbU (pink) and PsbV (blue) are visible at the *bottom*. (**c**) Dimeric PS II, view is onto the membrane plane from the cytoplasmic side, membrane extrinsic parts are omitted. The monomer–monomer interface is indicated by a black dotted line, the non-crystallographic *C2* axis relating the two monomers by a black ellipse. In monomer I (*left*) protein subunits are indicated and Chl, Car, Heme and PQ (olive) cofactors shown. In monomer II (*right*) TMH of D1, D2, CP43 and CP47 are named a-e/f and lipid and detergent molecules (yellow) are

light-driven cyclic electron flow through PS I and cyt b_6f, which does not produce NADPH.

II Lipids Localized in the Structural Models

A General Considerations

As we now have knowledge about the specific binding pockets for 34 individual lipids, namely, 14 MGDG, 8 DGDG, 6 SQDG, 6 PG molecules (Table 1) bound to membrane protein complexes of oxygenic photosynthesis, a detailed analysis of the specific binding pockets for each lipid species is possible. Due to the presence of the non-bilayer forming lipid DGDG the thylakoid membrane itself shows a pronounced asymmetry between the cytoplasmic and lumenal leaflets (see Chapter 13; Siegenthaler, 1998). This asymmetry is also reflected in the various protein structures (see Table 2). Analysis of integral lipid positions in all available crystal structures of the protein complexes of oxygenic photosynthesis reveals an exclusive location of PG and SQDG on the cytoplasmic side, an exclusive location of DGDG on the lumenal side and the presence of MGDG on both sides. The results for DGDG and PG are as expected, but for SQDG data from spinach thylakoids (showing a predominant presence of SQDG in the lumenal leaflet (Giroud and Siegenthaler, 1988; Siegenthaler, 1998)) do not fit with the structural data, perhaps due to differences between cyanobacterial and plant thylakoid membranes.

The head groups of all integral lipids localized in the complexes of oxygenic photosynthesis are located at the height of the border between the membrane and the adjacent aqueous phase. As this coincides with the stromal or cytoplasmic end of the TMHs, Tyr and Trp, which are typical residues found at the termini of a TMH, are present in the binding pockets of all lipid head groups (see Tables 3–5). The detailed lipid–protein interactions are dependent on the nature of the head group of the lipid. Between two and six amino acids form polar interactions with each head group, yielding a total of four to eight (in some cases up to 16) hydrogen-bond and salt bridge interactions between the protein and the lipid head. The average number of hydrogen bond and salt bridge interactions increases from 4.5 for the smaller MGDG to 8.5 for the larger DGDG. This situation is similar for the negatively charged PG and SQDG, where the smaller PG shows about 4.3 interactions but the average number of interactions for the larger head of SQDG is 6.5. The non-native detergent molecules show a lower number of polar protein interactions (about 3.3 on average), indicating a weaker binding to the complex compared to the natively bound lipids. Also the amino acid composition of the binding pocket is different depending on the lipid species. Positively charged residues Arg and Lys are nearly exclusively found in the binding pockets of the negatively charged SQDG and PG whereas MGDG and DGDG have relatively few Arg and nearly no Lys residues as their neighbors. On the other hand negatively charged residues Glu and Asp are only found in the binding pockets of MGDG and DGDG and not around PG and SQDG.

The found differences in binding pocket architecture together with the fact that the nature of the head group of each lipid bound in the protein complexes could be identified unambiguously in the electron density shows that each binding pocket is highly specific for one type of lipid, in other words that there is no indication for chemical heterogeneity concerning the occupation of binding sites by the lipid head groups.

For comparing the lipid content in the different membrane complexes one possibility is to use the number of lipids per TMH as a criterion. These values are given for the various complexes in Table 1. Although this ratio can depend on the resolution (e.g., 0.39 lipids/TMH for PS II at 3.0 Å and 0.69 lipids/TMH for PS II at 2.9 Å resolution) and the architecture of the complex it might also indicate different functional importance of lipids in the various complexes (e.g., only 0.125 lipids/TMH for PS I and 0.69 lipids/TMH for PS II) as will be discussed in later sections.

Table 2. Location of lipids within photosynthetic protein complexes.

Protein complex	MGDG	DGDG	PG	SQDG
PS I	Cytoplasm	–	Cytoplasm	–
PS II	Both	Lumen	Cytoplasm	Cytoplasm
cyt b_6f	Lumen	–	–	Cytoplasm
LHCII	–	Lumen	Cytoplasm	–

Table 3. Lipid and detergent positions in cyanobacterial PS II (PDB ID: 3bz1).

Nr	Lipid type	Orientation of head group/position in complex	Neighbouring subunits	Polar interactions with protein ≤3.5 Å/with cofactors	Hydrophobic interactions with protein ≤5 Å/with cofactors
1	DGDG	Lumenal intrinsic	D1, CP43, PsbI, PsbO	A-Trp97[*H], A-Glu98[*H,*H] C-Ser216[*A,*H], I -Lys5[*H], I-Tyr9[*H], O-Gly38[*H]	A-Phe93, A-Phe117, A-Leu121, A-Phe155, C-Pro217, C-Phe218, C-Trp223, C-Met281, C-Phe284, C-Ile285/Chl_{ZD1}, Chl41, Chl42, DGDG2
2	DGDG	Lumenal intrinsic	D1, CP43	C-Phe218O[*H], C-Gly219[**H], C-Gly220[*H,*H], C-Ser226[**H], C-Phe292[**H], C-Asn294[*H], C-Arg362[*H,*H,**H]	A-Ala152, A-Phe155, A-Ile160, A-Ile163, C-Pro217, C-Phe218, C-Phe284, C-Ile285, C-Cys288, C-Phe431, C-Phe435, C-Leu438/ DGDG1, Chl41
3	PG	Cytoplasmic intrinsic, PQ cavity	D1, D2, CP43	A-Arg140[P,#P,#P], C-Arg447[*H,*H,**H], D-Asn220[*A], D-Thr231[*P,*P]/ Chl44[*H,*H,**H,**H]	A- Phe273, A-Phe285, A-Val281, C-Trp36, C-Phe436, C-Trp443/Chl37, Chl44, Chl46, SQDG4
4	SQDG	Cytoplasmic intrinsic, PQ cavity	D1, D2, CP43 PsbK	A-Asn267[*H,*H], A-Ser270[*H,**H], C-Trp36[*S], D-Arg223 [#S, #S, #S]	A-Phe265, A-Phe274, A-Trp278, C-Trp35, C-Trp36, D-Phe232, K-Leu33, K-Phe37/LHG3, LHG22, Q_C, Car_{D2}, Car15, Chl44, DGDG5, DGDG6, MGDG7, MGDG18,
5	DGDG	Lumenal intrinsic, PQ cavity	D1, CP43, PsbJ	C-Tyr82[**H], C-Glu83[*H,*H], C-Gln84[**H,*H], C-Gly85[**H], C-Asn418[*H,*H,*H], C-Val420[*H], J-Tyr33[*A]/ Chl37Mg[§A], DGDG6[*H], MGDG19[*H,*H]	A-Phe197, A-Leu297, C-Leu404, C-Val432, J-Phe29/SQDG4, DGDG6, MGDG19, LHG22, Car15, Chl37, Chl44
6	DGDG	Lumenal intrinsic, PQ cavity	D1, CP43, PsbJ, cyt-c550	A-Asn301[**H], A-Ser305[*H], C-Asn405[**H,*H], C-Asn415[*H,*H,**H], C-Ser416[*H,*H,**H], J-Ala32[**H], J-Gly37[*H,*H], J-Ser38[*H], J-Ser39[*H], V-Gln60[*H]/DGDG5[*H]	A-Pro196, A-Leu200, A-Ala203, A-Trp278, A-Val281, A-Phe300, A-Phe302, C-Leu404, J-Phe29/SQDG4, DGDG5, MGDG7, Chl_{D2}, Chl37, Q_C
7	MGDG	Lumenal intrinsic, PQ cavity	D2, PsbF, PsbJ	D-Tyr67[*H], D-Gly70[*H], F-Gln41[*H,*H], J-Gly31[*H]	D-Leu45, D-Trp48, D-Leu49, D-Phe73, J-Phe28, J-Phe29, F-Leu26/SQDG4, DGDG6, Car_{D2}, Q_C, P_{D2}, Chl_{D2}
8	DGDG	Lumenal intrinsic	D2, CP47, PsbH	B-Tyr193[*H], B-Ser277[*H], B-Tyr273[*H], D-His87[*H,**H,**H], D-Ser165[*H], H-Val60[*H], H-Ser61[*H,*H], H-Trp62[*H,**H]	B-Phe246, B-Phe250, B-Val251, B-Trp257, B-Tyr258, B-Ala456, B-Phe463, D-Leu89, D-Leu116, D-Phe120, D-Ile123, D-Leu158, D-Leu162, D-Leu291, D-Trp167, H-Tyr49/Chl12, Chl21, Chl22

(continued)

Table 3. (continued)

9	MGDG	Cytoplasmic intrinsic	D1, D2, CP47, PsbL, PsbM	A-Asn234^{**H},B-Arg7^{*H}, D-Tyr141^{*H,*H}/ MGDG10^{*H,**H}	B-Trp5, B-Tyr6, B-Leu461, B-Phe464, D-Ile144, D-Phe269, D-Leu272, D-Phe273, D-Val276, D-Trp280, D-Met281, L-Leu23, L-Leu27, M-Phe14, M-Val17, M-Pro18/MGDG10, MGDG14, Chl17, Chl24, Chl26
10	MGDG	Cytoplasmic intrinsic	D1, D2, CP47, PsbL, PsbM	A-Ser232^{*H}, A-Asn234^{*H,**H}, B-Trp5^{*A,*A}, B-Tyr6^{*H}, L-Glu11^{*H,*H}, L-Asn13^{**H,**H}, L-Ser16^{H}/ MGDG9^{*H,*H}	D-Ala202, D-Trp266, D-Phe269, D-Phe273, L-Leu19, L-Leu22, L-Leu23, L-Ile24, L-Val26, M-Val17, M-Pro18, M-Phe21, M-Leu22/Chl_{D1}, Chl24, Chl27, Car3, Car5, MGDG9, MGDG11, MGDG14, Q_A
11	MGDG	Cytoplasmic intrinsic	D1, D2, PsbL, PsbT	D-Ala260^{*H}, D-Ser262^{*H,*H,*H,*H,*H}, D-Asn263^{*H,**H}, D-Trp266^{*A}, L-Thr15^{*H,*H}, L-Leu19^{*A}	A-Tyr126, D-Phe257, D-Phe261, D-Phe270, L-Leu19, L-Leu22, L-Val26, L-Leu29, T-Phe10, T-Ile13, T-Phe17, T-Ile21/Q_A, Phe_{D1}, **Car4**, **SQDG12**, P_{D1}, MGDG10, Chl_{D1}
12	SQDG	Cytoplasmic monomer interface	**D1**, CP47, **PsbI**, **PsbT**	**A**-Trp20^{*H,*H}, **A**-Asn26^{*H}, **A**-Arg27^{*A}, **A**-Leu28^{*A}, B-Trp113^{*S}, B-Tyr117^{*S}	**A**-Leu28, **A**-Val30, **A**-Ile38, **A**-Leu41, **A**-Leu42, B-Leu109, **I**-Val11, **I**-Phe15, **T**-Phe22/Car4, Car6, Chl16, Chl29, **Phe_{D1}**, **Chl_{D1}**, **MGDG11**, **Car_{D1}**, **MGDG20**, **Chl_{ZD1}**
13	SQDG	Cytoplasmic monomer interface	**CP47**, PsbL, **PsbL**, PsbM, PsbT, **PsbT**	**B**-Arg18$^{\#S,\#S}$, **L**-Asn4^{**S}, L-Arg14^{*H,*H,**H}, M-Tyr26^{*A}	**B**-Ala28, **B**-Leu29, **B**-Phe108, L-Leu17, L-Tyr18, L-Leu21, T-Leu16, T-Phe19, T-Phe23/MGDG17, **Chl27**, **Car3**, **Car4**, **Car5**
14	MGDG	Lumenal monomer interface	D2, CP47, PsbL, PsbM,	B-Tyr40^{**H}, B-Thr327^{*H}, B-Gly328^{*H}, M-Asn4^{**A}/Chl17$^{*H,\S A}$, **β-DM26**^{*H}	B-Phe196, B-Trp450, B-Phe453, B-Ala454, B-Val457, B-Phe458, B-Leu461, D-Met281, D-Ile284, L-Phe35, M-Ala10, M-Leu13, M-Phe14, M-Val17 / Chl17, Chl24, Chl26, Car3, Car5, MGDG9, MGDG10
15	MGDG	Lumenal monomer interface	**D1**, **D2**, CP47, **PsbO**	B-Ser76^{*A}, B-Trp78^{**A}, **D**-Arg304^{*H,*H,*H}/ **β-DM27**^{**H,**A}	B-Leu39, B-Leu42, B-Ala43, B-Trp75, B-Leu98, B-Ile101, **A**-Ile50, **A**-Ala54, **A**-Leu72, **A**-Leu102, **A**-Leu106/**Car_{D1}**, **DGDG23**
16	SQDG	Cytoplasmic intrinsic	D2, CP47, PsbH, PsbX	B-Lys227$^{\#S}$, B-Ala228*S, B-Arg230$^{\#S,\#S,*A}$, D-Lys23^{*H}, D-Trp32^{*A}/β-DM31^{*S,*S}	B-Leu229, B-Leu474, D-Phe15, D-Trp32, H-Leu39, X-Leu30, X-Phe34/ Chl21, Chl22, LMT31
17	MGDG	Cytoplasmic monomer interface	PsbM, **PsbM** **PsbL**	M-Glu30^{*H}, M-Ser31^{*H}, M-Gln33^{**H}	M-Leu16, M-Ile23, M-Tyr26, M-Val27, **L**-Val10, **M**-Val20, **M**-Phe21, **M**-Ile24, **M**-Leu25/SQDG13, **Chl27**
18	MGDG	Cytoplasmic intrinsic, PQ cavity	D1, D2, PsbE, PsbF	A-Tyr262^{**H}, E-Phe10^{*H,*H}, E-Ser11^{*H,*H,*H,**H}	A-Ile259, A-Phe260, A-Tyr262, A-Ala263, D-Phe27, D-Val28, D-Phe38, E-Phe10, F-Ala22, F-Val23, F-Leu26/Q_B, Q_C, SQDG4, LHG22, Car_{D2}, Car15, Chl_{D2}
19	MGDG	Lumenal intrinsic PQ cavity	CP43, PsbJ, PsbK, ycf12	/Chl37^{*H}, DGDG5^{*H,*H}	C-Leu433, J-Ile22, K-Val24, K-Val27, K-Val30, K-Leu31, y-Ile25 /DGDG5, Car15, Chl37, Chl44, Chl46

Table 3. (continued)

20	MGDG	Lumenal monomer interface	PsbI, **CP47**	I-Thr3[*H,**H/] DGDG23[*H,*H,**A], β-DM30[**H], β-DM32[*H]	I-Thr3, I-Leu4, I-Thr7/LMT30, LMT32, **SQDG12**, DGDG23, Car_{D1}, Chl_{ZD1}
21	MGDG	Lumenal membrane exposed	CP43, PsbZ	C-Trp97[**A], C-Asp107[*H,**H]	C-Val61, C-Trp97, C-Phe109, C-Val113, C-Val114, C-Val117, Z-Phe59 /Car16, Chl34, Chl35, Chl48
22	PG	Cytoplasmic intrinsic, PQ cavity	D1, CP43, PsbK	A-Tyr262[**H], A-Asn266[**H], C-Trp35[*A]	C-Trp35,K-Phe37, K-Phe45 /SQDG4, DGDG5, MGDG18, Car_{D2}, Car12, Car15
23	DGDG	Lumenal monomer interface	D1, **CP47**	**B**-Trp75[*H], **B**-Asp87[*H,**H]/ MGDG20[**H,*H], **β-DM28**[*H], β-DM32[*H]	A-Ile46, A-Ile50, A-Leu102, **B**-Trp75, **B**-Phe90, **B**-Trp91, **B**-Leu98, **B**-Val102/ LMT32, MGDG20, Car_{D1},**Car5**, **Car6**, **Chl16**
24	SQDG	Cytoplasmic membrane exposed	D2, PsbE, PsbF, PsbX, PsbY	D-Arg24[**H], D-Arg26[#S,#S], F-Val18[*S]	D-Trp21, F-Phe16, F-Trp20, F-Val21, X-Leu32, X-Val36, X-Leu37, X-Ile40/ Q_B, DGDG25
25	DGDG	Lumenal membrane exposed	D2, PsbE, PsbY	D-Asp100[*H,*H], D-Thr102[**H]/β-DM29[*A]	D-Tyr42, D-Phe101, E-Val46, E-Phe47, F-Pro29, F-Phe33/Car_{D2}, LMT29, Chl_{ZD2}, SQDG24
26	β-DM	Lumenal monomer interface	**CP47**, PsbM, **PsbM**, PsbT	M-Met1[*,**], **M**-Gln5[*,*,*,**]/ **MGDG14**[*]	**B**-Tyr40, M-Leu8, M-Ala12, **M**-Leu6, **M**-Ile9, T-Met1, T-Ile4, T-Phe8 /, **MGDG14**, **Car3**, **Car4**, **Chl17**, **Chl27**
27	β-DM	Lumenal monomer interface	D1, **CP47**, PsbT	**B**-Thr44[**]/ **MGDG15**[**,**]	A-Leu72, **B**-Leu39, **B**-Tyr40, T-Thr3, T-Ile4, T-Val7, T-Ala11/**Car4**, **Car5**
28	β-DM	Lumenal monomer interface	CP47	/ Chl16[*], **DGDG23**[*]	B-Trp91, B-Leu149, B-Phe162/Chl14, Chl15, Chl16, Chl28, Car6
29	β-DM	Lumenal, membrane exposed	D2, PsbX	X-Ile21[**], X-Ser25[*]/ DGDG25[*]	D-Leu92, D-Trp93, X-Val29, X-Leu30/ DGDG25, Chl_{ZD2}, Chl21
30	β-DM	Lumenal, membrane exposed (close to monomer interface)	PsbI	I-Thr3[**]/MGDG20[**]	I-Thr3, I-Thr7, I-Ile10, I-Val11, I-Phe14, I-Phe15/MGDG20
31	β-DM	Cytoplasmic intrinsic	D2, CP47, PsbH	B-Arg224[*,*,**], B-Lys227[*], D-Asp19[*]/SQDG16[*]	B-Leu225, D-Phe15, H-Trp15, H-Met31, H-Ala32, H-Met35/SQDG16, Chl22
32	β-DM	Lumenal monomer interface	D1, **CP47**, PsbI, PsbO	A-Ala100[**], **B**-Gly85[*], **B**-Asp87[*,*], O-Lys95[*]/MGDG20[*], DGDG23[*]	A-Ile96, A-Leu102, I-Met1, I-Leu4/ MGDG20, DGDG23, Car_{D1}, Chl_{ZD1}

Subunit or cofactor of the other monomer is in **bold**

[*]Potential hydrogen bond.

[**]Potential hydrogen bond if a distance longer than 3.5 Å is accepted.

[#]Potential salt bridge.

[§]Axial coordination of Chl Mg^{2+}.

[A]Interaction formed with carbonyl group of fatty acid.

[P]Interaction formed with phosphate group.

[S]Interaction formed with sulfate group.

[H]Interaction formed with other part of the lipid head group.

Table 4. Lipid and detergent positions in the cyt b_6f complex.

Nr	Lipid type	Orientation of head group/position in complex	Neighbouring subunits	Polar interactions with protein ≤ 3.5 Å/with cofactors	Hydrophobic interactions with protein ≤ 5 Å/with cofactors
		cyt b_6f from *M. laminosus* (PDB ID 2e74)			
1	SQDG	Cytoplasmic large cavity	petA, petC, petD	B-Trp32[*H,*H], B-Tyr38[**H], C-Lys275[#S], D-Arg16[#S] D-Asn20[*S]/UMQ7[*H]	D-Leu21, D-Phe24
2	DOPC	Cytoplasmic membrane exposed	petD	B-Val128[*P], B-Val129[*P], B-Asn118[*P]	B-Ile87,B-Leu96, B-Leu100, B-Leu108, B-Val104, B-Val111, B-Ile114, B-Ala129, B-Ile132, B-Leu143/Chl
3	DOPC	Lumenal intrinsic	petA, petB, petD, petG, petL, petM, petN	C-Gln38[**P]	A-Cys43, A-Met92, B-Thr47, B-Cys50, B-Ile51, E-Met1, E-Ala5, E-Tyr8, E-Ile9, F-Ala8, F-Leu11, F-Leu15, F-Val18, G-Leu9, G-Leu13, H-Val5, H-Trp8, H-Val9, H-Leu11, H-Leu12 H-Phe15/Car
4	UMQ	Lumenal intrinsic, partially membrane exposed	petA, petB, petC, petD	A-Glu75[*], A-Phe78[*], A-Trp80[**], C-Asn253[*]	A-Phe78, A-Trp80, A-Leu81, B-Phe48, B-Val52, B-Val56, C-Trp257, C-Phe261, D-Pro37
5	UMQ	Cytoplasmic monomer-monomer interface	petB, **petB**	A-Lys208[*,*], **A**-Leu12[*,*], **A**-Glu13[**]/**UMQ5**[*]	A-Leu116, A-Ile119, A-Ile123, A-Phe198, A-Leu201, A-Leu204, A-Met205, **A**-Phe8, **A**-Leu12/ **UMQ6**
6	UMQ	Cytoplasmic monomer-monomer interface	petC, petA[*]	A-Asn3[*], A-Asp6[*,**], A-Gln15[*,**], **C**-Lys96[**]/ UMQ7[**]	A-Tyr5, A-Ile14, A-Leu17, A-Ala18/ UMQ7, **UMQ5**
7	UMQ	Cytoplasmic monomer-monomer interface	petB, petD	A-Thr22[**]/SQDG1[*], UMQ6[**]	A-Ala18, A-Val21, B-Trp32/UMQ6
		cyt b_6f from *C. reinhardtii* (PDB ID 1q90)			

1	SQDG	Cytoplasmic large cavity	petA, petD, petC	A-Lys272[#S], D-Trp32[*H,*H], R-Arg42[#S,#S,#S], R-Asn46[*S]	A-Thr265, A-Leu269, D-Trp32, D-Leu37, D-Pro41, D-Leu45, R-Leu49, R-Ala53, R-Ile57
2	MGDG	Lumenal intrinsic	petB, petD, petG, petL, petM	L-Thr3[**H,**H], N-Glu69[*H,*H], L-Ile4[**A]	B-Ile39, B-Cys43, B-Met92, B-Met96, D-Thr47, D-Cys50, D-Val51, D-Leu54, G-Leu5, G-Ile9, G-Leu13, L-Ile4, L-Tyr7, M-Thr69, M-Thr72, M-Met73, M-Met76, M-Val79, N-Trp77, N-Thr80, N-Cys81, N-Phe84/Car
3	MGDG	Lumenal intrinsic, partially membrane exposed	petB, petD, petG, petM	D-Thr148[*A]	B-Val98, B-Phe102, D-Trp79, D-Tyr82, D-Pro83, D-Phe133, D-Leu134, D-Thr137, D-Leu138, D-Ala140, D-Val141, D-Ile145, D-Phe149, G-Cys7, G-Val10, G-Leu11, G-Val14, G-Pro15, G-Ile18, G-Ala19, G-Phe22, M-Ile66, M-Ala67/Chl

Subunit or cofactor of the other monomer is in **bold**

[*]Potential hydrogen bond.

[**]Potential hydrogen bond if a distance longer than 3.5 Å is accepted.

[#]Potential salt bridge.

[A]Interaction formed with carbonyl group of fatty acid.

[P]Interaction formed with phosphate group.

[S]Interaction formed with sulfate group.

[H]Interaction formed with other part of the lipid head group.

Table 5. Lipid positions in cyanobacterial PS I (PDB ID: 1jb0).

Nr	Lipid type	Orientation of head group/ position in complex	Neighbouring subunits	Polar interactions with protein ≤ 3.5 Å/with cofactors	Nonpolar interactions with protein ≤ 5 Å/with cofactors
1	PG	Cytoplasmic intrinsic	PsaA, (PsaE)	A-Arg575[*H], A-Trp592[*A], A-Ser723[*P], E-Gly50[**H]	A-Trp49, A-Asn50, A-His52, A-Ala53, A-Leu54, A-Phe403, A-Leu599, A-Ile725, A-Ala729, A-Val730, A-Val732, A-Ala733, A-Leu736, A-Leu737, A-Ile740/ ChlA1, ChlA4, ChlA6, ChlA9, ChlA26, ChlA28, ChlA40
2	MGDG	Cytoplasmic intrinsic	PsaB	B-Ala562[*H], B-Trp579[*A]	B-Trp21, B-Tyr22, B-Ile24, B-Ala25, B-Met26, B-Phe384, B-Phe582, B-Met586, B-Val709, B-Gln710, B-Leu713, B-Val714, B-Leu716, B-Ala717, B-Ser720, B-Val721, B-Ile724, B-Leu725/ ChlB1, ChlB3, ChlB4, ChlB6, ChlB24, ChlB26, ChlB39, Car19, Q_K-B
3	PG	Cytoplasmic intrinsic, marginally involved in monomer-monomer interface	PsaA	A-Phe336[*P], A-Thr337[*H,*P,*P]/ ChlPL1[§P]	A-Lys333, A-Gly334, A-Phe336, A-Thr337, A-His341/ChlA22, ChlA29, ChlA30, Car7, **ChlM1**
4	PG	Cytoplasmic intrinsic	PsaB, PsaX	B-Asn319[*P], B-Arg413[#P], X-Thr8[*H], X-Arg12[*P]	B-Phe318, B-Met320, X-Pro7, X-Thr8, X-Tyr9, X-Phe11, X-Arg12, X-Trp15/ChlB20, ChlB27

Subunit or cofactor of the other monomer is in **bold**
[*]Potential hydrogen bond.
[**]Potential hydrogen bond if a distance longer than 3.5 Å is accepted.
[#]Potential salt bridge.
[§]Axial coordination of Chl Mg^{2+}.
[A]Interaction formed with carbonyl group of fatty acid.
[P]Interaction formed with phosphate group.
[S]Interaction formed with sulfate group.
[H]Interaction formed with other part of the lipid head group.

B Photosystem II

1 General Structure

PS II occurs as a homodimer in the thylakoid membrane and is composed of at least 20 different protein subunits and nearly 100 cofactors per monomer, yielding a molecular mass of ca 350 kDa for each monomer (Guskov et al., 2009) (Fig. 1b and c). For reviews on the function of PS II see (Wydrzynski and Satoh, 2005; Barber, 2006; Kern and Renger, 2007). The two subunits ligating the cofactors for charge separation are called D1 and D2 (encoded by genes psbA and psbD, respectively). Both show five TMHs each, short loop regions on the stromal side and extended loop regions at the lumenal side. They are arranged in a heterodimer with the five TMHs of each subunit interlocking in a hand-shake motif with each other, similar to the arrangement of the two subunits L and M in the purple bacterial reaction center (PBRC) (Michel et al., 1986). They bind from the lumen to the cytoplasm/stroma four Chl*a* (P_{D1}, P_{D2}, Chl_{D1}, Chl_{D2}), two Pheo*a* ($Pheo_{D1}$, $Pheo_{D2}$), two plastoquinone (PQ) molecules (Q_A, Q_B) and a non-heme iron (Fe^{2+}), the latter being located on the pseudo-C_2 axis relating the two subunits. In addition two peripheral Chl*a*

(Chl_{ZD1} and Chl_{ZD2}), as well as two molecules of β-carotene (Car_{D1}, Car_{D2}), are bound to the D1/D2 heterodimer.

The two large subunits CP43 and CP47 (encoded by genes *psbC* and *psbB*) with six TMHs each are located on the two sides of the D1/D2 heterodimer and bind 13 and 16 Chl*a*, respectively. They also show extended lumenal protrusions, interacting with the lumenal regions of subunits D1 and D2. The membrane-intrinsic cyt b_{559}, composed of the two subunits PsbE and PsbF, each showing one TMH, is located on the side of the D1/D2 heterodimer opposite to the monomer-monomer interface. In addition 11 other small membrane-intrinsic subunits with one or two (in the case of PsbZ) TMH each are part of the cyanobacterial PS II. The lumenal region of PS II is completed by the addition of at least three membrane-extrinsic subunits. In the cyanobacterial system these are PsbO (33 kDa protein, manganese stabilizing protein), PsbU (12 kDa protein) and PsbV (15 kDa, cyt c_{550}). In the plant system instead of the latter two the subunits PsbP and PsbQ are found, although there is also some recent indication for the presence of a PsbQ-like protein in cyanobacterial PS II (Roose et al., 2007).

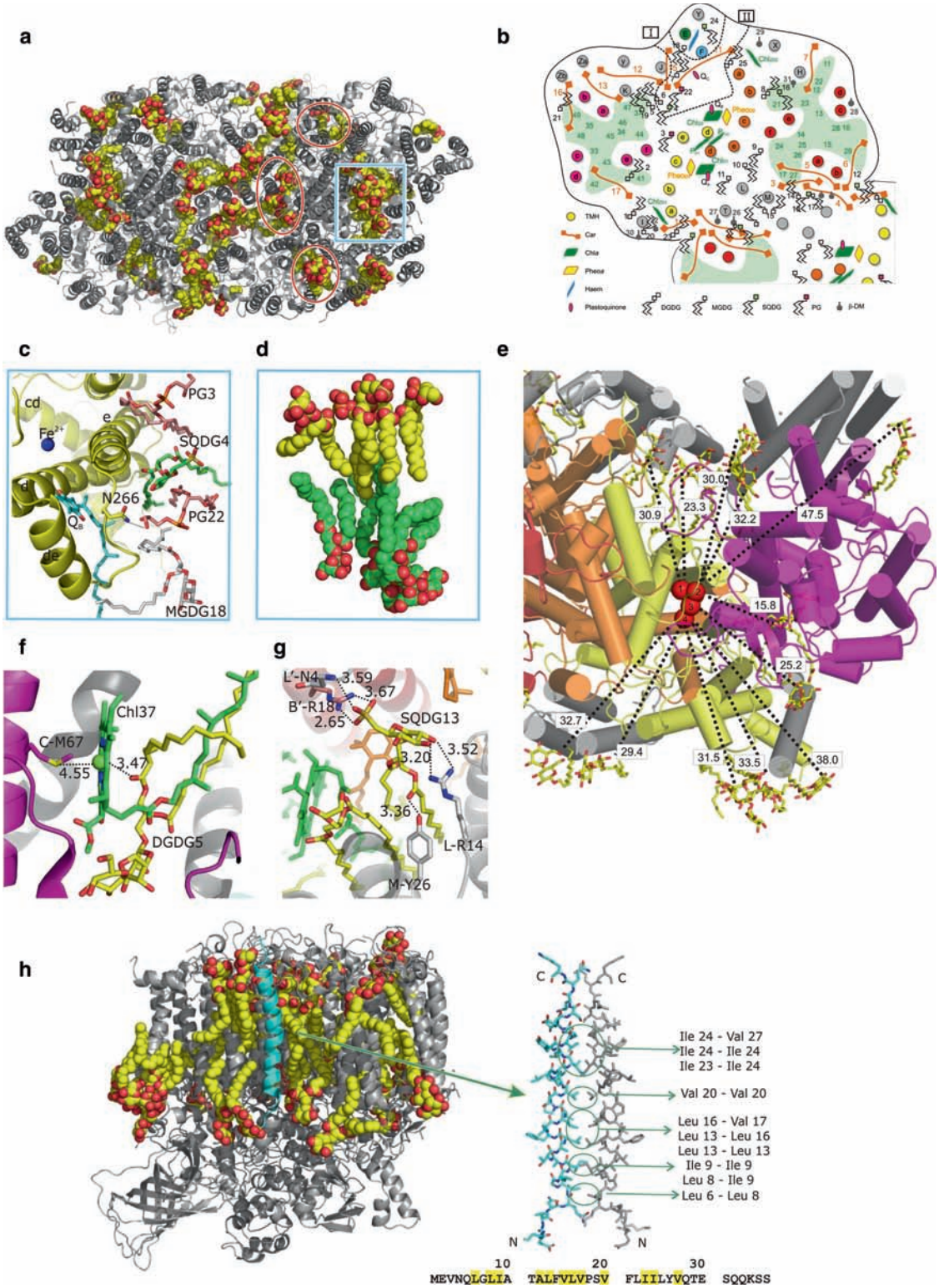
Photosystem II catalyses the light-driven oxidation of water at the lumenal side of the membrane (Renger, 2007; Brudvig, 2008). Here, the Mn_4Ca cluster is bound, ligated mostly by residues from subunit D1 (except CP43-Glu354). After each light-induced charge separation the oxidation state of the Mn_4Ca cluster is increased by one, and finally, after accumulation of four oxidation equivalents in the cluster, two molecules of water are oxidized forming dioxygen. In each charge separation the electron is transferred to the moveable electron acceptor Q_B, located at the stromal/cytoplasmic side of the complex. After acceptance of two electrons and double protonation the formed plastoquinol (PQH_2) leaves its binding pocket and diffuses into the plastoquinone pool located in the thylakoid membrane.

2 Lipid Positions within Photosystem II

For the dimeric PS II of *T. elongatus,* solubilized with the detergent n-dodecyl-β-D-maltoside (β-DM), the first structural information on integral lipids was obtained from data at 3.0 Å resolution (Loll et al., 2005, 2007), which showed the presence of 14 lipids and three β−DM per monomer in the complex. This picture was extended very recently, with improved 2.9 Å resolution structural model (Guskov et al., 2009) revealing the presence of 25 integral lipids and seven detergent molecules in each monomer (see Table 3 and Fig. 2a and b).

All of the lipids are located as expected with their head group facing towards the membrane surface and their fatty acid segments being within the membrane-spanning part of the complex. Twelve of the lipids and one detergent molecule have their head group oriented towards the cytoplasm whereas 13 lipids and six β-DM molecules are oriented with their head group pointing towards the lumenal side (Fig. 2b). A further classification is possible based on the lipid positions within the complex. Only three of the 25 lipids and one β-DM molecule are found at the membrane-exposed outside of the complex (annular lipids), with most of the remainder being located either at the interface between the two PS II monomers (seven lipids and five β-DM from each monomer) or embedded between different subunits within each monomer (15 lipids and one β-DM). These integral or "non-annular" lipids are arranged roughly in a belt surrounding the reaction center formed by the D1/D2 heterodimer and separating it partly from the other membrane-intrinsic subunits (Fig. 2a). Interestingly, these lipids are always grouped together in clusters, smaller ones formed by only two to three lipids and one large cluster consisting of eight lipids (Fig. 2d). This large cluster is composed of two layers of four lipids each, one oriented towards the lumenal phase and the other towards the cytoplasmic phase.

The composition of lipids as derived from the crystal structure of PS II corresponds very well to the average lipid composition of the thylakoid membrane. In each PS II 11 MGDG, seven DGDG, five SQDG and two PG molecules could be located (Guskov et al., 2009), corresponding to relative contents of 44%, 28%, 20%, and 8%, respectively. This composition reflects the highly conserved lipid composition of cyanobacterial thylakoid membranes, ≈45%, ≈25%, 15–25% and 5–15%, respectively (Sakurai et al., 2006; Chapter 8). The distribution of lipids is asymmetric with the head groups of negatively charged PG and SQDG being located exclusively at the cytoplasmic side, those of uncharged DGDG at


a
b
c
d
e
f
g
h
PG3
cd
e
Fe2+
SQDG4
d
N266
PG22
QB
ie
MGDG18
Chl37
C-M67
4.55
3.47
DGDG5
L'-N4
3.59
3.67
B'-R18
2.65
SQDG13
3.20
3.52
3.36
L-R14
M-Y26
30.0
30.9
23.3
32.2
47.5
15.8
25.2
32.7
29.4
31.5
33.5
38.0
Ile 24 - Val 27
Ile 24 - Ile 24
Ile 23 - Ile 24
Val 20 - Val 20
Leu 16 - Val 17
Leu 13 - Leu 16
Leu 13 - Leu 13
Ile 9 - Ile 9
Leu 8 - Ile 9
Leu 6 - Leu 8
N
C
10
20
30
MEVNQLGLIA TALFVLVPSV FLIILYVQTE SQQKSS

the lumenal side and those of MGDG at both sides, similar to the situation in the other photosynthetic membrane protein complexes (see Section II.A and Table 2). There are some studies on the content of lipids in PS II preparations from plants and cyanobacteria. For *T. elongatus* and *Thermosynechococcus vulcanus* between 10 and 60 lipids have been reported per PS II monomer (Ohno et al., 1986; Kern et al., 2005; Sakurai et al., 2006) and different compositions were found especially for the content of PG. The large deviations from the number of 25 lipids found in the structure might be due to differences in preparation, intrinsic errors in the quantification and the problem that the used analytical methods cannot distinguish between free, annular or integral lipids molecules. The observed enrichment of PG in PS II preparations (Sakurai et al., 2006) is not reflected in the structural data, possibly due to the presence of the additional PG molecules in the lipid shell around the complex not resolved in the crystal structure.

For PS II from higher plants no X-ray crystal structure is available to date. The best structural data available were determined by electron crystallography of two-dimensional crystals of a PS II subcomplex CP47RC, composed of D1, D2, CP47, cyt b_{559} and three additional single TMH subunits (Rhee et al., 1997, 1998; Rhee, 2001). The available resolution of 8 Å allowed positioning of TMH and tetrapyrrole rings of Chl/Pheo and heme in the electron density map. A detailed comparison of the found arrangement of TMHs and Chls for spinach and cyanobacteria showed that the positions of the TMHs of the small subunits in relation to D1/D2 are slightly shifted (by 2–4 Å) but that the general arrangement of TMHs in the region between D2 and CP47 is very similar in both systems (Barber and Nield, 2002; Büchel and Kühlbrandt, 2005). This suggests that the lipids present in this region in the cyanobacterial structure (DGDG8, MGDG9, MGDG10, MGDG11, SQDG16, SQDG24, DGDG25) could have corresponding counterparts in the higher plant PS II complex.

For the D1/CP43 side of the plant PS II complex less information is available but fitting of the cyanobacterial structural model into images obtained by single particle electron microscopy of intact dimeric PS II complexes from spinach showed that there is good agreement for the arrangement of TMHs of the core region in both organisms (Hankamer et al., 2001; Nield and Barber, 2006). Although the subunit composition is slightly different between plants and cyanobacteria, these data support the idea that the central part of the PS II complex, including the lipid-rich quinone exchange cavity next to the Q_B site, is very similar

Fig. 2. Lipids in PS II. (**a**) The membrane intrinsic part of the dimeric PS II complex, viewed from the cytoplasmic side, protein is shown in cartoon mode in grey, lipid and detergent molecules in space filling representation with carbons in yellow and oxygens in red. Lipid clusters between D1/D2 and CP43/CP47 are indicated by *red ellipses*, the region around the Q_B site is marked by a *blue rectangle*. (**b**) Schematic view of cofactor positions in PS II, shown is one monomer and part of the second monomer; the quinone exchange cavity is indicated by a *broken line* and the two portals by roman numerals. TMHs are shown as *circles* with coloring according to subunits (D1 yellow, D2, orange, CP47 red, CP43 magenta, PsbE green, PsbF blue). Other subunits are indicated by letters, for example, PsbE by "E", whereas ycf12 is labeled "y". Symbols for lipids with head upwards or downwards indicate location of the head group at the cytoplasmic or lumenal side, respectively. Chl positions in CP43 and CP47 are given by green numbers. (**c**) Lipids positioned around the Q_B binding site formed by subunit D1 (yellow). The non heme Fe^{2+} is shown as blue sphere, the PQ9 bound to the Q_B-site in light blue, PG in salmon, SQDG in green and MGDG in grey. (**d**) Bilayer like arrangement of eight lipids in the quinone exchange cavity, lipids with head group at the cytoplasmic side are shown with carbons in yellow, lipids with head group at the lumenal side with carbons in green. (**e**) Lipids on the lumenal side in vicinity of the Mn_4Ca cluster (*red spheres*). Coloring of subunit is as in panel (b), view is from the lumenal side onto the membrane plane. Distances from lipid head groups to the Mn_4Ca cluster in Å are indicated. (**f**) Coordination of the central Mg^{2+} of Chl 37 (green) by the glycerol moiety of DGDG5 (yellow). Surrounding protein is shown in magenta (CP43) and grey (other subunits). (**g**) Interactions of the polar head group of SQDG13 (yellow) with amino acid side chains from PsbL, PsbM, PsbL′ and CP47′. Possible hydrogen bond interactions are shown by *dotted lines* and distances are given in Å. (**h**) Lipids in the monomer-monomer interface, shown is one monomer looking onto the monomer–monomer interface along the membrane plane, cytoplasm at the *top*, lumen at the *bottom*. Proteins are shown in cartoon presentation in grey; lipid and detergent molecules in space filling representation, with carbons in yellow, oxygens in red, subunit PsbM is highlighted in cyan. Interactions between subunits PsbM (cyan) of monomer I and of monomer II (PsbM' [grey]) are shown on the *right*. N- and C-termini are labeled; specific protein–protein interactions are indicated by *circles* and involve residues highlighted (yellow) in the amino acid sequence of PsbM given at the *bottom.* Panels a-d and h are adapted from Guskov et al. (2009) (See Color Fig. 8 on Color Plate 6).

in cyanobacteria and plants. Biochemical analysis of spinach PS II yielded values between eight (Sakurai et al., 2006), ten (Murata et al., 1990) and 50–70 lipids (Kruse et al., 2000) per reaction center, reflecting the dependence of the results on the purification procedure and the quantification method. As the overall architecture of the plant and cyanobacterial PS II complex is quite similar a significantly lower number of lipids is not expected and might be the result of different purification procedures for the plant and the cyanobacterial PS II. In addition one group reported the enrichment of cardiolipin (CL) in PS II preparations from plants (Depalo et al., 2004; Ventrella et al., 2007) and found an increased thermal stability conferred to PS II complexes by addition of CL.

C *Cytochrome* b_6f *Complex*

1 *General Structure*

The cyt b_6f complex (for review, see Breyton, 2000; Allen, 2004; Cramer et al., 2004) is composed of four large subunits, cytochrome *f*, cytochrome b_6, the membrane-extrinsic Rieske iron–sulfur protein (ISP) and subunit IV, as well as several small membrane-intrinsic subunits (Fig. 1f). It occurs as a homodimeric complex in the thylakoid membrane with a molecular mass of about 220 kDa for the dimer.

Similar to the respiratory cyt bc_1 complex (Xia et al., 1997; Hunte et al., 2000) the cyt b_6f complex has two quinone binding sites, one for quinone oxidation and proton release (Q_p site) located at the lumenal or positive (*p*) side and one for quinone reduction and proton uptake (Q_n site) located at the cytoplasmic or negative (*n*) side. In the so called "Q-cycle" doubly reduced PQH_2 from the PQ pool binds to the Q_p site and in a bifurcated mechanism donates one electron to the Fe_2S_2 cluster in ISP, which subsequently reduces heme *f*, and uses the second electron to reduce heme b_p. The first reaction is coupled to the release of two protons, which means that for every electron transferred *via* the cyt b_6f complex to plastocyanin or cytochrome c_6, and from there to PS I, two protons are released into the aqueous phase at the lumenal side of the membrane. From heme b_p an electron is transferred vectorially across the membrane to heme b_n. The PQ molecule resulting from the *p*-side oxidation of PQH_2 now travels towards the cytoplasmic side and binds to the Q_n site. Here PQ is reduced in two steps, where reduced heme b_n and heme c_n are involved, and takes up two protons from the cytoplasmic aqueous phase to form PQH_2. The overall reaction at the cyt b_6f complex during linear electron transfer can be summarized as:

$$PQH_2 + 2H^+_{\text{cytoplasm}} + 2\ \text{Plastocyanin (ox)} \rightarrow PQ + 2\ \text{Plastocyanin (red)} + 4\ H^+_{\text{lumen}}$$

While hemes b_p, b_n, and *f* correspond to analogous prosthetic groups in the respiratory cyt bc_1 complex, the cyt b_6f complex has bound a unique heme *x* or heme c_n close to heme b_n, which might play a role in PS I-driven cyclic electron transport but also in Q_n site reduction of PQ. In addition one Chl and one Car (not present in the cyt bc_1 complex) are bound to the cyt b_6f complex with so far unclear function.

2 *Lipid Positions in the Cytochrome* b_6f *Complex*

Structural information for the cyt b_6f complex was obtained from two sources. One is the crystal structure of the complex isolated from *Chlamydomonas reinhartdtii*, obtained at 3.1 Å resolution in 2003 (Stroebel et al., 2003). At the same time the structure was also solved for the cyt b_6f complex from the cyanobacterium *Mastigocladus laminosus* at a resolution of 3.0 Å (Kurisu et al., 2003). This complex contained the quinone site inhibitor tridecylstigmatelin (TDS). In 2007 the native structure of cyt b_6f from *M. laminosus* was solved at 3.0 Å resolution, revealing the location of three lipid and four detergent (n-undecyl-β-D-maltoside, UMQ) molecules (Yamashita et al., 2007). For both the *M. laminosus* and *C. reinhartdtii* cyt b_6f the biologically active dimer was crystallized. Both structures showed the presence of two or three lipid molecules per monomer, and in both an SQDG was localized with its head group at the stromal side (Table 4, Fig. 3a and b). This SQDG is located within the large quinone exchange cavity (with dimensions of 30 × 25 × 15 $Å^3$) at the monomer–monomer interface. In addition, in the structure from *C. reinhartdtii* two MGDG molecules were found with their head groups pointing towards the lumenal side. They are located in between the TMH of PetM and PetL (MGDG2) and between PetM and TMH G of cyt b_6 (MGDG3), respectively, with one fatty acid chain each interacting either with a Car or a Chl and the other fatty acid

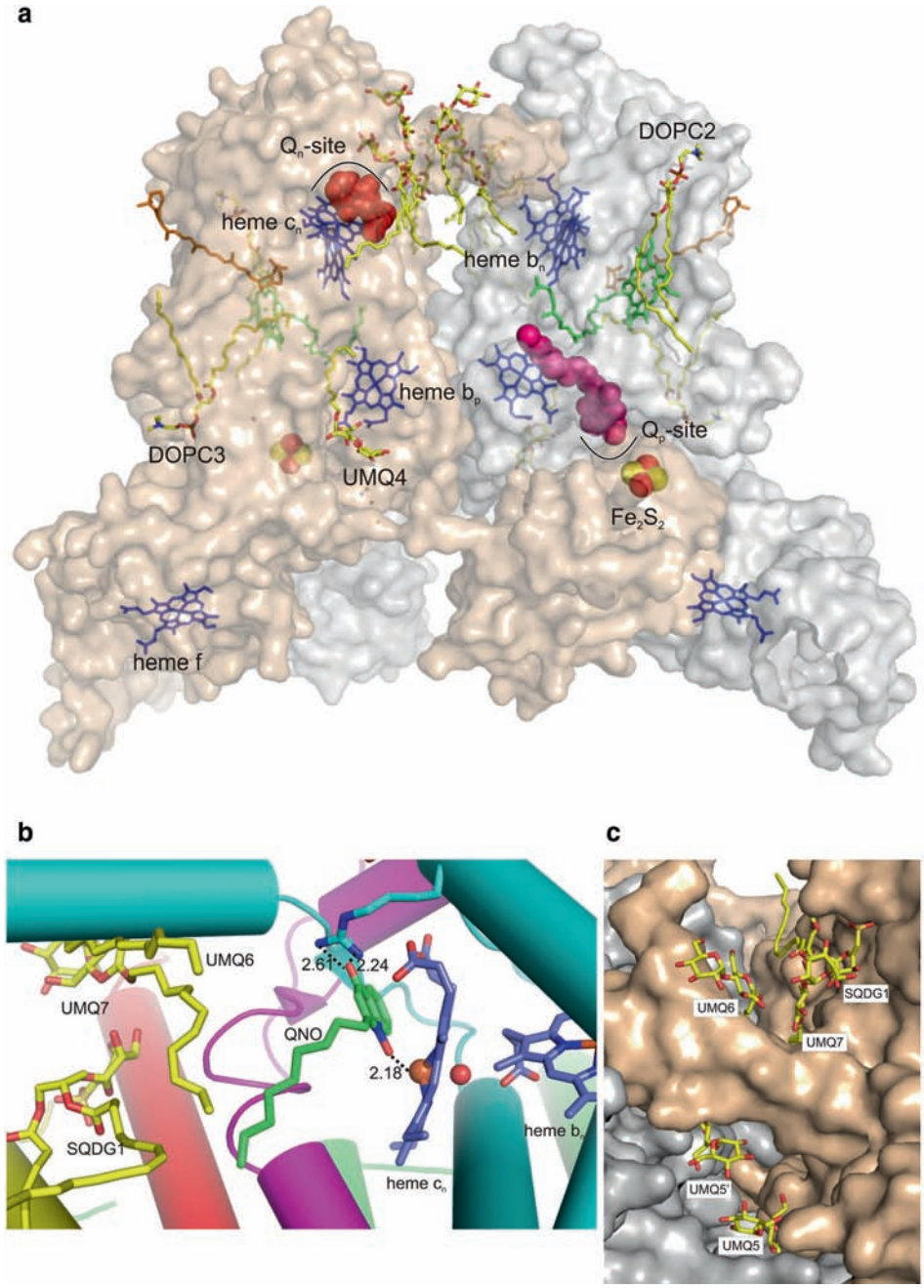


Fig. 3. The cyt b_6f complex from *M. laminosus*. (**a**) Side view of the dimeric cyt b_6f complex from the membrane interior into the quinone exchange cavity. Molecular surface as defined only by the polypeptide subunits of one monomer from the crystal structure of the complex with QNO (pdb 2E75) is shown in grey, corresponding surface of the second monomer from structure of the complex with TDS (pdb 2E76) in brown. The two inhibitors TDS (magenta), bound to the Q_p side, and QNO (red), bound to the Q_n side, as well as the Fe_2S_2 clusters (orange and red) are shown in sphere representation. Lipids and detergent molecules are shown in yellow, other cofactors in green (Chl), blue (Heme) and orange (Car). (**b**) The cytoplasmic quinone binding side Q_n and its surroundings (pdb code 2E76). Protein is shown in cartoon mode with cyt b_6 in cyan, cyt *f* in red, subunit 8 in green, and subunit 4 in magenta. The Q_n side inhibitor QNO (green), replacing PQ at the Q_n side, binds as an axial ligand to the central Fe of heme c_n (blue). Lipid SQDG1 and the two detergent molecules UMQ6 and UMQ7 (yellow) are in the direct vicinity of the Q_n side and their fatty acids are partially interacting with the aliphatic tail of QNO/PQ. (**c**) Top view onto a part of the monomer–monomer interface from the cytoplasmic side, surface of one monomer is shown in grey the other in brown. The lipid and detergent molecules in the monomer-monomer interface are shown in yellow (pdb code 2E74) (See Color Fig. 9 on Color Plate 8).

running along the membrane-exposed surface of the complex. In the native refined *M. laminosus* structure at 3.0 Å dioleylphosphatidylcholine 3 (DOPC), a non-native lipid added to aid crystallization (Zhang et al., 2003), was found in a position similar to MGDG2 in *C. reinhardtii* (see Table 4 and Fig. 3a), with one fatty acid tail coming close to the ionone ring of the carotenoid. DOPC2 was found at the membrane-exposed outside of the complex close to TMH F and G of subunit IV. Of the four molecules of the detergent UMQ present in the structure three are located at the monomer–monomer interface with their head groups on the cytoplasmic side (Fig. 3c) and one (UMQ4) with the head on the lumenal side partially exposed to the membrane exterior.

In the initial structure of cyt b_6f from *M. laminosus* two molecules of the synthetic lipid DOPC were located. These two DOPC molecules were found within the large cavity, one with its head group bound to the cytoplasmic side at a position equivalent to the SQDG found in *C. reinhardtii*, the other to the lumenal side. As addition of these artificial lipids was necessary for successful crystallization of the highly purified cyt b_6f complex (Zhang et al., 2003), it seems that these lipids (and their counterparts in the native non-solubilized complex) are necessary to provide stability to the large quinone exchange cavity.

D Photosystem I

1 General Structure

In cyanobacteria, PS I occurs as a homotrimer consisting of 11 or 12 different protein subunits. Each monomer of ca. 350 kDa has nearly 130 bound cofactors (Fig. 1d and e). For reviews on PS I see (Fromme et al., 2001; Saenger et al., 2002; Golbeck, 2006; Amunts et al., 2008; Krauß, 2008). PS I from higher plants is a monomer composed of a core, which is similar to cyanobacterial PS I and is surrounded by the light-harvesting complex LHCI (Ben-Shem et al., 2003; Amunts et al., 2007; Morosinotto and Bassi, 2008). Like PS II, PS I contains a heterodimer of pseudo-C_2 symmetry formed by two homologous subunits, PsaA and PsaB. In the inner-membrane space this heterodimer folds into 2 × 11 TMHs, which resembles the corresponding structure formed by CP43/D1/D2/CP47 in PS II (Schubert et al., 1998). The PsaA/PsaB heterodimer binds the majority of cofactors, which constitute the PS I core antenna, and the electron transfer chain, which facilitates transmembrane charge separation. The PsaA/PsaB bound part of the electron transfer chain obeys the pseudo-C_2 symmetry and is formed, from the lumen to the cytoplasm/stroma, by a heterodimer of Chl*a*′ and Chl*a* (eC-A1 and eC-B1), four Chl*a* (eC-B2, eC-A2, eC-A3, eC-B3), two phylloquinones (Q_K-A and Q_K-B), and the Fe_4S_4 cluster F_X located on the pseudo-C_2 axis. The symmetry is broken by the terminal acceptors in the electron transfer chain, the Fe_4S_4 clusters F_A and F_B, which are bound to subunit PsaC. Together with PsaD and PsaE, PsaC forms a ridge on the cytoplasmic/stromal side of PS I.

PS I catalyzes the light-driven reduction of ferredoxin or flavodoxin by plastocyanin or cytochrome c_6. Which protein is oxidized and which is reduced depends on the organism and the growth conditions. The initial process in the catalytic mechanism of PS I is light-induced transmembrane charge separation, which involves the electron transfer chain and generates an oxidized chlorophyll pair eC-A1/eC-B1 at the lumenal side, and reduced Fe_4S_4 clusters F_A or F_B at the cytoplasmic/stromal side of PS I. Upon binding to the oxidized lumenal side of PS I, reduced plastocyanin or cytochrome c_6 is oxidized by reducing eC-A1/eC-B1, whereas binding of oxidized ferredoxin or flavodoxin to the reduced cytoplasmic/stromal side of PS I results in reduction of the soluble electron carrier by reduced F_B, leaving F_A and F_B in their oxidized states.

2 Lipid Positions within Photosystem I

In the 2.5 Å resolution crystal structure of trimeric PS I from *T. elongatus* four different lipid molecules could be localized in each monomer, one MGDG and three PG molecules (Jordan et al., 2001; Table 5 and Fig. 4a). Their arrangement follows the pseudo-C_2 symmetry of PsaA/PsaB and their head groups are located on the cytoplasmic side. The molecules of the first pair (PG1 and MGDG2) are located within the core of the monomer close to the electron transfer chain, whilst the lipids of the second pair are located at the periphery of PsaA/PsaB, one close to the monomer–monomer interface (PG3) and one more towards the membrane exposed surface

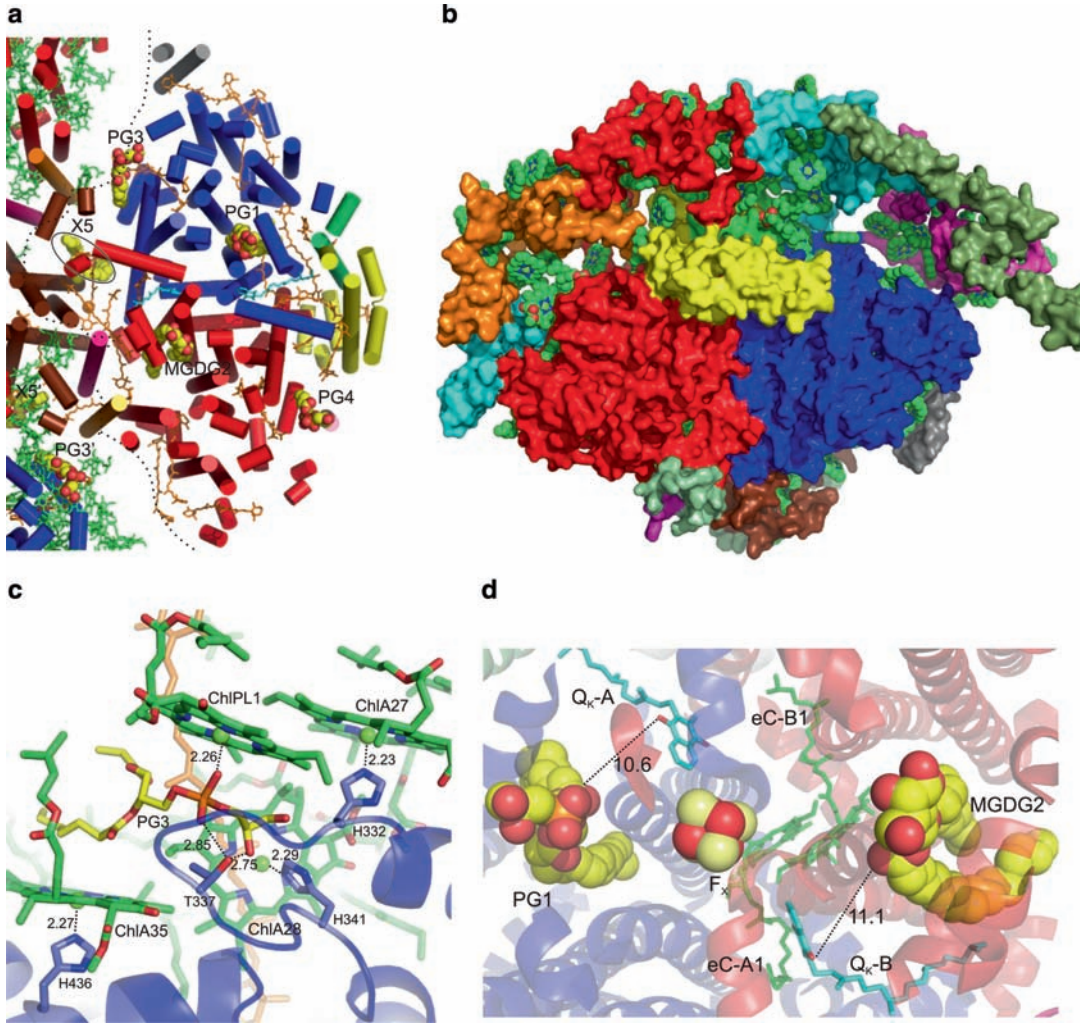


Fig. 4. Lipids in PS I. (**a**) Lipid positions in cyanobacterial PS I (pdb 1jb0), view is from the cytoplasm onto the membrane plane, protein is shown in cartoon mode with coloring of subunits and cofactors as in Fig. 1e. The four lipids PG1, MGDG2, PG3 and PG4, as well as the glycerol moiety of the putative fifth lipid X5, are shown in space filling representation. The position of the fifth lipid is also indicated by a *black ellipse*. (**b**) Molecular surface of the plant PS I complex (pdb 2o01) as defined by the polypeptide subunits, view is from the lumenal side onto the membrane plane. The subunits common with the cyanobacterial system are colored as in Fig. 1e, the array of LHCI complexes arranged in a half moon shape around one side of the PS I core is shown in magenta (Lhca3), cyan (Lhca2), red (Lhca4) and orange (Lhca1) with the lumenal subunit PsaN (olive) partly connecting Lhca3 and Lhca2. The Chls are shown in space filling representation with carbons in green, nitrogens in blue, oxygens in red. Clearly visible is the void region between LHCI and the PS I core, which is most likely filled by ca. 30 lipids. (**c**) Coordination of Chl PL1 (green) by lipid PG3 in cyanobacterial PS I. Possible hydrogen bond interaction between the lipid head group and the protein and ligation of the central Mg^{2+} of the Chl are indicated by black dotted lines, distances are given in Å. (**d**) Arrangement of lipids PG1 and MGDG2 (in space filling representation, carbons yellow) symmetrically to the Fe_4S_4 cluster F_X (shown as *spheres*) and next to phylloquinones Q_K-A and Q_K-B (in stick representation, carbons in cyan) with distances given in Å. Subunits PsaA (blue) and PsaB (red) are shown in cartoon mode (See Color Fig. 10 on Color Plate 9).

(PG4), but shielded from it by the small subunit PsaX. The electron density map at 2.5 Å resolution showed a fifth lipid-like structure, which was not modeled but might belong to a galactolipid, of which the diacylglyceride unit is clearly visible (P. Jordan, P. Fromme, H.T. Witt, W. Saenger and N. Krauß, 2001, unpublished). This molecule is exposed to the membrane very close to the

trimerization domain. It is located on the lumenal side and is probably in contact with the first TMH of PsaL and the loop region between TMH g and TMH h of PsaA (circle in Fig. 4a). That the "fifth lipid" is neither SQDG nor PG can be inferred from the lack of any high electron density as it should be visible within the head group if sulfur or phosphorous were present. This is in agreement with the observation based on X-ray crystallography that in the protein complexes of oxygenic photosynthesis PG and SQDG are found to be exclusively located on the cytoplasmic side (see above and Table 2).

Due to its lower resolution of 3.4 Å, the crystal structure of plant PS I does not show any lipids (Amunts et al., 2007). However, after superpositioning the plant and the cyanobacterial PS I structure the structural model of plant PS I reveals voids at the positions where lipids are located in the cyanobacterial structure, suggesting that these could also be occupied by lipid molecules in the plant complex.

The likelihood of the same lipid binding sites being present in both the cyanobacterial and plant PS I complexes can be assessed by comparing structural details. At the binding site of PG1, the guanidinium moiety of Arg578 of PsaA in the plant PS I structure would clash with the phosphodiester group of this lipid, as it adopts a different conformation than the corresponding Arg575 of PsaA in the cyanobacterial complex. Moreover, the loop connecting β-strands C and D of PsaE, which interacts with the membrane-intrinsic subunits PsaA and PsaB in cyanobacterial PS I and covers the polar head group of PG1 from the cytoplasmic side, is much shorter in plant PS I and thus would not contribute to the binding pocket of a lipid at an equivalent position in this complex. Overall, it seems to be possible that a lipid binds to a similar position in plant PS I, but its polar head group might be less ordered and/or in a slightly different conformation than in cyanobacterial PS I.

MGDG2 would fit well in plant PS I, the only exception being the galactosyl residue, which would cause a steric clash with the bulky side chain of Trp70 of PsaC, which corresponds to Gly69 in cyanobacterial PsaC.

PG3 would cause steric clashes in plant PS I, which seem to be insignificant because they might disappear after slight rearrangements of amino acid side chains. The Chl (ChlPL1), which is coordinated by the lipid's phosphodiester group in cyanobacterial PS I, is replaced by a nearby Chl in plant PS I (Chl1151, having no possible protein ligand) of different orientation and position, the central Mg^{2+} of which could not be axially coordinated by the lipid if the lipid were at the same position as in cyanobacterial PS I.

It is likely that in plant PS I the binding sites corresponding to PG1, MGDG2 and PG3 in cyanobacterial PS I are occupied by ordered lipid molecules. The only exception is the binding site of PG4. This lipid would cause steric clashes with a cytoplasmic loop in PsaB of plant PS I, which has an amino acid sequence of different length and a totally different conformation than the corresponding loop in cyanobacterial PS I. Furthermore, plant PS I has no subunit PsaX, which contributes significantly to the binding of PG4 in cyanobacterial PS I. In plant PS I, this lipid site would be exposed to a larger space between the PS I core and the surrounding LHCI (Melkozernov and Blankenship, 2005). This space corresponds to extended voids separating the PS I core and LHCI in the crystal structure of plant PS I (Amunts et al., 2007), which are most likely occupied by less ordered lipid molecules. Due to its dimensions about 30 lipid molecules can be expected to reside in this interface region (see Fig. 4b).

The lipid composition as it was derived from the crystal structure of cyanobacterial PS I is in agreement with studies on plant PS I-LHCI and cyanobacterial PS I core complexes showing that both only contain MGDG and PG, and indicating that PG is tightly bound to the PS I core from both species, whereas MGDG seems to be more involved in the LHCI (Makewicz et al., 1995, 1996). More recently, analysis of plant PS I by a combination of high performance liquid chromatography and electrospray ionization mass spectrometry revealed a more complex lipid composition, comprising MGDG, DGDG, PG, SQDG and PC but no stoichiometry of the different lipids could be derived (Yao et al., 2006).

E Light Harvesting Complex II

Light harvesting complex II (LCH II) acts as an external antenna to PS II in algae and plants (Allen and Forsberg, 2001; Van Amerongen and Dekker, 2003; Van Amerongen and Croce, 2008)

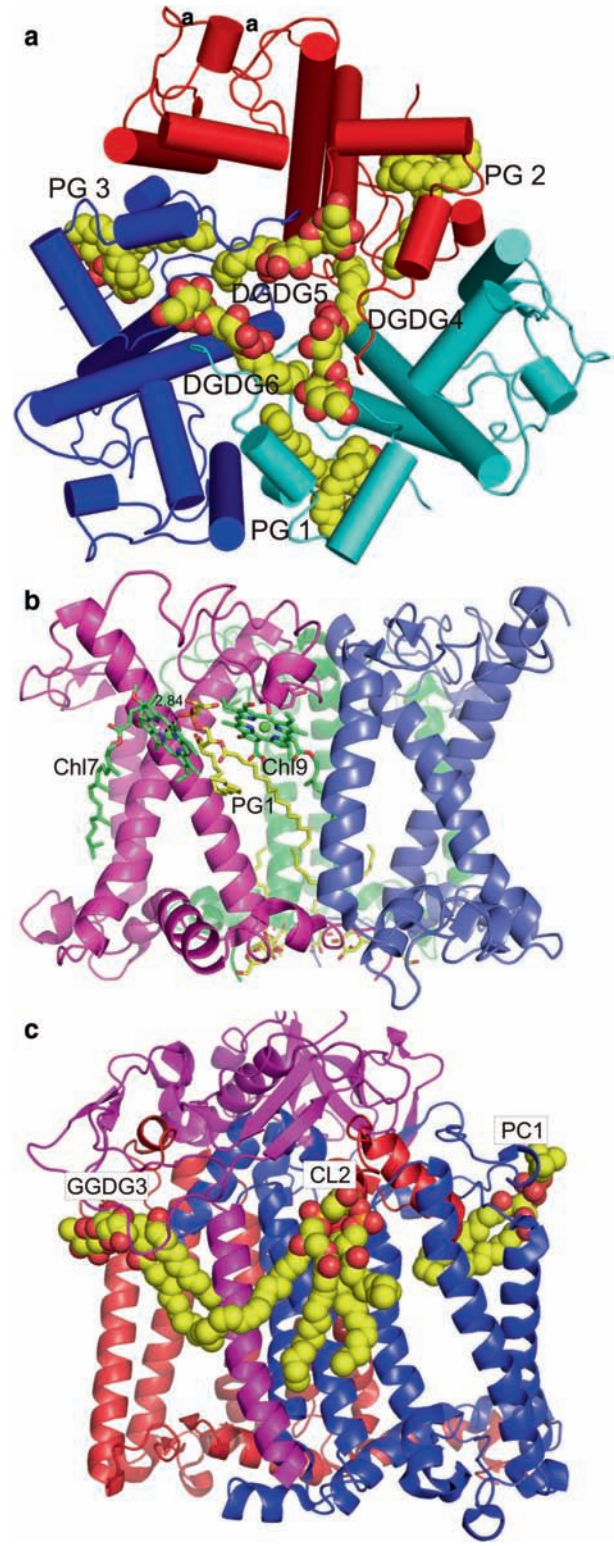


Fig. 5. Lipids in other membrane protein complexes. (**a**) Structure of trimeric LHCII from pea (pdb 2bwh), view is from the lumenal side onto the membrane. Protein from each monomer is shown in different color, lipids are shown in space filling mode with carbons in yellow and oxygen in red. The central position of DGDG in the middle of the trimer is easily visible. (**b**) View along the membrane plane, cytoplasm at top of trimeric LHCII. Visible is the ligation of the central Mg^{2+} of Chl 7 (green) by the oxygen of PG (yellow) and the interaction of PG with Chl 9, maybe providing indirect stabilization to the LHCII trimer. (**c**) Structure of PBRC from *Rb. sphaeroides* (pdb 2j8c) with subunits H (magenta), L (red) and M (blue) in cartoon mode. The three lipids identified in the structure are shown in space filling representation with carbons in yellow and oxygen in red, view is along the membrane plane with cytoplasm at *top* (See Color Fig. 11 on Color Plate 10).

and is the most abundant membrane protein in nature (Peter and Thornber, 1991). The crystal structures of LHCII from pea (Standfuss et al., 2005) and spinach (Liu et al., 2004) have been solved at 2.5 and 2.7 Å resolution, respectively (Barros and Kühlbrandt, 2009). LHCII is organized as a homotrimer in the native state, with each monomer formed by a single polypeptide chain having three TMHs (Fig. 1g). In addition to the 14 Chls and four carotenoids bound to each monomer several lipid molecules could be modeled in the two crystal structures (Fig. 5a). In both structures a PG with its head group at the cytoplasmic side was found. It is located at the interface between the monomers and is directly ligating the central Mg^{2+} of one Chl molecule (Fig. 5b). In the structure from pea one DGDG per monomer was found at the trimerization axis with its head group pointing towards the lumenal side (Standfuss et al., 2005). The cavity accommodating these three lipids was empty but a DGDG in a different location was found in the spinach structure (Liu et al., 2004). This DGDG is located at the outside of the trimer and is involved in crystal contacts between different LHCII trimers in the crystal lattice. As these crystal contacts are non-native (with two neighboring trimers showing a tilt of the membrane plane with respect to each other) it is unlikely that this DGDG molecule will adopt the same position in the native membrane bound LHCII. The spinach LHCII structure also revealed the position of one detergent (n-nonyl-β-D-glucoside, BNG) molecule per monomer. It is located at the membrane-exposed outside of the trimer with its head group at the cytoplasmic side, forming a hydrogen-bonding interaction with the phytol ester group of one Chl, and its long alkyl chain partially shielding the Chl chlorin ring from the membrane. This position could well be occupied by an annular lipid in the native membrane-embedded state of the complex.

F Comparison with Other Membrane Protein Complexes

1 Bacterial Reaction Center

There are more than 50 different structures available in the Protein Data Bank for the PBRC, originating from the three species *Blastochloris viridis*, *Rhodobacter sphaeroides* and *Thermochromatium tepidum*. All of them contain the two large subunits L and M (PufL, PufM), each possessing five TMH, and the subunit H (PuhA) with one TMH and a large membrane-extrinsic domain at the cytoplasmic side (Deisenhofer and Michel, 2004; Fyfe and Jones, 2005; Jones, 2007). No lipids have been included in the structures for the *Bcl. viridis* PBRC but some include detergent molecules. In contrast several structures for the *Rb. sphaeroides* PBRC show one CL attached to TMH c and e of subunit M with its head group at the cytoplasmic side and close to the cytoplasmic end of the TMH of subunit H (McAuley et al., 1999; Jones et al., 2002; Koepke et al., 2007) (Fig. 5c). This lipid head group forms interactions between TMH c, e of subunit M and subunit H, mostly via water bridges, and appears to have a structural role. By mutating one residue in the CL binding pocket the thermal stability of PBRC was reduced (Fyfe et al., 2004), indicating that this CL is indeed important for supporting the inter-subunit contacts in the PBRC.

In the structure for the *Rb. sphaeroides* PBRC with the highest resolution reported to date (1.87 Å) (Koepke et al., 2007, pdb: 2j8c) a phosphatidylcholine (PC) and a glucosylgalactosyldiacylglycerol (GGDG) were found in addition to the CL described above (Fig. 5c), all with their head group at the cytoplasmic side. The PC is found close to TMH a and the N-terminal loop region of subunit M. The GGDG was found in a roughly symmetry related position close to TMH a of subunit L and the side of the TMH of subunit H not exposed towards the CL. Its head group is inserted between loops of subunits L and H, though no hydrogen bonds are observed for specific lipid binding. In *Tch. tepidum* PBRC a phosphatidylethanolamine (PE) was found at the same position as the GGDG (Nogi et al., 2000) and the acyl chains of these two lipids share nearly the same site. In addition detergent molecules were found in a similar position in some other structures from *Rb. sphaeroides* PBRC. Camara-Artigas et al. (2002) also reported the presence of one GGDG and one PC in their *Rb. sphaeroides* PBRC structure at 2.2 Å resolution. Although in this structure the head group of GGDG was modeled in an unusual position in the middle of the membrane interior and a different orientation of the PC compared to the Koepke et al. (2007) work was found. These differences might originate

from differences in sample preparation or could be caused by insufficient resolution, rendering the modeling of flexible regions of lipids and detergent molecules very challenging. For a recent review on lipids identified in the various PBRC structures see (Jones, 2007).

In vivo the PBRC is surrounded by the cylindrical light harvesting complex LH1, forming a nearly complete circle around the RC (Law and Cogdell, 2008). The low resolution crystal structure of this LH1-RC complex (Roszak et al., 2003), as well as atomic force microscopy-based modeling studies (Fotiadis et al., 2004), showed that there is an interface region between both proteins, which is most probably filled by lipids (see also Section III.C.3.). In a recent work the phospholipid content of detergent washed LH1-RC complexes from *Rb. sphaeroides* was determined to be about 80–90 phospholipids per RC (Dezi et al., 2007), several of them probably located in the interface region.

Due to a common evolutionary origin all three RCs, the PBRC, PS I and PS II share a similar arrangement of the ten central TMHs around the pseudo-C_2 axis relating PsaA/PsaB, D1/D2 and PufL/PufM (Michel and Deisenhofer, 1988; Schubert et al., 1998). When comparing the location of lipids arranged around this common motif between all three complexes it becomes evident that the position of the CL in the PBRC coincides with the position of MGDG2 in PS I and MGDG9 in PS II, and that the positions of the PC and the GGDG with respect to the L/M heterodimer as found by Koepke et al. (2007) are equivalent to the positions of MGDG18 and MGDG11 with respect to the D1/D2 core in PS II (Guskov et al., 2009). When continuing this comparison between PS I and PS II, it can also be seen that the positions of PG3 (PS II) and PG1 (PS I), which are related to MGDG9 (PS II) and MGDG2 (PS I) by the pseudo-C_2 symmetry, roughly coincide. There are some indications that in the PBRC there could be a similar additional lipid-binding site at a position symmetrical to the CL binding site (see Jones, 2007). This binding site could possibly coincide with the position of PG3 in PS II and PG1 in PS I (see also Section III.B.3). Also when visualizing the lipid belt surrounding the D1/D2 core in PS II and comparing it with the arrangement in the LH1-PBRC complex one can envisage that several of the other lipid positions in PS II could correspond to potential lipid locations in the open space between PBRC and the LH1 ring.

2 ATP Synthetase

There is no X-ray structural information available for the membrane-intrinsic part of the chloroplast or cyanobacterial F_0F_1 ATP synthetase. Electron microscopy and atomic force microscopy studies have shown that the F_0 part, formed by a variable number of copies of subunit C (10–15), forms a ring with a large cavity inside (Seelert et al., 2000; Pogoryelov et al., 2005; Lau et al., 2008; Nakamoto et al., 2008). In the x-ray structure of the C_{11} ring of the Na^+-translocating ATP synthase from *Ilyobacter tartaricus* (Meier et al., 2005) one acyl chain originating from the solubilizing detergent was identified per C subunit, indicating that this region is very probably filled by lipids in the native state. Biochemical, cross-linking and electron microscopy data on C-rings from different species suggest the presence of several lipids inside the ring. In the case of the *E. coli* F-ATP synthetase several PE molecules were found to be in close vicinity to several amino acid residues pointing towards the interior of the ring, sealing it (Oberfeld et al., 2006).

In the case of the ATP-synthetase this lipid patch seems to have a purely sealing function, preventing passage of ions and protons across the bilayer and therefore sustaining the membrane potential. There is also a recent report on the F_0 ring from spinach, suggesting the presence of additional lipophilic cofactors (Chl and Car) within the C-ring (Varco-Merth et al., 2008). As no specific binding site for Chl or Car could be deduced from the sequence of the C-subunit it might be possible that these molecules are solvated within the lipid phase inside the C-ring.

III Functions of Lipids

A Mediating Protein–Protein Interactions and Oligomerization

1 Photosystem II

The head groups of 18 of the intrinsic lipids found in PS II have polar contacts (hydrogen

bonds or salt bridges) with at least two different subunits, and three of them (DGDG1, DGDG6 and SQDG13) even connect four different subunits each via polar interactions (Table 3, Fig. 2g). This large number of intersubunit contacts shows the importance of lipid head groups in promoting assembly and providing stability for the PS II complex. In most cases these contacts are formed with residues at the ends of TMH of membrane-intrinsic subunits, but for the lumenally-located DGDG1 and DGDG6 contacts with loop regions of the membrane-extrinsic protein subunits PsbO and PsbV are found, indicating the possibility of (a small) involvement of lipids in the optimal binding of these subunits (see also Section III.B.4).

The dimer-interface in the homodimeric PS II complex is dominated by the presence of lipids and only very few protein–protein interactions between the two monomers are observed. These are mainly contributed by the small membrane-intrinsic subunit PsbM. The TMH of PsbM is located close to the pseudo-C_2 axis relating the monomers in the homodimer and shows a high content of leucine residues (Fig. 2h). The TMHs of PsbM in one monomer and its symmetric counterpart in the second monomer can therefore closely interact via a motif typical of membrane-spanning leucine zippers (Gurezka et al., 1999). In addition there are some polar interactions at the N-terminus of the TMH between the two symmetric residues Gln5 and at the C-terminus between two pairs of symmetry related residues (Ser31 and Gln33) from the two monomers. The only other possible direct protein–protein contact between the monomers is found at the lumenal side between a protruding loop of subunit PsbO (from one monomer) and a membrane-extrinsic part of subunit CP47 (from the other monomer). Therefore only a total of 5–6 polar protein–protein contacts exist between the two monomers, which is surprisingly low considering the size of the protein complex. Due to the arrangement of PsbM sticking somewhat out of the roughly planar surface defined by the remaining polypeptides of one monomer and facing the second monomer there is a large space between the two monomers, which is filled by a total of 14 lipids, seven from each monomer (Fig. 2b and h). In addition, there are eight β–DM molecules that may have replaced galactolipids during purification, suggesting the presence of even more lipids in this region when PS II is embedded in the thylakoid membrane.

This overall weak protein–protein interaction and the strong involvement of lipids in the interaction between the monomers suggest that the lipids are an important constituent of this interface and are employed for special reasons in this position.

Whereas the other oligomeric membrane protein complexes in the thylakoid membrane (PS I, LHCII and cyt b_6f) do not normally dissociate into monomers (see Sections III.A.2 through III.A.4) the PS II complex experiences a constant disassembly and reassembly process (Baena-Gonzalez and Aro, 2002; Rokka et al., 2005; Vass and Aro, 2008). This fast degradation and reassembly is necessary to provide replacement of damaged parts of PS II caused by side reactions within the reaction center, namely the formation of long lived triplet states on Chl molecules and the subsequent generation of singlet oxygen by the reaction of these triplet states with molecular oxygen (Krieger-Liszkay, 2005). The formed singlet oxygen is extremely reactive and leads to oxidative damage of cofactors and the protein. In order to circumvent this damage problem a complicated repair system is employed in PS II, which enables the selective replacement of the subunit D1, which is most prone to damage. In the course of this exchange, termed D1 turnover, it is thought that the damaged dimeric PS II complex first dissociates into monomers, followed by detachment of subunit CP43 (Barbato et al., 1992; Rokka et al., 2005). As the estimated half-life of subunit D1 in the thylakoid membrane under high light conditions can be shorter than 30 min an easy way of separating the dimers into monomers has to be employed.

It seems that lipids are an ideal component for providing both the specific interaction necessary for association of two monomers into a dimeric complex, as well as the required flexibility to avoid a too strong binding for efficient dissociation back into monomers. Six of the 14 lipids in the monomer–monomer interface are arranged similarly, forming contacts with protein subunits from both monomers. Six other lipids are arranged to mediate interactions between protein from one monomer and a lipid (or detergent) molecule attributed to the second monomer. Only the remaining two lipids are found to only form contacts within the same monomer. As an example SQDG13 is

located at a position enabling hydrogen-bonding interactions of its head group with subunits PsbM, CP47 from second monomer and PsbL from both monomers (see Fig. 2g and Table 3).

There are some indications that PG in particular may be involved in the dimerization of PS II. Kruse et al. (2000) found for dimeric spinach PS II that digestion of PG by adding phospholipase resulted in formation of monomeric PS II. A similar effect was observed in *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*) by blocking the biosynthesis of PG through genetic manipulation. In this case a higher percentage of monomeric PS II and a destabilization of dimeric PS II was observed (Gombos et al., 2002; Laczkó-Dobos et al., 2008). In contrast, a recent study on *T. vulcanus* PS II showed no effect of phospholipase and lipase treatment on dimeric PS II cores (Leng et al., 2008) in line with the observation that dimeric PS II from *T. elongatus* did not monomerize upon treatment with phospholipase (Loll et al., 2007). In accordance, no molecule of PG could be located at the monomer–monomer interface in the structure of the cyanobacterial PS II (Guskov et al., 2009). In addition, the detergent molecules localized at the monomer–monomer interface have their head groups at the lumenal side of the membrane, indicating that in the native state these positions are probably not occupied by PG, which would rather prefer the positively charged cytoplasmic side (see Table 2). However, it cannot be excluded that additional lipids are present on the cytoplasmic side, which are not yet resolved in the 2.9 Å electron density.

A very recent report showed an additional role of PG in stabilizing the D1-CP43 interaction as upon depletion of PG in *Synechocystis* mutant strains an increased amount of monomeric CP47RC complexes was found, indicating only loose attachment of CP43 to the D1/D2 core in these cells (Laczkó-Dobos et al., 2008). This corresponds well with the location of the two PG molecules in the PS II structural model, which are placed at the interface region between D1 and CP43. A destabilization of the D1-CP43 interaction by depletion of PG could also be an indirect cause for monomerization of the dimeric PS II complex. In addition the localization of both PG molecules at the outside of subunit D1 (Fig. 2b and c) is in good agreement with a study showing tight association of PG with D1 in cyanobacteria (Kruse and Schmid, 1995). Furthermore, studies of *C. reinhardtii* mutants depleted in PG suggested that PG might be essential for the correct insertion of D1 into the thylakoid membrane and assembly of the complex during replacement of D1 (Pineau et al., 2004).

Interestingly the rate of D1 processing, which is necessary after insertion of a newly synthesized copy of D1 into the PS II complex and prior to complete assembly of the Mn_4Ca cluster, was found to depend also on the degree of saturation of thylakoid lipids, as unsaturated fatty acids supported efficient processing of D1 in *Synechocystis* (Kanervo et al., 1997).

2 *Cytochrome* b_6f *Complex*

In the cyt b_6f complex only the SQDG located in both the *M. laminosus* and the *C. reinhardtii* structures seems to play a role in supporting protein-protein interactions, as its head group is interacting with residues from the cyt *f*, cyt b_6 and subunit IV. A tight interaction between the two monomers in the homodimeric complex is ensured by the fact that the ISP crosses between the two monomers with its single TMH forming part of one monomer and its lumenal membrane-extrinsic region being part of the second monomer. In addition to this direct link between the two monomers there are additional tight protein–protein interactions found at the monomer–monomer interface, being more closed at the lumenal and more open at the cytoplasmic/stromal side. The N-terminus of the cyt b_6 is folded into a short and a longer surface helix, lying on the cytoplasmic membrane surface and interacting with the cyt b_6 of the second monomer. An open space is present between the longer surface helices of the two monomers, which in projection is of rectangular shape with dimensions of approximately 10 × 15 $Å^2$ and could possibly accommodate two lipid head groups in the native state. In the refined native *M. laminosus* structure (Yamashita et al., 2007) two detergent molecules per dimer could be modeled in this region (UMQ5, see Fig. 3c and Table 5). Their head groups form hydrogen-bonding interactions to amino acid side chains from both monomers, thereby supporting the interaction of the N-terminal surface helix of one monomer with the C-terminal TMH d of the second monomer. On the lumenal side the TMHs

of cyt b_6 from both monomers interact with each other, leaving no space for lipids in the monomer–monomer interface.

From the available structural information it can be concluded that in the case of the cyt b_6f complex lipids play only a very minor role in promoting the monomer–monomer interaction. It rather seems that the arrangement is optimized for very stable association of the two monomers in the dimer, and to encase some lipids within the complex on the stromal side of the membrane to provide a lipophilic environment for quinone diffusion, see Section III.C.2. Furthermore lipids could enhance the stabilization of the monomers by providing some additional interactions between different subunits within each monomer.

3 Photosystem I

In PS I, PG4 is involved in hydrogen bonding and hydrophobic interactions with both PsaB and PsaX, such that the phospholipid might be essential for binding of the small peripheral subunit PsaX to the PS I core. A weak polar interaction between the primary hydroxyl group of the terminal glycerol moiety of PG1 and the polypeptide backbone of PsaE adds to numerous interactions between this lipid and PsaA, suggesting that PG1 has a minor role in mediating interactions between these protein subunits of PS I. The "fifth lipid", which was not part of the refined crystal structure of the cyanobacterial PS I (see Section II.D.2), might slightly contribute towards stabilization of the interface between PsaL in the trimerization domain and PsaA within the same monomer.

That PG is involved in trimerization of PS I was suggested by studies involving long-term PG depletion of cyanobacterial cells, which resulted in complete degradation of PS I trimers into monomers (Domonkos et al., 2004; Sato et al., 2004). The same studies showed that PS I trimers were restored by supplementation of PG. In the crystal structure of cyanobacterial PS I only one lipid, PG3, weakly interacts with a neighboring monomer (Jordan et al., 2001). It seems to be extremely unlikely that this interaction, with ChlM1 of the next monomer, significantly contributes to the stability of the PS I trimer. The formation of PS I trimers is stabilized by numerous direct monomer–monomer interactions involving several protein subunits (PsaL, PsaI, PsaB, PsaA, PsaM) and cofactors in the trimerization domain, which is located in the centre of the trimer. The only lipid molecule found in the crystal structure in a position close to the trimerization domain is the "fifth lipid", which is not in any direct contact with neighboring PS I monomers. This lipid might contribute to stabilization of the trimerization domain by mediating interactions between PsaL and PsaA of the same monomer, but it is not a PG. Taken together, the crystal structure does not provide any evidence that PG is directly involved in trimerization of cyanobacterial PS I. The observed long time span required for degradation of PS I trimers by PG depletion indicates that the degradation process is associated with removal of intrinsic PG. As suggested earlier (Domonkos et al., 2004; Sato et al., 2004) this might destabilize the overall structural integrity of PS I monomers and indirectly affect the monomer–monomer interfaces, finally leading to disintegration of the trimers.

4 Light Harvesting Complex II

For the LHCII complex the location of PG and DGDG in the interior of the trimeric complex and close to the monomer–monomer interface supports the finding that lipids are important for trimer formation in LHCII (see Fig. 5a and b). The head group of each DGDG forms contacts with the backbone of the C-terminal loop of one monomer and the TMH c of the next monomer, thereby connecting two adjacent monomers, whereas the PG is more involved in coordination of Chl*a*7 and forming the binding pocket of Chl*b* 9 and has no direct contact to the adjacent monomer (Fig. 5b). An explanation for the finding that PG is necessary for the formation of stable LHCII trimers (as upon digestion of PG by phospholipase the dissociation of trimers into monomeric LHCII was observed (Nussberger et al., 1993)) could be that by loss of PG the binding of the two Chl 7 and 9 is disrupted leading to an indirect destabilization of the trimer.

B Mediating Protein–Cofactor Interactions

1 Chlorophylls

The interaction between fatty acid chains from lipids and the hydrophobic phytol chains of Chl

is found regularly in PS I and PS II. In addition there are many examples of fatty acid tails contributing to the hydrophobic binding pocket of the chlorin ring of Chl molecules (see below). In contrast only few examples are known to date of a direct interaction between the head groups of Chl and lipid molecules, but some are present in PS I, PS II and LHCII.

In cyanobacterial PS II, 11 of the 35 Chl*a* per monomer have a lipid in the vicinity of their chlorin ring, two more have a β-DM, which might be replaced by a lipid in the native state, giving a total of 13 Chl (roughly one third of all Chl) with a lipid group forming part of the binding pocket of the chlorin ring (Guskov et al., 2009). In most cases the lipid contributions to the Chl binding pockets are from the fatty acid moieties and are of apolar character. But in the case of six Chl (Chl7, 17, 24, 27, 37, 44) the glycerol moiety and the head group of a lipid contributes to the chlorin ring binding pocket and even polar interactions between the head group of the respective lipid and the chlorin ring or the carbonyl oxygen of the phytyl ester are formed. The involved lipids are MGDG1, PG3, DGDG5, MGDG9, MGDG14, and MGDG17. Examples for typical Chl–lipid interactions are shown in Figs. 2f, 4c, and 5b.

The chlorin rings of 15 out of the 96 Chl*a* per monomer of cyanobacterial PS I are in contact with lipids (Jordan et al., 2001). For 12 of these Chl the fatty acid chains of the lipids are an integral part of the chlorin ring binding pockets. In case of three Chl the glycerol moiety or the polar or charged lipid head group also contributes to the chlorin ring binding pocket. One of these, ChlPL1, is axially coordinated by PG3 (see below), and another one, ChlB26, is involved in a polar interaction with the galactosyl moiety of MGDG2 via the carbonyl oxygen of its phytyl ester. Hydrophobic interactions between the lipids' fatty acid chains and the Chls phytyl chains also contribute to the integration of Chl into PS I. The conformationally flexible phytyl chains of a number of chlorophylls and the fatty acid chains of the peripheral lipids PG3 and PG4 are not (or only partially) visible in the electron density map, probably because they exhibit highly dynamic structures. However, a number of these "invisible" chains might interact with each other, such that the 12 Chl, which are found to be involved in hydrophobic interactions with fatty acid chains of lipids in the PS I crystal structure, only represent a lower limit.

In PS II there are two Chls, Chl17 and Chl37, which show a lipid possibly ligating the central Mg^{2+}. In the case of Chl17 the carbonyl oxygen of one of the fatty acid esters of MGDG14 is located 3.9 Å from the central Mg^{2+}. The only other possible axial ligation of this Mg^{2+} would be via the sulphur of CP47-Met37, but here the distance is even larger (4.6 Å). The distances in both cases are rather long and not typical for the distances found for normal axial ligands of the Mg^{2+} (around 2.3–2.5 Å for His-Mg^{2+}) but the found lipid-Mg^{2+} distance is in the range found for some other indirect ligand-Mg^{2+} interactions in PS II (4.1–4.2 Å), indicating that in this case MGDG14 could be the indirect ligand of Chl17.

As there was disk-like density in the 8 Å data from spinach PS II for all the Chls of the CP47 in the cyanobacterial structures (except Chl27) (Rhee et al., 1998; Büchel and Kühlbrandt, 2005) it is very likely that Chl17 is present in the spinach system too in a position similar to that in the cyanobacterial structure. As the region of CP47 (around Met37 and Phe61) forming the binding pocket of this Chl is highly conserved between plants and cyanobacteria it can be assumed that in the case of higher plants this Chl could be ligated similarly via a lipid.

The Chl at the symmetry-equivalent position in CP43, Chl37, similarly shows no direct ligand for the central Mg^{2+}. The glycerol moiety and one fatty acid of DGDG5 is blocking one face of the chlorin ring with the fatty acid carbonyl group in a position similar to the situation found for MGDG14, leading to an O–Mg distance of 3.5 Å (Fig. 2f). At the other face of the chlorin ring the two nearest amino acids CP43-Trp63 (at 5.4 Å) and CP43-Met67 (at 4.5 Å) are not capable of providing direct ligation of the central Mg^{2+}.

The central Mg^{2+} ion of ChlPL1 in PS I has one oxygen atom of the negatively charged phosphodiester group of PG3 at 2.3 Å distance as the direct axial ligand (see Fig. 4c). Unlike the other two PG molecules in cyanobacterial PS I, the anionic head group of PG3 has no cationic amino acid side chain in its near environment for local compensation of its negative charge, which might strongly affect the Chls spectral properties.

The PG localized in both LHCII structures (Liu et al., 2004; Standfuss et al., 2005) provides direct

axial ligation of the Mg^{2+} of one of the Chls, with an oxygen of its phosphate group being at 2.3 Å distance from the Mg^{2+} (Fig. 5b). Only few amino acids Trp16, Lys179, Lys182 (H-bond to carbonyl of methyl ester group of Chl), Asn183 and Leu186 are contributing to the binding pocket of this Chl*a*, the majority of the pocket being formed by the next Chl, the modeled detergent, the head and one fatty acid of the PG and the xanthophyll molecule. The mode of Chl coordination is very similar to the one found in PS I, although in the case of LHCII one Lys residue is present as additional ligand to the phosphate group of the PG. This Lys can neutralize the negative charge at the phosphate group more strongly compared to the situation for PG3 in PS I. As a consequence the shift of the spectral properties of the ligated Chl might be smaller compared to ChlPL1 in PS I, where no counterbalancing positively charged amino acid is found near the PG's phosphate group.

Based on the found structural arrangement of lipids in the vicinity of Chl described above, several ways in which a nearby lipid could influence the properties of a Chl can be envisaged. One obvious possibility is that the presence of a negative charge from the head group of SQDG or PG could influence the electronic distribution over the π-system of a nearby Chl, or the energies of its excited electronic states, thereby tuning its spectral and redox properties. Using lipids as ligands for the Mg^{2+} of a Chl gives the opportunity to incorporate Chl into regions of a structure where no direct ligation by the protein is possible, thereby increasing the possible number of Chl arrangements within a protein framework. This might be utilized for some of the Chl molecules found in the connection region between LHCI and the PS I core in the plant system (Fig. 4b) (Amunts et al., 2007), for which no obvious protein ligand is present in the structural model, and maybe similarly for the Chl found to be present in the interior of the membrane spanning C-ring of chloroplast ATP synthetase (Varco-Merth et al., 2008). In the light of these structural findings one might speculate that ligation of Chl by lipid head groups can occur temporarily during assembly of Chl–protein complexes, and therefore the lipids could play a chaperone-like role in these processes.

In some cases hydrogen bonding interactions between lipid head groups and substituents of the chlorin ring were found. In this case lipids could be employed to fine tune the redox potential of the Chl if no hydrogen bond interaction by the protein is possible for steric or sequence reasons. In addition by the presence of the fatty acids of lipids in the binding pocket compared to a situation where only protein is forming the binding pocket of the chlorin ring the overall hydrophobicity of the binding pocket could be increased, leading to a more solvent-like environment for the Chl and therefore the composite absorption spectrum of all Chl bound to one protein complex could be broadened, compared to a situation where only protein-bound Chls are present.

2 Carotenoids

In PS II from *T. elongatus* a total of 12 Car per monomer are embedded in the complex (Guskov et al., 2009). Nine of them (75%) have fatty acids of a lipid contributing to their binding pocket. The number of lipids contributing to each binding pocket lies between one and five. For example, the binding pocket of Car15 is formed by only six amino acids, covering roughly less than one third of the binding pocket. The other two thirds of the binding pocket are contributed nearly exclusively by fatty acids from five different lipids. Interestingly also the two special carotenoids Car_{D1} and Car_{D2}, bound to D1 and D2 respectively, and thought to be involved in possible secondary electron transfer reactions, are found in a lipid-rich environment with four or five lipids, respectively, contributing about 50% to the Car binding pockets.

In the cyt b_6f complex only one carotenoid could be identified in the electron density and was modeled only partially with one ionone ring tightly enclosed by protein and the second ionone ring missing, as it is protruding into the membrane phase. The binding pocket is mostly contributed by protein and only one fatty acid from MGDG1 is in contact with the ionone ring of the Car. Therefore the influence of lipids on the binding of this Car is only minor.

A total of 22 Car molecules are an integral part of a PS I monomer from *T. elongatus* (Jordan et al., 2001). On average only about 40% of their binding pockets is provided by the protein subunits. The major portion of the Car binding pockets is contributed by Chl, whereas the role of lipids in stabilizing Car in the complex is only marginal.

Only the central polyene chain of Car7 and the ionone ring of Car19 make a few contacts with one fatty acid moiety each in PG3 and MGDG2, respectively.

Xanthophyll is located in a hydrophobic cavity within LHCII and is in contact with fatty acids from both lipids (DGDG and PG) located in the structure, as well as with various Chl, but shows only very few protein contacts (Standfuss et al., 2005). The two luteins do not have a lipid in their binding pocket and show more protein contacts. The neoxanthin is located on the outside of the complex, pointing into the membrane, with most of it exposed to the lipid phase but no specific interactions with structurally-resolved lipids are found.

In summary, lipids are employed in some cases for binding of carotenoids into protein complexes but the lipid–carotenoid interactions are less specific compared to the situation for Chl.

3 Quinones and Phylloquinones

Interestingly the only lipids found at roughly similar positions in PS I and PS II are the two lipids close to the quinone/phylloquinone pair (see Section II.F.1 and Figs. 2b, c, and 4d). The asymmetry of lipid head groups close to the quinone side is conserved between both systems. The Q_A/Q_K-B side has uncharged sugar lipids, whereas the Q_B/Q_K-A side shows negatively charged PG and SQDG lipids. When superpositioning PS I and PS II the head group of PG1 (PS I) is located in-between the positions of the head groups of PG3 (PS II) and SQDG4 (PS II), with one fatty acid of PG1 (PS I) overlapping with one fatty acid of PG3 (PS II) and the other overlapping with one fatty acid of SQDG4 (PS II). Similarly, the head group of MGDG2 (PS I) is found in an intermediate position between the head groups of MGDG9 and MGDG10 in PS II, with the two fatty acids in similar positions to that occupied by one of the fatty acids of MGDG9 and MGDG10, each, in PS II. In PS II the asymmetry between the two quinone sites is further enhanced by the location of a third charged lipid, PG22, in the vicinity of the Q_B site (Fig. 2c).

The primary protein–cofactor interactions are very similar for Q_K-A and Q_K-B in PS I, and also similar for Q_A compared to Q_B in PS II, due to the high conservation between PsaA and PsaB in PS I and PsbA and PsbD in PS II. Therefore, asymmetry in the lipid environment could be important for modulating the properties of the two quinones/phylloquinones in different ways depending on their location in the system. This could affect the redox potentials of the quinones, allowing a fine tuning of electron transfer from Q_A to Q_B in PS II by the lipid environment. In PS I a slight shift in redox potential between Q_K-A and Q_K-B could be responsible for changing the electron transfer rates along the A or B branch respectively and could allow predominant electron transfer along one of the branches (Rappaport et al., 2006; Redding and Van der Est, 2006).

Interestingly there are some indications that the lipid binding site close to the two quinones might even be conserved in the case of the PBRC (Jones, 2007). Similarly to PS II the PBRC structure from *Rb. sphaeroides* shows a lipid (the negatively charged CL) in the vicinity of Q_B (Fig. 5c) at a roughly equivalent position to that of PG3 (PS II). In addition there is experimental evidence for such a secondary effect of lipids on the properties of quinones in the PBRC. Addition of CL to solubilized PBRC influenced the redox potential of Q_A (Rinyu et al., 2004) and reconstitution of isolated PBRC into liposomes with different lipid composition lead to changes in the electron transfer rate from Q_A to Q_B and the charge recombination rate, explained by a change in the redox potentials of both Q_A and Q_B depending on the type of lipid used for reconstitution (Nagy et al., 2004).

In PS II, depletion of PG in *Synechocystis* leads to changes in the redox potential of Q_B, slowing down the Q_A-Q_B electron transfer rate (Gombos et al., 2002) and in mutants of *C. reinhardtii* depleted in SQDG the interaction of the Q_B site with artificial electron acceptors was changed, indicating a conformation change due to the absence of SQDG (Minoda et al., 2003).

The location of lipids in the vicinity of quinone binding sites seems to be a general motif. In the cyt b_6f complex the SQDG is located in the vicinity of the Q_n site (Stroebel et al., 2003) and similarly in yeast cyt bc_1 complex a phospholipid is found close to the corresponding Q_n site (Lange et al., 2001). In addition to influencing the properties of the quinones the main role of these lipids could be to support the entry/exit of quinones into their binding pockets, see Sections III.C.1 and III.C.2.

4 Influencing the Water Splitting Reaction in Photosystem II

There are several reports of changes in water splitting activity of PS II induced by changes in the lipid composition of the thylakoid membrane (Reifarth et al., 1997; Steffen et al., 2005; Sakurai et al., 2007; Chapters 11 and 12). The catalytic site of water oxidation, the Mn_4Ca cluster, is located at the lumenal side of PS II close to the membrane surface but shielded from the lumen by large loop regions of the four subunits D1, D2, CP43 and CP47, as well as by the three membrane-extrinsic subunits PsbO, PsbU and PsbV. In the structural model no lipid was found in the direct vicinity of the Mn_4Ca cluster. The closest lipids are DGDG2, DGDG5 and DGDG6 (Fig. 2e), all of them at distances of 15 Å or more from the Mn_4Ca cluster. This indicates that the observed effects, for example of DGDG depletion on water oxidation, are indirect, perhaps due to conformational changes on the lumenal side of the PS II complex induced by depletion of a lipid located on that side. This might also be related to changes in binding of the membrane-extrinsic subunits of PS II upon depletion of DGDG, as there are two examples of interactions of DGDG head groups with PsbO and PsbV (see Section III.A.1.).

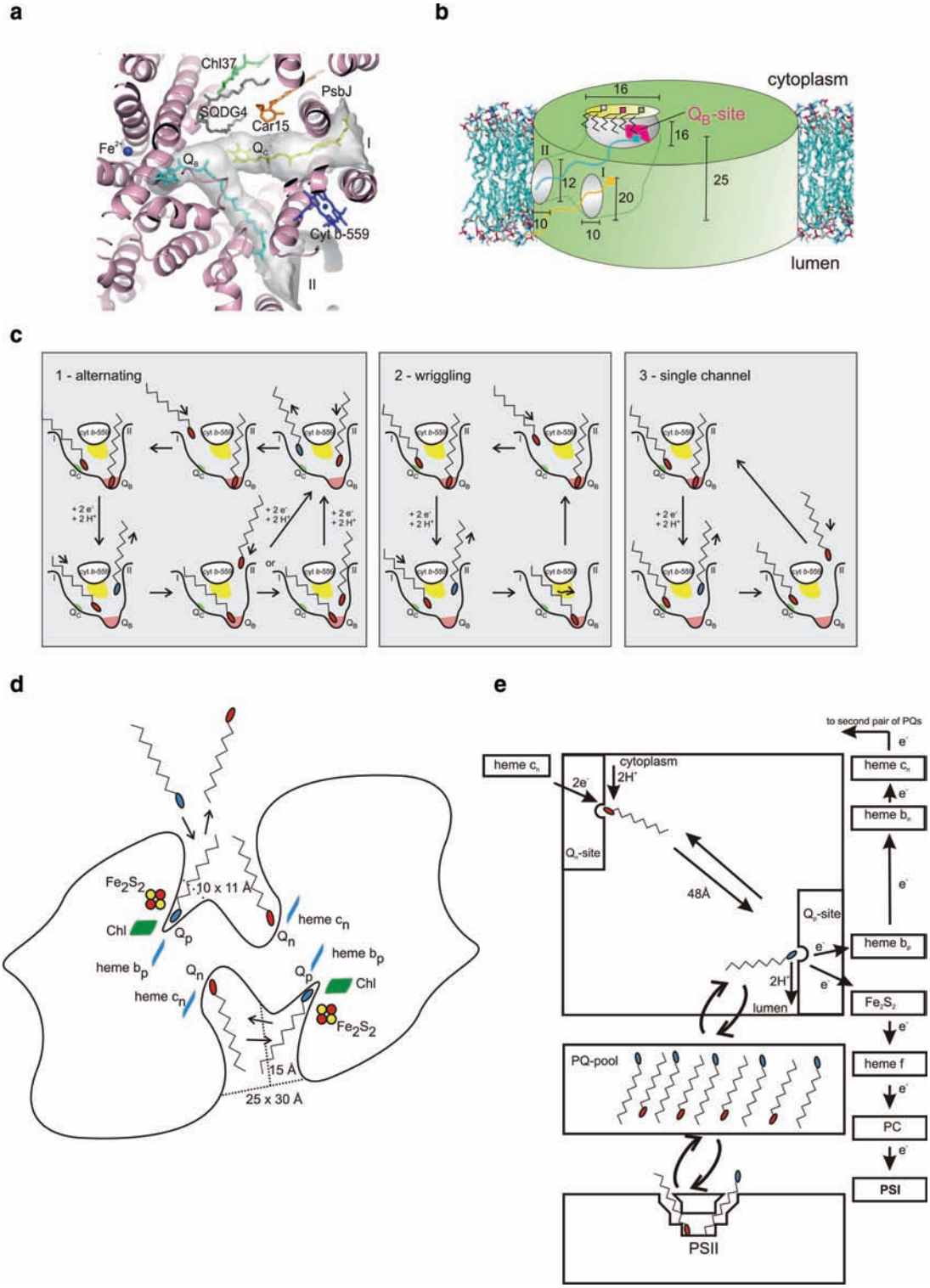
C Providing Lipophilic Regions within the Protein Complex

1 Quinone Exchange in Photosystem II

For rapid turnover of the enzyme it is necessary that the final electron acceptor plastoquinone is always available in sufficient amounts. In particular an efficient oxidation of the primary quinone Q_A is important, as accumulation of the semiquinone Q_A^- could lead to charge recombination reactions and finally formation of deleterious triplet states at the primary donor Chls (^{3}P680). But PS II faces the problem that the quinone binding site, in contrast to several other quinone binding proteins, is not located towards the membrane-exposed surface of the complex but is rather buried deep within the complex in the D1/D2 heterodimer. The solution found in PS II is to include part of the membrane inside the protein complex. Specifically a large cluster of at least eight lipids (with space for maybe one or two additional lipids) is found next to the Q_B site, and this resembles a lipid bilayer structure with four lipids each on the lumenal and cytoplasmic side (Fig. 2d). This membrane "island" within the protein ("protein intrinsic lipid bilayer") is to our knowledge the first crystallographic structure of a lipid bilayer.

As PQ9 is well solvated within the thylakoid membrane itself (forming the PQ pool), the lipid region within the protein seems to be an ideal environment for the solvation of quinones. Accordingly it was possible to locate an additional quinone Q_C within this lipid pool, suggesting that this lipophilic cavity could not only act as transfer pathway but also as storage pool for PQ9 in PS II (Fig. 6a). The connection of this small protein-intrinsic PQ pool with the thylakoid membrane is provided by two portals, one between TMHs of PsbE/F and J and the second between the TMH of PsbF and TMH a of D2. Both portals open towards the membrane interior at a height approximately in the middle of the membrane and are large enough (dimensions of 10 × 20 and 10 × 12 Å^2 respectively) to allow passage of PQ9 (Fig. 6b). As quinones in a lipid bilayer orient their tail along the plane of the membrane in

Fig. 6. Quinone exchange in PS II and cyt b_6f. (**a**) Calculated channels (I and II, grey) for PQ/PQH_2 transfer between PQ-pool and Q_B-, Q_C-sites in PS II. Shown are the PQs in the Q_B-site (light blue), and Q_C-site (yellow), non-heme Fe^{2+} (*blue sphere*), Car15 (orange), Chl37 (green), SQDG4 (grey), cyt b_{559} heme (dark blue), and surrounding proteins (pink). (**b**) Schematic view of the PQ/PQH_2 exchange cavity in PS II and the two entry/exit portals connecting Q_B- and Q_C-sites to the PQ-pool in the thylakoid membrane. Approximate dimensions given in Å, Q_B and Q_C colored in cyan and yellow, respectively, Q_B site highlighted in pink, the three lipids forming the "cork" (head groups for PG22, SQDG4, MGDG18 as red, green, white squares) nearly closing the cavity towards the cytoplasm are indicated. (**c**) Possible mechanisms for the PQ/PQH_2 exchange between the Q_B-site of PS II and the PQ-pool, view from cytoplasm. Channels I and II open towards the PQ-pool. PQ with red, PQH_2 with blue head group. Q_B-site highlighted pink, Q_C-site green and labeled; yellow patch indicates hydrophobic region formed by fatty acids of MGDG7, MGDG18 and phytol chain of Chl_{D2}. Small arrows symbolize movements of PQ (red) and PQH_2 (blue) molecules. (**d**) Schematic view of the quinone exchange cavity in cyt b_6f, view is from the cytoplasm onto the membrane plane. Arrows indicate movement of PQ/PQH_2, Heme and Chl are indicated as in Fig. 2b, Fe_2S_2 cluster by circles. Approximate dimensions are given in Å. (**e**) Scheme for the PQ/PQH_2 and electron transfer between PS II, the PQ pool, cyt b_6f and PS I. Panels a-c are adapted from Guskov et al. (2009) (See Color Fig. 12 on Color Plate 11).


a
Chl37
PsbJ
SQDG4
Car15
Fe2+
Qc
Qb
I
II
Cyt b-559
b
cytoplasm
16
QB-site
16
II
I
12
20
25
10
10
lumen
c
1 - alternating
2 - wriggling
3 - single channel
+ 2 e-
+ 2 H+
or
d
Fe2S2
10 x 11 Å
Chl
Qp
Qn
heme cn
heme bp
heme bp
Qn
Qp
Chl
heme cn
Fe2S2
15 Å
25 x 30 Å
e
to second pair of PQs
heme cn
cytoplasm
2e-
2H+
Qn-site
48Å
Qp-site
heme bp
2H+
lumen
Fe2S2
heme bn
heme f
PC
PSI
PQ-pool
PSII

the middle between the two layers (Hauss et al., 2005) the position of the openings in the middle of the bilayer seems to be well suited for entry/exit of quinones. As outlined in Guskov et al. (2009) the combination of one binding site (the Q_B site), one additional PQ9 bound in a "waiting" position (Q_C) within the PQ/PQH_2 exchange cavity and two possible entry/exit channels connecting the exchange cavity with the membrane interior is compatible with three different mechanisms for plastoquinone exchange (Fig. 6c). In the "alternating mechanism" PQ/PQH_2 would enter and leave the complex via both portals in an alternating way. In this case each PQ/PQH_2 molecule would leave the complex the same way it initially entered it. In the "wriggling mechanism" one portal would be used exclusively for entry of PQ into the complex and the other for exit of PQH_2 into the membrane. After binding to the Q_B site the isoprenoid chain of the PQ would have to wriggle around from one channel to the other before it can leave the complex again. In the "single channel" mechanism only one channel is used for entry and exit of PQ/PQH_2 and the Q_C, which is located in the other channel, would not participate in PQ/PQH_2 exchange but could rather fulfill other functions, for example in secondary electron transfer in combination with the cyt b_{559} (Bondarava et al., 2003; Kaminskaya et al., 2007) or in quenching of singlet oxygen (Kruk and Trebst, 2008).

2 *Quinone Exchange in Cytochrome* b_6f

In the cyt b_6f complex two quinone-binding sites are present in each monomer, one plastoquinol oxidation site Q_p located towards the lumenal surface of the membrane and a plastoquinone reduction site Q_n located closer towards the cytoplasmic surface of the membrane. In the in vivo active dimeric form of the cyt b_6f the Q_n site of one monomer is connected via a large and wide quinone exchange cavity with the Q_p site of the second monomer (Fig. 3a), providing a pathway for PQ/PQH_2 to travel between both sites depending on its oxidation state. The cavity is 30 Å high, 15 Å deep and 25 Å wide at the base near the n-side/cytoplasmic side in *M. laminosus* (Fig. 6c), and the cavity walls are formed by TMHs c, d, f, a^*, e^* of cyt b_6 and the TMH of the iron sulfur protein from the second monomer (ISP^*).

As derived from the position of the p-side inhibitor 2n-nonyl-4-hydroxy-quinoline-N-oxide (NQNO) in the refined *M. laminosus* structure (Yamashita et al., 2007) the head group of the quinone in the Q_n side can act as ligand to the Fe of the heme c_n (formerly designated heme *x*) and its isoprenoid tail is in contact with fatty acids of the SQDG and the alkyl chain of detergent UMQ7 (Fig. 3b). The Q_p side (Fig. 3a), as visualized by the position of the p-side inhibitor TDS in the *C. reinhardtii* and the refined *M. laminosus* structures (Stroebel et al., 2003; Yamashita et al., 2007), has a small entry portal of 11 × 10 Å formed by helices from cyt b_6 and subunit IV and is close to the Fe_2S_2 cluster of the ISP of the other monomer within the dimeric complex (Fig. 6d). The isoprenoid tail of PQ9 bound to the Q_p site can be expected to point into the large exchange cavity, partly interacting with the aliphatic phytol chain of the Chl, as well as with fatty acid chains of lipids, most likely present in the cavity in the native state.

At the cytoplasmic side the surface helices of the N-termini of subunit cyt b_6 from both monomers encase an open space of rectangular shape and a size of approximately 10 × 15 $Å^2$. This would be sufficient to accommodate at least two (or maybe four) lipid head groups, which could be located here in the membrane-embedded native state of the complex. In the recently improved structure of cyt b_6f from *M. laminosus* one detergent molecule (UMQ5) per monomer was found in this region (Yamashita et al., 2007), partially sealing the cavity towards the cytoplasm (Fig. 3c). The fatty acids of the lipids, which would be present at the same position in the native state of the complex, would fill up most of the quinone cavity, giving it a membrane-like interior ideally suited for diffusion of PQ from the thylakoid membrane into the complex and between the two binding sites, which are approximately 48 Å from each other (Figs. 3a and 6d).

3 *Quinone Exchange in the PBRC-LH1 Complex and in the Cytochrome* bc_1 *Complex*

In vivo the PBRC is surrounded by the LH1-antenna ring. A first low resolution crystal structure of this RC-LH1 complex from *Rhodopseudomonas palustris* was solved at 4.8 Å resolution (Roszak et al., 2003), showing the dimensions of the ring

and the orientation of the RC within the LH1 ring. The membrane-exposed surface of the PBRC shows a pronounced asymmetry with a concave surface at the side that contains the entry of the Q_B binding site. This concave shape might be necessary to provide a hydrophobic lipid-filled area between the Q_B site and the LH1 ring, acting as an antechamber similar to the quinone exchange cavity described for PS II. The analysis of the quinone content of detergent washed LH1-RC complexes from *Rb. sphaeroides* showed that this complex contains about 15 ubiquinone molecules per RC (Dezi et al., 2007), supporting the role of the lipid-filled area between the LH1 ring and the RC as an antechamber for quinone exchange. This would also fit into the picture of the role for subunit PufX in the PBRC, which is thought to have a single TMH and modifies the LH1 ring to allow passage of PQ between the RC-LH1 complex and the cytochrome bc_1 complex (the side of quinol oxidation), as reviewed by Holden-Dye et al. (2008). So in both systems, PS II and PBRC, lipids could be essential to provide an area of quinone storage/exchange to enable fast turnover of the reaction center.

In the cyt b_6f, as well as in the yeast and bovine bc_1 complexes, the same arrangement of the quinone binding sites next to wide hydrophobic cavities is found. From the chemical nature of the amino acids lining the walls of these cavities, and from the presence of some lipid molecules in these cavities in the available structures (Lange et al., 2001; Huang et al., 2005; Solmaz and Hunte, 2008), it can be concluded that these cavities are filled completely by lipids when the complexes are embedded in their native membrane environment. It seems that also in these cases lipids associated (more or less strongly) to the protein complexes fulfill the function of providing a region for quinone exchange.

4 Possible Oxygen Diffusion in Photosystem II

It is well known that lipid bilayers can solvate oxygen and are permeable for oxygen (Ligeza et al., 1998; Subczynski and Wisniewska, 2000). Measuring the oxygen concentration profile within a bilayer using spin labels at different positions revealed an accumulation of oxygen in the middle of the bilayer within the hydrophobic environment of the fatty acids (Marsh et al., 2006). As oxygen is constantly produced in the lumen during oxygenic photosynthesis (Renger, 2007) it has to leave the thylakoids via the lipid bilayer. Xenon, known to show a similar behavior as dioxygen, is used routinely in protein crystallography for mapping of oxygen channels inside protein structures (Prange et al., 1998; Svensson-Ek et al., 2002; Luna et al., 2008).

X-ray diffraction data collected from Xe-derivatized PS II crystals yielded information on the location of Xe sites within the complex (Guskov et al., 2009). A total of 19 Xe binding sites were found per PS II dimer (Fig. 7a). All the Xe sites are located in hydrophobic environments formed by fatty acids from lipids, phytol chains from Chl molecules, carotenoids or hydrophobic amino acids (Val, Gly, Leu, Ala, Ile, Phe, Trp). With the exception of one site located in the hydrophobic interior of the β-barrel of PsbO, all sites are located in the membrane-spanning part of PS II at approximately the middle of the membrane, at the monomer–monomer interface, in the interface between D1 and CP43 or D2 and CP47, or close to the binding site of Q_C. All of the Xe sites were found at a distance of more than 17 Å to the redox-active cofactors involved in charge separation and transmembrane electron transfer, and for several of the Xe sites connective pathways to the lumenal and cytoplasmic phase could be traced (Fig. 7b) (Guskov et al., 2009). This suggests that the hydrophobic patches in the interior of PS II, formed partly by lipids, could allow diffusion of the dioxygen formed at the lumenal side through PS II to the cytoplasmic side. As these patches have a higher affinity to dioxygen compared to the other regions of the PS II complex, their arrangement could provide a means to keep dioxygen away from the reaction center region, preventing oxidative damage to the highly reactive pigments involved in charge separation and electron transfer.

This proposed function of the hydrophobic patches within PS II could be an additional reason for the high lipid content of PS II when compared to PS I, the cyt b_6f complex and other protein complexes (see Section II.A), as PS II is both the source of oxygen and the protein complex most susceptible to oxidative damage in the thylakoids.

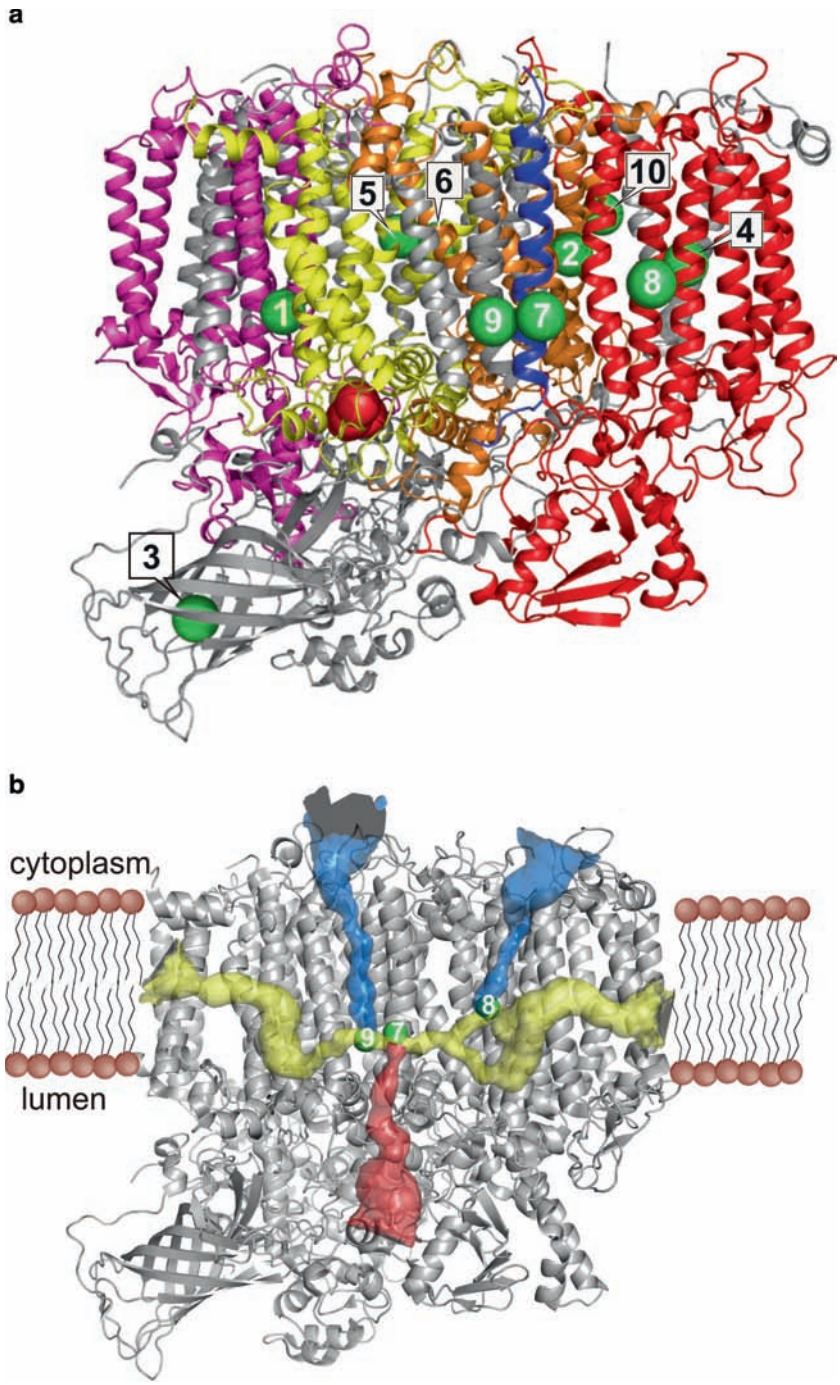


Fig. 7. Xe positions and possible oxygen diffusion in PS II. (**a**) Location of Xe sites (*green spheres* labeled 1–10) in the PS II monomer looking onto the monomer–monomer interface along the membrane plane, cytoplasm at the top, lumen at the bottom. All 20 protein subunits are shown in cartoon mode with D1 in yellow, D2 in orange, CP47 in red, CP43 in magenta and PsbM in blue, the remaining subunits in grey, cofactors, apart from the Mn_4Ca cluster (*red* and *yellow spheres*), are omitted. (**b**) Possible diffusion channels for Xe (and maybe oxygen) connecting Xe sites 7, 8 and 9 with the lumen (red), the cytoplasm (blue) and the membrane interior (yellow) (See Color Fig. 13 on Color Plate 12).

IV Conclusions and Perspectives

In this chapter we have given an overview of the structural information on lipids bound to membrane proteins of oxygenic photosynthesis. The amount of structural information available allows the deduction of several possible functions of these lipids incorporated in the various complexes. One general conclusion is that lipids can be termed a novel group of cofactors, similar to carotenoids or chlorophylls, as they do not simply provide a hydrophobic background to embed membrane proteins, but rather they seem to fulfill specific roles within the protein complexes. Based on the structural data it is possible to derive some principles of lipid arrangement, for example the exclusive location of the charged lipids PG and SQDG on the cytoplasmic/stromal side of the protein complexes or the presence of lipid molecules in the vicinity of quinone binding pockets. Due to their involvement in intersubunit and intermonomer contacts lipids are likely to fulfill important functions in assembly and disassembly, as well as oligomerization of the different complexes, although to a variable degree depending on the type of the respective protein complex. The crystallographic structures together with mutagenesis and spectroscopic studies show that there are various interactions between lipids and quinones or chlorophylls, indicating a direct influence of lipids on these cofactors. Interestingly the direct ligation of Chl by lipid head groups found in several cases might be a general principle and could also be employed as an intermediate during assembly of Chl–protein complexes, i.e. lipids could act in a chaperone-like way in these processes. Apart from influencing other cofactors, protein-intrinsic lipids also provide tailored hydrophobic regions in the interior of the protein complex, which might be necessary for transfer of other molecules (e.g., quinones and oxygen). In this respect the different "greasiness" of the various complexes might be related to the different requirement for such hydrophobic regions depending on the different function of each of the protein complexes.

The structural information on integral lipids in the photosynthetic membrane protein complexes is still incomplete, due to limited available resolution (e.g., in cyt b_6f and cyanobacterial PS II) and to the absence of diffracting crystals (for example for plant PS II). But it is likely that this situation will improve in the coming years and that the localization of more lipid molecules in all these complexes will be revealed. In the meantime the existing structural information is already sufficient to address several questions in more detail. Up to now mutagenesis experiments on the role of lipids in photosynthesis were rather restricted to general modifications of the whole thylakoid membrane composition. But the detailed knowledge about the specific binding pockets for individual lipids in the various complexes now available will allow for the design of more directed mutagenesis studies in the near future, addressing the specific role of individual integral lipids by changing the specificity of a lipid binding pocket. In addition theoretical calculations on the properties of the redox active cofactors can now be conducted with inclusion of the nearby lipids to evaluate the specific effect individual lipids can exert, for example, on the redox properties and absorption spectra of chlorophylls.

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Chapter 11

The Role of Phosphatidylglycerol in Photosynthesis

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Summary

Since the first identification of phosphatidylglycerol (PG) in the green alga *Scenedesmus* by Benson and Maruo in 1958, this lipid has been recognized as a ubiquitous phospholipid that is present in almost all organisms. PG is an anionic phospholipid, with a negatively charged phosphate group at neutral pH. In photosynthetic organisms, such as cyanobacteria and higher plants that perform oxygenic photosynthesis, the majority of PG is found in thylakoid membranes, which are the site of photosynthetic light reactions and electron transport. Thylakoid membranes are composed predominantly of glycolipids, such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG), and PG is the only major phospholipid that is present in thylakoid membranes. Thus, it has been suggested that PG might play an important role in the primary processes of photosynthesis in thylakoid membranes. Recent x-ray crystallographic analyses of the cyanobacterial photosystem I (PS I) and photosystem II (PS II) complexes that are involved in the photosynthetic transport of electrons in thylakoid membranes have identified four and 25 lipid molecules per monomer in the respective complexes. Three and two of these lipid molecules are PG in the PS I and PS II complexes, respectively. These findings suggest that lipids, including PG, might play important roles not only in the formation of the lipid bilayers of thylakoid membranes but also in the folding and assembly of the protein subunits in each complex. Genetic and biochemical studies of the role of PG, using mutants and genetically manipulated strains of cyanobacteria and higher plants with levels of PG different from those in the corresponding wild-type strains, have confirmed that PG is essential for the growth of cyanobacteria

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and higher plants, moreover, that it is crucial to the photosynthetic transport of electrons, the development of chloroplasts, and tolerance to chilling. In this review, we summarize our present understanding of the biochemical and physiological roles of PG in photosynthesis.

I Introduction

In 1958, Benson and Maruo (1958) discovered phosphatidylglycerol (PG) in a green alga, *Scenedesmus*. PG is now considered to be a ubiquitous phospholipid that is present in almost all organisms (Benson and Maruo, 1989; Dowhan, 1997; Shibuya, 1992). Figure 1 shows the structure of PG. The phosphate group in PG is negatively charged at neutral pH and, thus, PG is classified as an anionic phospholipid.

In non-photosynthetic prokaryotes, such as *Escherichia coli*, PG functions as an important but not indispensable participant in cellular metabolism (Shibuya, 1992; Matsumoto, 2001). Non-photosynthetic eukaryotes, such as *Saccharomyces cerevisiae*, synthesize PG exclusively in mitochondria and use PG for the biosynthesis of cardiolipin (CL; Carman and Henry, 1999), which is located in the inner membranes and at sites of contact between the inner and outer membranes of mitochondria (Schlame et al., 2000). CL itself is involved in the assembly and stabilization of the protein complexes that are required for the respiratory transport of electrons and oxidative phosphorylation within the mitochondria (Schlame et al., 2000; Schlame, 2008; Braun and Wu, 1994). It has been reported that CL plays a role in the release of cytochrome *c* (Cyt *c*) from mitochondria during apoptosis (Ott et al., 2007). Recent studies with CL-deficient HeLa cells, in which expression of the gene for CL synthase was blocked by RNA interference, indicated that CL deficiency induces the release of Cyt *c* from mitochondrial membranes and accelerates apoptosis (Choi et al., 2007). However, Huang et al. (2008) reported, by contrast, that CL deficiency is associated with decreased oxidation of CL and the increased resistance of cells to apoptosis. Thus, it is unclear whether CL deficiency accelerates or decelerates apoptosis. In addition, it has been demonstrated that fatty acids bound to CL are remodeled by an acyltransferase, tafazzin (Xu et al., 2006a, b). Mutations in tafazzin are associated with Barth syndrome, which is an X-linked disease associated with cardiomyopathy and skeletal muscle weakness (Chicco and Sparagna, 2007; Schlame and Ren, 2006).

In higher plants, PG is a genuine constituent of all extraplastidial membranes, but it is present in low amounts, namely, it represents only a few percent of total glycerolipids in each membrane. By contrast, PG is the major phospholipid in plastidial membranes (Joyard et al., 1998). In cyanobacteria and in the chloroplasts of higher plants that perform oxygenic photosynthesis, the majority of PG is found in thylakoid membranes, which are the site of photosynthetic light reactions and electron transport (Joyard et al., 1998). Thus, it has been suggested that PG might play an important role in the primary processes of photosynthesis in thylakoid membranes. Table 1 shows the composition of lipids in thylakoid membranes from two cyanobacteria, *Synechocystis* sp. PCC 6803 and *Thermosynechococcus vulcanus*, and from a higher plant, *Spinacia oleracea* (spinach). Although other biological membranes are mainly composed of phospholipids (see Chapter 4), thylakoid membranes are composed predominantly of glycolipids, such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG) (see Chapter 3; Siegenthaler, 1998). PG is the only major phospholipid found in thylakoid membranes (Block et al., 1983; Dorne et al., 1990; Wada and Murata, 1998, 2007). It is likely that PG is involved in indispensable interactions with the components of the photosynthetic

Abbreviations: ACP – Acyl-carrier protein; CL – Cardiolipin; CDP-DG – CDP-diacylglycerol; Cyt – Cytochrome; DGDG – Digalactosyldiacylglycerol; ER – Endoplasmic reticulum; X:Y(Z) – Fatty acid containing X carbon atoms with Y double bonds, in the *cis*-configuration, at position Z counted from the carboxyl terminus; FAS – Fatty acid synthase; G3P – Glycerol 3-phosphate; LHC – Light-harvesting complex; LPA – Lysophosphatidic acid; MGDG – Monogalactosyldiacylglycerol; PA – Phosphatidic acid; PC – phosphatidylcholine; PE – Phosphatidylethanolamine; PG – Phosphatidylglycerol; PGP – Phosphatidylglycerophosphate; PS I – Photosystem I; PS II – Photosystem II; SQDG – Sulfoquinovosyldiacylglycerol

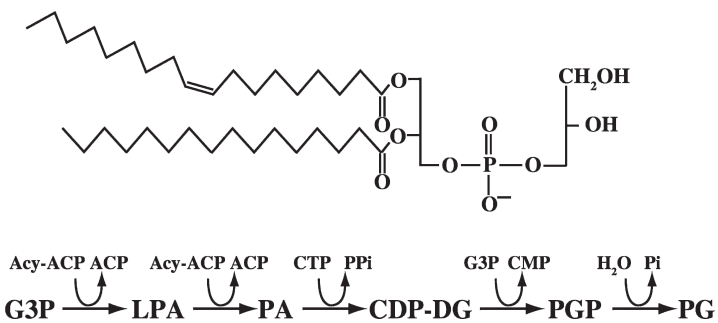

Fig. 1. Structure of PG (1,2-diacyl-*sn*-glycero-3-phospho-1′-*sn*-glycerol) and a schematic representation of the biosynthetic pathway to PG in cyanobacteria. Fatty acids are esterified to the *sn*-1 and *sn*-2 positions of the glycerol backbone.

Table 1. Composition of lipids in thylakoid membranes from two species of cyanobacteria, *Synechocystis* sp. PCC 6803 and *Thermosynechococcus vulcanus*, and from a higher plant, *Spinacia oleracea.*

Source	Lipid					Reference
	MGDG	DGDG	SQDG (mol %)	PG	Others	
Synechocystis sp. PCC 6803	37	20	29	14	0	Sakurai et al., 2006
Thermosynechococcus vulcanus	44	26	25	6	0	Sakurai et al., 2006
Spinacia oleracea	53	27	7	7	6[a]	Dorne et al., 1990

[a]This value is a sum of 2% of phosphatidylinositol and of trace amounts (<1%) of trigalactosyldiacylglycerol, tetragalactosyldiacylglycerol, and diacylglycerol.

complexes in thylakoid membranes (Domonkos et al., 2008; Trémolières and Siegenthaler, 1998) and and that, in photosynthesis, PG plays a specific role in which glycolipids cannot replace it (Sato, 2004; Wada and Murata, 2007).

In higher plants, PG is synthesized in, at least, three subcellular compartments: in plastids such as chloroplasts; in the endoplasmic reticulum (ER); and in mitochondria (Moore, 1974, 1982; Mudd and Dezacks, 1981; Ohlrogge and Browse, 1995). PG synthesized in chloroplasts plays an important role in photosynthesis. By contrast, PG synthesized in mitochondria is utilized for the biosynthesis of the CL that is required for the assembly and stabilization of the protein complexes that are involved in respiratory electron transport and oxidative phosphorylation in mitochondria (see Chapter 4; Frentzen, 2004), as is also the case in non-photosynthetic eukaryotes (Schlame, et al., 2000, Schlame, 2008; Chapter 4). Although, in higher plants, PG is also synthesized in the ER, the role of ER-synthesized PG remains to be clarified.

In this review, we summarize our present understanding of the crucial role played by PG in photosynthesis, focusing on oxygenic photosynthesis in cyanobacteria and higher plants.

II Biosynthesis of Phosphatidylglycerol

A Cyanobacteria

The fatty acids that are required for the biosynthesis of PG are synthesized via sequential reactions that are catalyzed by acetyl-CoA carboxylase and fatty acid synthase (FAS) (see Chapter 2). Acetyl-CoA is used as a substrate for acetyl-CoA carboxylase and as a primer for fatty acid synthesis. Acetyl-CoA carboxylase catalyzes the formation of malonyl-CoA from acetyl-CoA and CO_2. Overall, to produce a 16- or 18-carbon fatty acid from acetyl-CoA and malonyl-CoA, at least 30 enzymatic reactions are required. All the enzymatic reactions for biosynthesis of fatty acids, with the exception of the conversion of acetyl-CoA to malonyl-CoA, are catalyzed by FAS. The FAS of fungi and animals, designated type I FAS, is a multi-functional enzyme that has all the functional domains necessary for fatty acid synthesis and acyl-carrier protein (ACP; Wakil et al., 1983). By contrast, the FAS of bacteria, including cyanobacteria, and of plants is composed of several subunits that catalyze individual steps

in fatty acid synthesis, and it is designated type II FAS (see Chapter 2; Ohlrogge and Browse, 1995). The malonyl-CoA generated by acetyl-CoA carboxylase is converted to malonyl-ACP by malonyl-CoA: ACP transacylase, a component of FAS. Then, malonyl-ACP is converted to butyryl-ACP (C_4) via sequential reactions that are catalyzed by other components of FAS, namely, 3-ketoacyl-ACP synthase, 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase. The resultant butyryl-ACP is further elongated with an acetyl unit of malonyl-ACP via the same reaction cycle, which is repeated until the chain length of the fatty acid reaches C_{16} or C_{18}. Finally, palmitoyl-ACP (16:0-ACP) and stearoyl-ACP (18:0-ACP) are synthesized and used for the biosynthesis of PG.

The biosynthesis of PG from glycerol 3-phosphate (G3P) and acyl-ACP in cyanobacteria is summarized schematically in Fig. 1 (see also Chapter 8; Murata and Nishida, 1987; Wada and Murata, 1998, 2007). In the first reaction, G3P acyltransferase transfers an acyl group from an acyl-ACP, such as 18:0-ACP or 16:0-ACP, to the *sn*-1 position of glycerol 3-phosphate to generate 1-acylglycerol 3-phosphate (lysophosphatidic acid, abbreviated as LPA). Then LPA is further acylated by LPA acyltransferase, which transfers an acyl group from 16:0-ACP to the *sn*-2 position of LPA to generate phosphatidic acid (PA). The PA synthesized by this two-step acylation is converted to CDP-diacylglycerol (CDP-DG) by CDP-DG synthase, which transfers the CMP moiety from CTP to PA. The resultant CDP-DG reacts with glycerol 3-phosphate to produce PG phosphate (PGP) and CMP in a reaction catalyzed by PGP synthase. The last step in this pathway is the release, by PGP phosphatase, of the phosphate group from PGP to generate PG. The molecular species of PG synthesized in this series of reactions contain saturated fatty acids exclusively at both the *sn*-1 and *sn*-2 positions of their glycerol moieties (Sato and Murata, 1982a, b). As a consequence of the substrate preferences of G3P acyltransferase and LPA acyltransferase, the newly synthesized PG has 18:0 or 16:0 at the *sn*-1 position and 16:0 at the *sn*-2 position of its glycerol moieties (Murata et al., 1992a; Weier et al., 2005; Okazaki et al., 2006). The saturated fatty acids at the *sn*-1 position of PG are desaturated by individual acyl-lipid desaturases, which introduce a double bond at a specific position in the fatty acids bound to PG at the *sn*-1 position. By contrast, 16:0 at the *sn*-2 position of PG is not susceptible to desaturation (see Chapter 8; Wada and Murata, 1989; Murata and Wada, 1995).

B Higher Plants

In higher plants, the fatty acids required for the biosynthesis of PG are synthesized in plastids by acetyl-CoA carboxylase and FAS (type II), which generates 16:0-ACP and 18:0-ACP as major products (see Chapter 2; Ohlrogge and Browse, 1995). 18:0-ACP is desaturated to 18:1(9)-ACP by 18:0-ACP desaturase, which is located in the stroma and introduces a double bond at the Δ^9 position of 18:0-ACP (Shanklin and Somerville, 1991). The resultant 18:1(9)-ACP and 16:0-ACP are used for the biosynthesis of PG. As noted above, PG is synthesized in at least three subcellular compartments, namely, in plastids such as chloroplasts, in the ER, and in mitochondria. The biosynthetic pathway in each organelle is essentially the same as that in cyanobacteria (Fig. 1). In plastids, 18:1(9)-ACP and 16:0-ACP are esterified to glycerol 3-phosphate to generate PA. As a result of the substrate preferences of the G3P acyltransferase and LPA acyltransferase, the *sn*-1 position of the PA molecular species is occupied by 18:1(9) or 16:0 and the *sn*-2 position is occupied exclusively by 16:0 (Nishida and Murata, 1996). The PA that is synthesized in plastids is converted to CDP-DG, which is then converted to PGP by PGP synthase and, finally, to PG upon dephosphorylation by PGP phosphatase. The 18:1(9) fatty acid, but not the 16:0 fatty acid, at the *sn*-1 position of the resultant PG is desaturated to yield more-unsaturated molecular species by the acyl-lipid desaturases of the plastids (Browse and Somerville, 1991; Somerville et al., 2000), whereas the 16:0 at the *sn*-2 position is desaturated to Δ^3-*trans*-16:1 fatty acid, which has been found, to date, only in the PG from plastids (Browse et al., 1985; Nishida and Murata, 1996; Roughan and Slack, 1984; Roughan et al., 1987). PG synthesized in plastids has a typical prokaryotic structure with C_{16} fatty acids esterified at the *sn*-2 position of the glycerol backbone. Although the biosynthesis of PG in plastids may take place

in the inner envelope membranes, location of enzymes involved in the biosynthesis of PG has not been determined.

Some of the acyl-ACP that is synthesized in plastids is hydrolyzed to free fatty acids by a thioesterase in the stroma and is then converted to acyl-CoA in a reaction catalyzed by acyl-CoA synthetase, which is located in envelope membranes. The various acyl-CoAs are transported to the ER where they are used for the biosynthesis of PG. As also found in other organisms, the G3P acyltransferase and LPA acyltransferase in the ER use acyl-CoA, but not acyl-ACP, as their substrate in the synthesis of LPA and PA (Moore, 1982; Roughan and Slack, 1984). The PA that is synthesized in the ER includes 18:1(9) or 16:0 at the *sn*-1 position and 18:1(9) at the *sn*-2 position of its glycerol moieties. The PA that has been synthesized in the ER is converted to CDP-DG by CDP-DG synthase and is then used for the synthesis of PG, just as it is in plastids. The 18:1(9) fatty acid, but not 16:0 fatty acid, of the resultant PG is desaturated, to yield more-unsaturated molecular species, by acyl-lipid desaturases that are located in the ER (Browse and Somerville, 1991; Somerville et al., 2000). PG synthesized in ER has a typical eukaryotic structure with C_{18} fatty acids at the *sn*-2 position of the glycerol backbone. Since no eukaryotic structure of PG was found in chloroplasts, it is likely that PG synthesized in ER is not transported to chloroplasts (Bishop et al., 1985; Dorne and Heinz, 1989).

In mitochondria (Griebau and Frentzen, 1994), mitochondrial G3P acyltransferase and LPA acyltransferase use acyl-ACP to synthesize LPA and PA, respectively, as they do in plastids. However, the origins of the fatty acids used for the biosynthesis of acyl-ACP in mitochondria remain to be clarified. It seems likely that fatty acids, after their synthesis in plastids, are transported to mitochondria as acyl-CoAs and are then converted to acyl-ACPs in mitochondria. By contrast to the PG that is synthesized in plastids and in the ER, the PG synthesized in mitochondria is utilized for the biosynthesis of CL (see Chapter 4; Katayama et al., 2004; Nowicki et al., 2005), which is located in the inner membranes of the mitochondria. CL interacts with the protein complexes that are involved in respiratory electron transport and oxidative phosphorylation (Schlame et al., 2000). In mitochondria, PG is present in low amount, thus, it is likely that PG synthesized in mitochondria is only used as an intermediate for the biosynthesis of CL.

III Phosphatidylglycerol in Photosynthetic Membranes

Thylakoid membranes in cyanobacterial cells and chloroplasts are mainly composed of glycerolipids and proteins. As major glycerolipids, they contain glycolipids, MGDG, DGDG, and SQDG, and a phospholipid, PG. These lipids play important roles in the formation of lipid bilayer of thylakoid membranes. Thylakoid membranes contain several protein–cofactor complexes involved in photosynthesis, namely, the photosystem I (PS I) complex, the photosystem II (PS II) complex, the cytochrome b_6f complex (Cyt b_6f), and ATP synthase (Melis, 1991; Malkin and Niyogi, 2000). Recently, it has been suggested that lipids in thylakoid membranes play important roles not only in the formation of lipid bilayers, but also in the construction of the complexes. Lipid molecules in these complexes, with the exception of ATP synthase, have been analyzed by x-ray crystallography (see Chapter 10; Guskov et al., 2009; Jordan et al., 2001; Kurisu et al., 2003; Loll et al., 2005; Yamashita et al., 2007) and lipid molecules extracted from purified PS I and PS II complexes have also been analyzed by thin-layer chromatography and gas chromatography (Sakurai et al., 2006; H. Kubota, I. Sakurai, K. Katayama, N. Mizusawa, S. Ohashi, M. Kobayashi, P. Zhang, E.-M. Aro and H. Wada, unpublished). Table 2 shows the lipid molecules that have been identified by x-ray crystallography and by chromatographic analysis of the lipids extracted from such complexes.

PS I is responsible for the reduction of $NADP^+$, which generates the NADPH that is required as a reductant for the Calvin–Benson cycle (carbon fixation), and PS I is composed of approximately 15 protein subunits and many cofactors, such as chlorophylls and lipids (see Chapter 10; Jordan et al., 2001). Jordan et al. (2001) analyzed the structure of the PS I complex from *Thermosynechococcus elongatus* by x-ray crystallography at 2.5 Å resolution, and they identified one molecule of MGDG and three molecules of PG per monomer of the complex. In the complex, one of the three PG molecules and the MGDG molecule were located symmetrically, relative to one another,

Table 2. Lipid molecules identified in protein–cofactor complexes from thylakoid membranes by x-ray crystallographic analysis and by biochemical analysis of lipids extracted from the complexes.

Protein complex	Source		Number of lipid molecules	Reference
X-ray crystallography				
	Thermosynechococcus elongatus			
PS I		PG	3	Jordan et al., 2001
		MGDG	1	
PS II		PG	1	Loll et al., 2005
		MGDG	6	
		DGDG	4	
		SQDG	3	
PS II		PG	2	Guskov et al., 2009
		MGDG	11	
		DGDG	7	
		SQDG	5	
LHCII	*Spinacia oleracea*	PG	1	Liu et al., 2004
		DGDG	1	
LHCII	*Pisum sativum*	PG	1	Standfuss et al., 2005
		DGDG	1	
Cyt b_6f	*Mastigocladus laminosus*	PG	2	Kurisu et al., 2003
Cyt b_6f	*Mastigocladus laminosus*	PC	2	Yamashita et al., 2007
		SQDG	1	
Cyt b_6f	*Chlamydomonas reinhardtii*	SQDG	1	Stroebel et al., 2003
		MGDG	2	
Lipid analysis				
	Synechocystis sp. PCC 6803			
PS I		PG	2	H. Kubota, I. Sakurai, K. Katayama, N. Mizusawa, S. Ohashi, M. Kobayashi, P. Zhang, E.-M. Aro and H. Wada, unpublished data
		MGDG	2	
		DGDG	1	
		SQDG	1	
PS II		PG	6	Sakurai et al., 2006
		MGDG	6	
		DGDG	3	
		SQDG	5	
	Thermosynechococcus vulcanus			
PS II		PG	8	Sakurai et al., 2006
		MGDG	8	
		DGDG	6	
		SQDG	6	

The number of lipid molecules per monomer is shown for each protein complex.

suggesting that these two lipid molecules might specifically be important in the formation of the photochemical reaction center of the PS I complex. A second PG molecule was located in the vicinity of the monomer–monomer interface in the trimeric structure of the PS I complex, suggesting that this PG molecule might participate in the trimerization of the PS I complex. This hypothesis is supported by the observation that monomers of the PS I complex accumulated in a PG-deficient mutant of *Synechocystis* sp. PCC 6803, as described below. A third PG molecule was found between the PsaB and PsaX subunits, suggesting that it might contribute to the binding of PsaX to the PS I complex.

Recently, we generated strains of *Synechocystis* sp. PCC 6803 strains that expressed a His-tagged PsaF or PsaJ subunit of PS I, hoping to use them for the rapid and simple purification of PS I complexes (H. Kubota, I. Sakurai, K. Katayama, N. Mizusawa, S. Ohashi, M. Kobayashi, P. Zhang, E.-M. Aro and H. Wada, unpublished). Using these strains, we were able to purify PS I complexes by Ni-affinity column chromatography, with subsequent ultracentrifugation on a glycerol

density gradient. Analysis of lipids extracted from the purified trimeric complex of PS I by thin-layer chromatography and gas chromatography identified six lipid molecules per monomer in the PS I complex (Table 2). Although the number of lipid molecules was close to that found in the crystal structure of PS I from *T. elongatus*, the lipid composition of PS I from *Synechocystis* sp. PCC 6803 differed from that of PS I from *T. elongatus*. Specifically two MGDG, one DGDG, one SQDG and two PG per monomer were identified in the PS I complex from *Synechocystis* sp. PCC 6803. These observations suggest that the lipid composition of PS I depends on the cyanobacterial species and that some lipid molecules are interchangeable with other classes of lipids.

PS II is responsible for the extraction of electrons from water molecules and it consists of approximately 20 protein subunits in addition to many cofactors, such as pigments, metals, and lipids (see Chapter 10; Murata et al., 1984; Nanba and Satoh, 1987; Boekema et al., 1995; Hankamer et al., 2001). The spatial arrangement of protein subunits and cofactors in PS II has been clarified by x-ray crystallographic analysis (Zouni et al., 2001; Kamiya and Shen, 2003; Ferreira et al., 2004; Loll et al., 2005; Guskov et al., 2009). In the most recently determined crystal structure of PS II at 2.9 Å resolution, 25 lipid molecules (11 MGDG, seven DGDG, five SQDG, and two PG) per monomer were assigned in the PS II complex from *T. elongatus* (see Chapter 10; Table 2; Guskov et al., 2009). Compared to the PS I complex, the PS II complex contains a relatively large number of lipid molecules. Seven of the 25 lipid molecules were located at the monomer–monomer interface, three are at the periphery of PS II, seven form clusters with two to three lipids in the lipid belt around D1 and D2, and the remaining eight lipids are arranged as a bilayer island forming the plastoquinone–plastoquinol exchange cavity (Guskov et al., 2009). The 15 lipid molecules located in the vicinity of the photochemical reaction center might provide some structural flexibility within the reaction center.

The PS II complex is inactivated by light and, as has been demonstrated in considerable detail, the inactivated PS II complex is repaired via a multi-step process that includes degradation of the D1 protein, synthesis of the precursor to the D1 protein, reassembly of the PS II complex, processing of the precursor to D1 to yield mature D1, formation of the manganese cluster, and dimerization of the PS II complex (Aro et al., 1993; Andersson and Aro, 2001; Nishiyama et al., 2006). The removal of the degraded D1 protein from the damaged complex and the insertion of the newly synthesized precursor to D1 into the damaged PS II complex are critical to the repair process, and a flexible environment around the reaction center, created by abundant lipid molecules, might facilitate this replacement of the D1 protein. Only two PG molecules were identified in the crystal structure of PS II. However, our analysis of extracted lipids from purified PS II complexes indicated that the PS II complexes from *T. vulcanus* and *Synechocystis* sp. PCC 6803 contain eight and six PG molecules per monomer, respectively, with PG being the most abundant lipid (Table 2; Sakurai et al., 2006). These results suggest that more PG molecules are present in the PS II complex than have been identified by crystallographic techniques (Sakurai et al., 2006).

The crystal structure of light-harvesting complex II (LHCII) from pea (Standfuss et al., 2005) and spinach (Liu et al., 2004) have been resolved at 2.5 and 2.7 Å resolution, respectively. In both LHCIIs, it was found that one PG and one DGDG molecule were bound to each monomer of the complex. Liu et al. (2004) demonstrated that the complex formed a trimer, which, in turn, aggregated to form an icosahedral sphere that consisted of 20 LHCII trimers. The DGDG molecules were located at the contact surfaces between adjacent trimers, suggesting that they might be required for maintenance of the icosahedral structure. The PG molecules were buried at the interfaces between the monomers and might, thus, be required for the trimerization of monomers. The putative requirement for PG in the trimerization of monomers is supported by the finding that treatment of the complex with phospholipase resulted in the dissociation of trimers into monomers (Nußberger et al., 1993). It has also been proposed that the PG molecules might play a direct structural role in the binding of one of the antenna chlorophylls.

Kurisu et al. (2003) crystallized the Cyt b_6f complex from *Mastigocladus laminosus* and found two phosphatidylcholine (PC) molecules per monomer of the complex after exogenous PC had been added during purification and crystallization of the complex. In the most recently determined

crystal structure, three lipid molecules (two PC and one SQDG) per monomer were assigned in the Cyt b_6f complex from *M. laminosus* (Yamashita et al., 2007). Stroebel et al. (2003) crystallized the Cyt b_6f complex from *Chlamydomonas reinhardtii* and found one SQDG molecule and two other lipid molecules (probably MGDG) but no PG. These findings suggest that the PG might not be part of the Cyt b_6f complex. However, it remains possible that PG might play a role in this complex because lipid molecules can dissociate from various complexes during the purification and crystallization of such complexes.

IV The Role of Phosphatidylglycerol in Photosynthesis

A Cyanobacteria

Among the genes for the five enzymes that are involved in the biosynthesis of PG in *Synechocystis* sp. PCC 6803 (see above), two genes for LPA acyltransferase (Weier et al., 2005; Okazaki et al., 2006), the *cdsA* gene for CDP-DG synthase (Sato et al., 2000), and the *pgsA* gene for PGP synthase (Hagio et al., 2000) have been identified. Genes for G3P acyltransferase and PGP phosphatase have not yet been identified. The identification of the genes that encode enzymes required for the biosynthesis of PG has allowed the generation, by targeted mutagenesis, of mutants of *Synechocystis* sp. PCC 6803 that are incapable of synthesizing PG. Sato et al. (2000) inactivated the *cdsA* gene for CDP-DG synthase and Hagio et al. (2000) inactivated the *pgsA* gene for PGP synthase in *Synechocystis* sp. PCC 6803. The resultant mutants were incapable of synthesizing PG and required an exogenous supply of PG for growth under photoautotrophic conditions. Other phospholipids, such as PC and phosphatidylethanolamine (PE), failed to support the growth of the mutant cells (Hagio et al., 2000). *Synechocystis* sp. PCC 6803 can grow under light-activated heterotrophic growth conditions, with cultures being incubated in complete darkness apart from 5 min of dairy illumination (Anderson and McIntosh, 1991). The growth of cells under these conditions is dependent on the presence of glucose in the growth medium but not on photosynthesis. The above mentioned *pgsA* mutant was able to grow under light-activated heterotrophic growth conditions in the presence of PG but not in its absence. These findings indicate that *Synechocystis* sp. PCC 6803 cells are able to take up PG from the growth medium and that PG is indispensable for their growth, even when such growth is independent of photosynthesis.

We used the above-mentioned mutants to investigate the role of PG in photosynthesis (Hagio et al., 2000; Sato et al., 2000, 2004; Gombos et al., 2002; Sakurai et al., 2003, 2007; Domonkos et al., 2004). The amount of PG in mutant cells decreased when cells that had been grown in the presence of PG were transferred to medium without PG. We were able to investigate the role of PG in photosynthesis by monitoring phenomena that occurred after the transfer of cells to medium without PG. Figure 2 shows what we observed when cells were deprived of PG. The photosynthetic activity of the *pgsA* and *cdsA* mutants fell dramatically, with a simultaneous decrease in the PG content of thylakoid membranes, in the absence of PG. However, the activity returned to the original level upon re-addition of PG to the growth medium. The decrease in photosynthetic activity that was induced in the absence of PG was attributed to a decrease in PS II activity but not to a decrease in PS I activity, suggesting that PG might play an important role in PS II (Hagio et al., 2000; Sato et al., 2000). We examined each step in the transport of electrons in PS II in *pgsA* mutant cells in an attempt to identify the functional site of PG (Gombos et al., 2002). Measurements of the fluorescent yield of chlorophyll indicated that the reduced form of plastoquinone Q_A (Q_A^-) accumulated in our *pgsA* mutant cells after removal of PG from the medium. Thermoluminescence measurements also suggested the accumulation of the $S_2Q_A^-$ state in the absence of PG. These results indicate that electron transport activity from Q_A to Q_B in PS II fell when the mutant cells were deprived of PG (Gombos et al., 2002), and they are consistent with the finding that the Q_B-mediated transport of electrons was inhibited by treatment of PS II complex with phospholipase A_2, which results in the digestion of PG (Sakurai et al., 2006; Leng et al., 2008).

Recently, Laczko-Dobos et al. (2008) generated a novel mutant of *Synechocystis* sp. PCC 6803 by

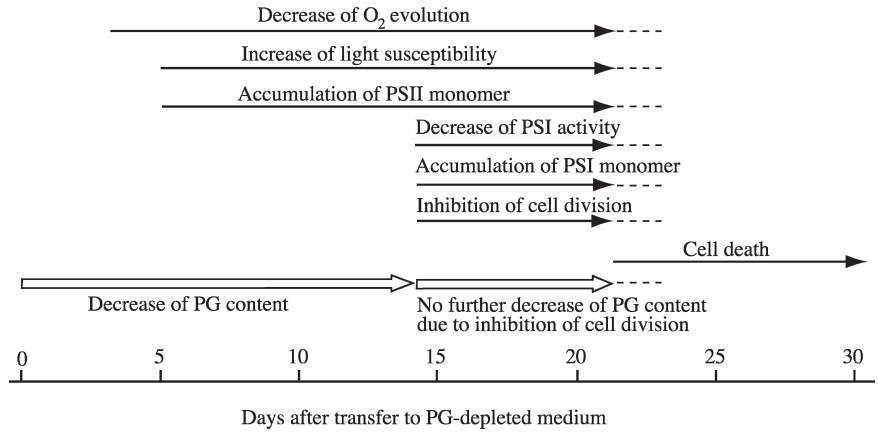

Fig. 2. Phenomena observed after removal of PG from the culture medium of *pgsA* mutant cells of *Synechocystis* sp. PCC 6803. Cells grown in standard medium supplemented with 20 μM PG were transferred to the same medium without PG at zero time and incubated as indicated.

disrupting the *cdsA* gene for CDP-DG synthase in PAL mutant cells, which lack phycobilisomes. Their mutant is unable to synthesize PG and lacks phycobilisomes. The results that they obtained with their mutant are similar to those that we obtained with our *pgsA* mutant. In addition, Kruse and Schmid (1995) demonstrated, in an immunological study with antibodies against PG, that PG is specifically associated with the D1 protein. These findings suggested that PG might play an indispensable role in maintaining the structure of the Q_B-binding site of the D1 protein. Further characterization of the *pgsA* mutant revealed that PG depletion had a negative effect not only on the electron acceptor side of PS II, via inhibition of the transport of electrons from Q_A to Q_B but also on the donor side (Sakurai et al., 2007).

To understand why the donor side of PS II was impaired in our *pgsA* mutant we purified the PS II complex from *pgsA* mutant cells. Analysis of the purified PS II complexes indicated that PS II from PG-depleted *pgsA* mutant cells had only half of the oxygen-evolving activity of the PS II from wild-type cells. We also observed the dissociation of the extrinsic proteins PsbO, PsbV, and PsbU, which are required for stabilization of the Mn-cluster, from PS II of PG-depleted mutant cells. In turn, this dissociation of extrinsic proteins induced the release of Mn from PS II, leading to a decrease in the oxygen-evolving activity. The released PsbO reassociated with PS II when PG was added back to the culture medium of the PG-depleted mutant cells, even when protein synthesis de novo was inhibited. It is likely that the dissociation of extrinsic proteins that we observed in our analysis of the PS II purified from the mutant cells also occurs in vivo because the photosynthetic activity of the PG-depleted *pgsA* mutant cells was suppressed by heat treatment and, also, by incubation in darkness, as observed similarly in Δ*psbO*, Δ*psbV* and Δ*psbU* mutant cells. In mutant cells that lack PsbO, PsbV or PsbU, oxygen-evolving activity is easily inactivated by heat treatment (Nishiyama et al., 1994, 1997; Shen et al., 1995; Clarke and Eaton-Rye, 1999; Kimura et al., 2002), and Mn atoms in the Mn cluster are reduced under dark conditions and released from PS II in mutant cells that lack PsbO or PsbV (Burnap et al., 1996; Shen et al., 1998). These earlier findings, in combination with our more recent results, suggest that PS II of the PG-depleted mutant cells cannot functionally bind certain extrinsic proteins, perhaps PsbO or PsbV, and that re-binding of extrinsic proteins is dependent on PG.

The PS II monomer accumulated in PG-depleted *pgsA* mutant cells and, therefore, we suggested that PG might be involved in the dimerization of PS II (Sakurai et al., 2003). However, the requirement for PG in the binding of extrinsic proteins to the PS II core complex raised the possibility that the accumulation of monomers might result from the dissociation of extrinsic proteins and that PG might not be involved directly in the dimerization

of PS II. Preliminary data indicated that the formation of dimers was severely impaired in Δ*psbO* mutant cells and partially impaired in the Δ*psbV* and Δ*psbU* mutant cells (Sakurai et al., 2007), suggesting that monomerization of the PS II complex in *pgsA* mutant cells might have been induced indirectly by the dissociation of extrinsic proteins.

Our findings with the *pgsA* mutant led us to postulate that PG molecules might be located near the Q_B-binding site and at the interface between the extrinsic proteins and the PS II core. Our biochemical analysis of lipids extracted from PS II complexes of wild-type and *pgsA* mutant cells indicated that approximately six molecules of PG were bound to each PS II monomer in wild-type cells, whereas three molecules of PG were bound to each PS II monomer in PG-depleted mutant cells (Sakurai et al., 2007). The three PG molecules that disappeared from PS II in the mutant cells might have been located near the binding site of Q_B and at the interface between the extrinsic proteins and the PS II core. The most recently determined crystal structure of the PS II complex from *T. elongatus* revealed that two PG molecules were present per PS II monomer (see Chapter 10; Guskov et al., 2009). The identified PG molecules in the PS II complex are localized in the plastoquinone–plastoquinol exchange cavity, their head groups face the cytoplasm, and one of the PG molecules is close to the Q_B site. These are consistent with our finding that the deprivation of PG influences the structural integrity of Q_B site, but it seems unlikely that the identified PG molecules would be able to influence the binding of extrinsic proteins to the PS II core. Nevertheless, it is possible that some molecules of PG remain to be identified within the crystal structure and that they play roles in the binding of extrinsic proteins to the PS II core.

The absence of PG results in impairment of the transport of electrons from Q_A to Q_B in PS II and in dissociation of extrinsic proteins from the PS II core, leading to a decrease in PS II activity. In addition, extended deprivation of PG induces a decrease in the activity not only in PS II but also of PS I (Fig. 2; Domonkos et al., 2004). At the same time as the decrease in PS I activity, we observed dramatic structural changes in the PS I complex. An analysis of purified PS I complexes revealed that trimers were no longer detectable and that the accumulated monomers did not contain the PsaL subunit, which is known to be responsible for the formation of trimers (Chitnis and Chitnis, 1993), even though the PsaL protein was detected in thylakoid membranes. The trimeric structure of PS I was restored by re-addition of PG, even in the presence of lincomycin, an inhibitor of protein synthesis. The accumulation of monomers of PS I after deprivation of PG and the reformation of trimers after re-addition of PG were also observed in the *cdsA* mutant (Sato et al., 2004). These findings indicate that free PsaL is present in thylakoid membranes even after long-term deprivation of PG and, moreover, that PG plays an indispensable role in the PsaL-mediated assembly of the PS I complex. Given these findings, we can anticipate that PG should be located at the interface between monomers and should be involved in the formation of trimers of PS I. This scenario would support the observation that one of three PG molecules identified in the crystal structure of PS I is located at the interface between monomers.

B Higher Plants

Phosphatidylglycerol is the only major phospholipid present at significant levels in the thylakoid membranes of higher plants, as it is in those of cyanobacteria (Block et al., 1983; Dorne et al., 1990; Wada and Murata, 1998). Since PG is negatively charged at neutral pH, it seems plausible that PG might mediate essential interactions among the protein complexes in thylakoid membranes and, moreover, that PG might play a specific role in photosynthesis in which it cannot be replaced by glycolipids such as MGDG and DGDG.

The role of PG in photosynthesis was studied by treating thylakoid membranes with phospholipases that specifically degrade PG. Jordan et al. (1983) showed that elimination of approximately 70% of the original PG from thylakoid membranes isolated from *Pisum sativum* (pea), by treatment with phospholipase A_2, almost completely blocked the photosynthetic transport of electrons in PS II without any significant effect on the photosynthetic transport of electrons in PS I. Similarly, Droppa et al. (1995) showed that treatment of thylakoid membranes from pea with phospholipase C, which degraded approximately half of the original PG, almost completely eliminated the photosynthetic transport of electrons in PS II.

These findings are consistent with those obtained with the *pgsA* mutant of *Synechocystis* sp. PCC 6803 (Hagio et al., 2000; Gombos et al., 2002). Phospholipases are bulky proteins that may have difficulties to access hidden PG molecules inside of protein complexes. The sensitivity of the PS II in thylakoid membranes to the treatment with phospholipases could indicate that PG is a rather fluid component of the PS II complex. Moreover, Kruse et al. (2000) found that PS II complexes isolated from spinach were converted from dimers to monomers by phospholipase A_2 and that monomers were reversibly converted to dimers upon addition of PG. On the basis of these findings, Kruse et al. (2000) proposed that PG is required for formation of dimers of the PS II complex. However, we recently performed similar experiments with PS II dimers prepared from *T. vulcanus* and found that treatment of these dimers with phospholipase did not induce monomerization of PS II but instead, it inhibited the transport of electrons from Q_A to Q_B, as found also in *pgsA* mutant cells (Leng et al., 2008). Dimers of PS II from *T. vulcanus* are very stable and extrinsic proteins do not dissociate from the PS II core during treatment with phospholipase (Leng et al., 2008). By contrast, PS II dimers from spinach are unstable and extrinsic proteins are released from the PS II core upon incubation of these PS II dimers with phospholipase (Kruse et al., 2000). Thus, it seems likely that PG is not involved directly in the dimerization of PS II. The conversion of dimers of spinach PS II to monomers is probably caused by the dissociation of extrinsic proteins from PS II cores after the digestion by phospholipase of the PG that is required for the binding of extrinsic proteins. The digestion of PG might prevent the binding of the extrinsic proteins that stabilize the dimers to induce the accumulation of monomers.

In addition to biochemical approach, molecular genetic studies of the role of PG in photosynthesis have been reported. Dubertret et al. (1994) isolated mutants of *Chlamydomonas reinhardtii* that were incapable of synthesizing molecular species of PG containing Δ^3-*trans*-hexadcenoic acid [16:1(3t)] as a result of a defect in desaturation at the Δ^3 position of 16:0 esterified to the *sn*-2 position of PG. Analysis of their mutants suggested that molecular species of PG containing 16:1(3t) might be essential for the assembly of the trimeric form of LHCII (Dubertret et al., 1994, 2002). However, this finding is not consistent with a report that, in a mutant of *Arabidopsis thaliana*, LHCII trimers formed even in the absence of the molecular species of PG that was able to bind 16:1(3t) (McCourt et al., 1985). Thus, it is unclear which molecular species of PG are involved in the formation of trimers of LHCII. Nevertheless, it is clear that PG itself is required for the formation of LHCII trimers. Hobe et al. (1994, 1995) investigated the interaction of PG with the LHCII apoprotein. They reported that the negative charge on PG interacts with the amino-terminal region, from amino acid positions 16 to 21 from the amino terminus, of the LHCII apoprotein and that this region plays a crucial role in the trimerization of the LHCII complex. Furthermore, Maanni et al. (1998) suggested that the formation of a trimeric LHCII complex might be important for grana stacking of thylakoid membranes.

Mutants of *A. thaliana* with a defect in the biosynthesis of PG have also been used for investigations of the functions of PG. Xu et al. (2002) isolated a mutant with a defect in the gene (*PGP1*) for PGP synthase, which is located in plastids and mitochondria. The level of PG in leaves of mutant plants was approximately 25% lower than that of wild-type plants. Moreover, mutant plants had pale-green leaves and a slightly lower capacity for photosynthesis than wild-type plants, suggesting that PG might play an important role in the development of chloroplasts and in the photosynthetic machinery. Hagio et al. (2002) and Babiychuk et al. (2003) isolated *pgp1* null mutants of *A. thaliana*, whose growth required the addition of sucrose to the standard growth medium and whose leaves were a pale yellow-green. In these mutant plants, the level of PG was approximately 10% of that in wild-type plants, and the development of chloroplasts in the leaf cells of mutant plants was severely impaired, as shown in Fig. 3. These findings suggested that PG might be indispensable for the development of chloroplasts and, in particular, for the development of thylakoid membranes.

Tropical and subtropical plants exhibit a distinct physiological damage when they are exposed to low temperatures (Lyons, 1973). This phenomenon is referred to as chilling injury. Because the phenomenon is observed at temperatures above 0°C,

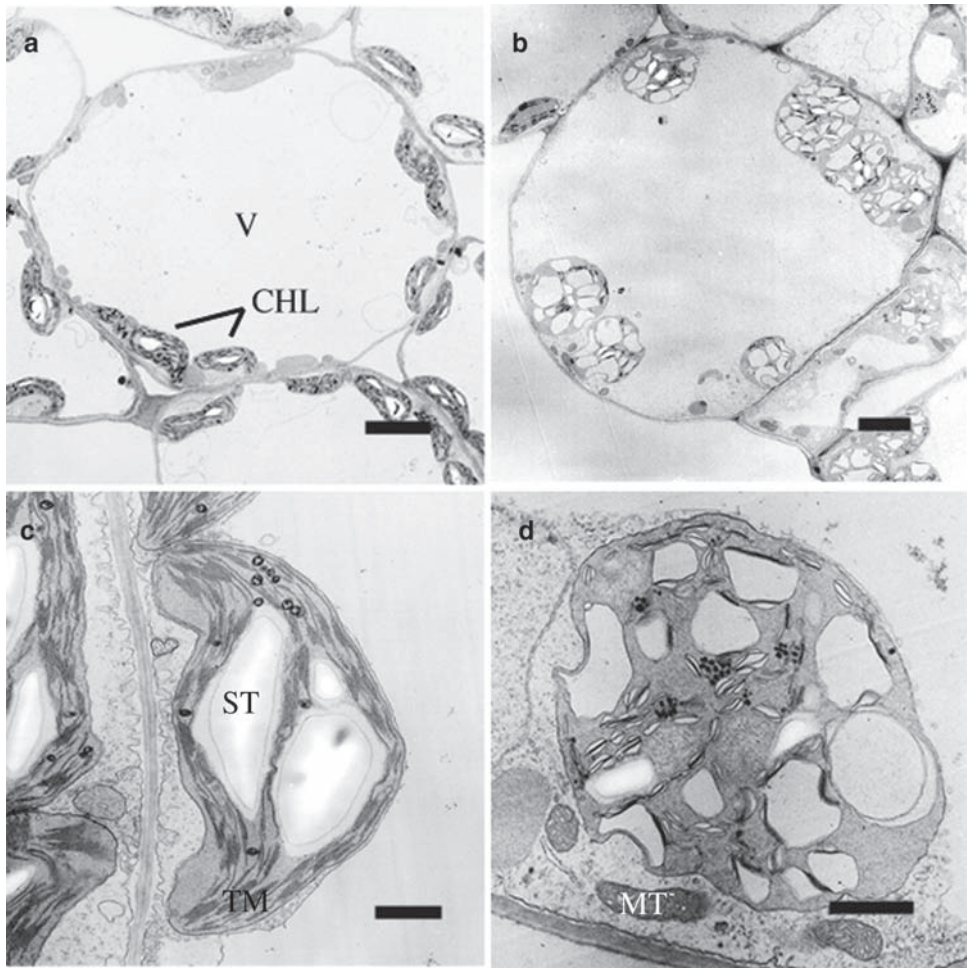

Fig. 3. Ultrastructure of mesophyll cells and chloroplasts of the wild type and the *pgp1* null mutant of *A. thaliana*. Mesophyll cells (**a** and **b**) and chloroplasts (**c** and **d**) of the wild type (**a** and **c**) and the *pgp1* null mutant (**b** and **d**) are shown. Abbreviations: CHL, chloroplast; MT, mitochondrion; ST, starch; TM, thylakoid membrane; V, vacuole. *Bars* in (**a**) and (**b**) and those in (**c**) and (**d**) represent 10 and 2 μm, respectively (adapted from Hagio et al. (2002) and reproduced with permission of Oxford Journals).

chilling injury is quite distinct from freezing injury, which is mainly associated with physical disorders caused by ice formation. As described below, PG present in chloroplasts is related to the chilling injury of higher plants. In the mechanism proposed by Lyons (1973) and Raison (1973) to explain the chilling sensitivity of plants, the primary event in chilling injury is the formation of a lipid-gel phase in cell membranes at low temperatures, followed by a series of events that lead to cell death. When a membrane enters a phase-separated state in which the gel and liquid-crystalline phases co-exist, the membrane becomes permeable to small electrolytes at the border of the two phases, with resultant diminution of the ion gradients across the membrane that are essential for the maintenance of physiological activity (Nishida and Murata, 1996). The temperature for the transition of membrane lipids from a liquid-crystalline state to a gel state depends on the extent of unsaturation of the fatty acids bound to the lipids. Lipids with only saturated fatty acids, such as 16:0 and 18:0, have a phase-transition temperature above 40°C, while those with fatty acids that include a *cis*-unsaturated bond have a phase-transition temperature of approximately

0°C (Phillips et al., 1972). The introduction of a second *cis*-unsaturated bond causes the phase-transition temperature to drop to close to −20°C (Phillips et al., 1972). A *trans*-unsaturated bond does not shift the phase-transition temperature downward as effectively as a *cis*-unsaturated bond (Kenrick and Bishop, 1986). These findings suggest that, if certain molecular species of lipids can raise the membrane phase-transition temperature above 0°C, they are most likely to be fully saturated or *trans*-monounsaturated species. To identify membrane lipids with phase-transition temperatures above 0°C, Murata and Yamaya (1984) extracted glycerolipids from chilling-sensitive and chilling-resistant plants and measured their phase-transition temperatures. Among all the glycerolipids extracted from leaf cells, only the PG from chilling-sensitive plants had a phase-transition temperature above 0°C, while none of the glycerolipids from chilling-resistant plants had a phase-transition temperature above 0°C. The PG from chilling-sensitive plants contained high levels of fully saturated or *trans*-monounsaturated molecular species (Murata et al., 1982; Murata, 1983; Raison and Wright, 1983; Tasaka et al., 1990). Chilling-sensitive and chilling-resistant plants contained 16:0-16:0 and 16:0-16:1(3t) species of PG of a range of levels from 3% to 19% and from 26% to 65% of the total PG, respectively (Murata, 1983). These values suggest strongly that the level of these two molecular species of PG is closely associated with the chilling sensitivity of plants.

Considering the details of the biosynthesis of PG (Mudd and Dezacks, 1981; Moore, 1982; Andrews and Mudd, 1985) and the desaturation to 16:1(3t) of 16:0 bound to the *sn*-2 position of PG (Browse and Somerville, 1991; Ohnishi and Thompson, 1991), researchers expected that 16:0-16:0 and 16:0-16:1(3t) species of PG would be synthesized in chloroplasts and that the levels of such species of PG might depend on the substrate selectivity of the G3P acyltransferase in the stroma of the chloroplasts (Frentzen et al., 1983; Murata, 1983; Murata and Tasaka, 1997). In fact, the substrate selectivity of acyltransferases from chilling-resistant plants was found to be weighted in favor of *cis*-unsaturated fatty acids, whereas, in chilling-sensitive plants, these enzymes were unable to discriminate between saturated and *cis*-unsaturated fatty acids (Frentzen et al., 1987; Nishida et al., 1987). Such observations suggest that the genetic manipulation of these enzymes might be effective strategy for conversion of chilling-sensitive plants to chilling-resistant plants.

To manipulate G3P acyltransferase genetically Murata and his colleagues cloned the cDNAs for the enzyme from a chilling-sensitive plant, *Cucurbita moschata* (squash; Ishizaki et al., 1988), and from a chilling-resistant plant, *A. thaliana* (Nishida et al., 1993), and introduced these cDNAs into *Nicotiana tabacum* (tobacco) under the control of the 35S promoter of tobacco mosaic virus. As anticipated, the extent of the unsaturation of fatty acids in PG was altered in the transgenic plants (Murata et al., 1992b). Detailed analysis of the transgenic tobacco plants revealed that the cDNA had been stably integrated into the tobacco genome in each case and that the expressed proteins were transported into chloroplasts, where they were processed to yield mature proteins. An analysis of the lipids from the leaves of these transgenic tobacco plants revealed that PG was the only lipid in which the extent of unsaturation of fatty acids had been significantly altered by expression of the cDNA for the respective acyltransferase. In transgenic tobacco plants that had been transformed with the cDNA for the squash enzyme, levels of saturated and *trans*-unsaturated molecular species of PG rose, whereas transformation with the cDNA for the enzyme from *A. thaliana* caused a small but significant decrease in the levels of these molecular species. Fourier transform infrared spectroscopy revealed that the critical temperature for the appearance of rigid domains in lipids within thylakoid membranes increased upon introduction of the squash cDNA and decreased upon introduction of the cDNA of *A. thaliana* (Szalontai et al., 2003).

Murata and his colleagues also examined the chilling sensitivity of the transgenic tobacco plants (Murata et al., 1992b). Exposure of whole plants to a temperature of 1°C for 10 days had a marked effect on the leaves of the transgenic tobacco plants, inducing chlorosis and leaf deterioration to a greater or lesser extent. Such damage was even more conspicuous when the plants were kept at 25°C for 2 days after the 10-day chilling treatment. Leaves of wild-type plants and of transgenic plants generated with the empty vector were only partially chlorotic, whereas leaves of transgenic plants that harbored the squash cDNA

were severely damaged or completely dead. However, transgenic plants generated with the cDNA from *A. thaliana* were much more resistant to chilling than both the wild-type plants and the control plants that had been transformed with the empty vector. The differences in chilling sensitivity were closely correlated with the extent of unsaturation of PG and with the shift in the critical temperature for the appearance of rigid domains in the thylakoid membranes of these plants (Szalontai et al., 2003).

The transformation of tobacco plants with cDNA for G3P acyltransferase from squash affected photosynthesis at various temperatures (Moon et al., 1995). Leaves of transformed plants were more sensitive to photoinhibition than those of wild-type plants. Moreover, recovery of photosynthesis from photoinhibition in the leaves of wild-type plants occurred more rapidly than that in the leaves of the transgenic tobacco plants. These results suggested that unsaturation of PG in thylakoid membranes might have stabilized the photosynthetic machinery against photoinhibition at low temperatures by accelerating the recovery of PS II. Although the analysis of transgenic tobacco plants, as described above, was performed with heterozygous lines, Murata's group also established homozygous lines of transgenic tobacco plants that overexpressed the cDNA for G3P acyltransferase from squash and used them for further analysis (Sakamoto et al., 2004). In the homozygous plants, saturated plus *trans*-monounsaturated molecular species accounted for 65% of the total PG, whereas they accounted for only 24% in wild-type plants. Chilling stress suppressed the growth and development of young seedlings of transgenic plants more severely than those of wild-type plants, and this observation suggested that changes in the proportion of *cis*-unsaturated PG might have affected not only mature leaves but also developing and growing plants. When the young transgenic seedlings reached maturity, chilling stress also damaged the inflorescences. In particular, the abscission of flower buds and inflorescence meristems from transgenic plants occurred more frequently than that from wild-type plants (Sakamoto et al., 2004). Thus, it seems likely that a decrease in the relative level of *cis*-unsaturated PG might enhance the sensitivity of reproductive organs to chilling.

Wolter et al. (1992) performed a similar experiment by introducing a gene for G3P acyltransferase from *Escherichia coli* into *A. thaliana*. The encoded enzyme preferentially transfers saturated fatty acids to the *sn*-1 position of glycerol 3-phosphate. The transformation of *A. thaliana* with this bacterial gene increased the level of saturated plus *trans*-monounsaturated PG and converted *A. thaliana* into a chilling-sensitive plant.

The Δ^9 desaturase in the cyanobacterium *Anacystis nidulans* (*Synechococcus* sp. PCC 6301) introduces a *cis* double bond at the Δ^9 position of both 16:0 and 18:0 that have been esterified to membrane lipids. It was postulated, therefore, that saturated molecular species of PG would be desaturated to unsaturated molecular species of PG and that chilling sensitivity would decrease if the gene for the Δ^9 desaturase of *A. nidulans* were to be introduced into higher plants. Ishizaki-Nishizawa et al. (1996) cloned the gene for this enzyme and introduced it stably into tobacco under the transcriptional control of the 35S promoter of Cauliflower mosaic virus. The expressed enzyme was targeted to chloroplasts and the level of saturated fatty acids bound to membrane lipids was lower in the transgenic plants than in the wild type. As expected, the transgenic plants exhibited significantly greater resistance to chilling than control plants that had been transformed with the empty vector.

V The Role of Phosphatidylglycerol in Non-Photosynthetic Processes

As described above, the *pgsA* mutant of *Synechocystis* sp. PCC 6803 failed to grow not only under photoautotrophic growth conditions but also under light-activated heterotrophic growth conditions in the absence of PG (Hagio et al., 2000). This finding indicates that PG is required even for the photosynthesis-independent growth of *Synechocystis* sp. PCC 6803.

In bacteria, PG is used as a substrate for the lipid modification of lipoproteins, which are an abundant class of peripheral proteins that are anchored to membranes. Lipoproteins are synthesized as precursors and processed as follows: first, the diacylglyceryl moiety is transferred, by prolipoprotein diacylglyceryltransferase, from PG to the sulfhydryl group of a specific cysteine residue in

the amino-terminal region of a precursor (Gan et al., 1993; Sankaran and Wu, 1994). The modified precursor is processed by signal peptidase II, which removes a signal sequence at the amino-terminal cysteine residue that has been modified with diacylglycerol (Tokunaga et al., 1982). As a result, the cysteine residue with its diacylglycerol modification becomes the amino-terminus of the mature protein. Finally, the amino group of the amino-terminal cysteine is acylated by apolipoprotein N-acyltransferase (Gupta et al., 1993). Since the initial identification of lipoproteins in *Escherichia coli*, many lipoproteins have been identified in almost all bacteria and have been found to play important roles in many processes, such as the uptake of nutrients and secretion of proteins (Braun and Wu, 1994; Sutcliffe and Russell, 1995). The genomes of *E. coli* K12 and *Bacillus subtilis* contain hundreds of genes for putative lipoproteins (Babu and Sankaran, 2002; Juncker et al., 2003). It is very likely that cyanobacteria also have many lipoproteins and that they too play important and/or essential roles in cyanobacteria.

Bacterial lipoproteins include a conserved signal sequence (lipoprotein signal peptides; Juncker et al., 2003; Wu and Tokunaga, 1986). To identify putative lipoproteins in cyanobacteria, we searched the genome database of *Synechocystis* sp. PCC 6803 using the hidden-Markov-model algorithm, LipoP (www.cbs.dtu.dk/services/LipoP/; Juncker et al., 2003), which is commonly used in searches for bacterial lipoproteins. We found close to 40 putative lipoproteins in *Synechocystis* sp. PCC 6803 (T. Ujihara, K. Katayama, I. Sakurai, N. Mizusawa and H. Wada, unpublished). Half of these putative lipoproteins were substrate-binding proteins of ABC transporters and three of them were PsbP, PsbQ and Psb27, which are extrinsic proteins that stabilize the manganese cluster in PS II. In spite of the predicted presence of many putative lipoproteins, thereis was no evidence to indicate whether they are real lipoproteins that are modified with lipids. Recently, we developed a method for analysis of lipoproteins by mass spectrometry (Ujihara et al., 2008). By this method we examined the lipid modification of PsbQ and Psb27 of *Synechocystis* sp. PCC 6803 and found that a sulfhydryl and an amino group of PsbQ are modified with a diacylglycerol and a palmitic acid moiety, respectively, whereas the corresponding sulfhydryl group of Psb27 is modified with diacylglycerol but the amino group is only partially modified with a palmitic acid moiety (T. Ujihara, K. Katayama, I. Sakurai, N. Mizusawa and H. Wada, unpublished). Nowaczyk et al. (2006) analyzed Psb27 of *T. elongatus* and found that it is modified with a diacylglycerol and a palmitic acid moiety, suggesting that lipid modification of Psb27 in *T. elongatus* might be different from that in *Synechocystis* sp. PCC 6803. In *Synechocystis* sp. PCC 6803, the three proteins PsbP, PsbQ and Psb27 are not essential for growth, but they are necessary for regulation of PS II activity (Kashino et al., 2006; Roose and Pakrasi, 2008; Sveshnikov et al., 2007; Thornton et al., 2004).

The presence of lipoproteins in cyanobacteria suggests that PG might be required for modification of lipoproteins, as reported in other bacteria, and that lack of PG might inhibit the processing of precursors to lipoproteins and induce damage that ultimately leads to cell death. We recently identified three genes in *Synechocystis* sp. PCC 6803, namely, *lgt*, *lspA* and *lnt*, which encode prolipoprotein diacylglyceryltransferase, signal peptidase II, and apolipoprotein N-acyltransferase, respectively. These enzymes are required for the modification of lipoproteins, and we have been trying to disrupt these genes. Although we have been able to disrupt the *lnt* gene, we have failed to obtain mutants with disrupted *lgt* and *lspA* genes (T. Ujihara, K. Katayama, I. Sakurai, N. Mizusawa and H. Wada, unpublished). Thus, it is likely that *lgt* and *lspA* are essential for the growth of *Synechocystis* sp. PCC 6803. This finding is consistent with the observation that lack of PG inhibits the growth of *Synechocystis* sp. PCC 6803 under both photosynthesis-dependent and -independent growth conditions, as observed in the *pgsA* mutant. It seems likely that precursors to lipoproteins are not processed in *pgsA* mutant cells because of a lack of PG and, thus, some functional lipoproteins that are essential for growth of *Synechocystis* sp. PCC 6803 are not generated. Further analysis of lipoproteins might allow us to identify the functions of PG in non-photosynthetic processes.

In *E. coli*, PG is involved in DNA replication and in the SecA-dependent translocation of proteins across inner membranes (Shibuya, 1992; Dowhan, 1997). A requirement for PG in such processes has not been studied in cyanobacteria or in higher plants. Studies focused on such processes are necessary if we are fully to understand the importance of PG in non-photosynthetic processes.

VI Conclusions and Future Perspectives

Phosphatidylglycerol is found in almost all organisms and is essential for oxygenic photosynthesis in higher plants and cyanobacteria. In cyanobacteria, PG plays an important role in (i) trimerization of the PS I complex; (ii) structural integrity of the Q_B-binding site of the D1 protein in the PS II complex; (iii) the binding of extrinsic proteins, which stabilize the manganese cluster in the PS II complex; and (iv) modification of lipoproteins. In higher plants, PG is required for (i) trimerization of LHCII; (ii) the development of thylakoid membranes in chloroplasts; and (iii) biosynthesis of CL in mitochondria as a precursor.

X-ray crystallographic analysis has identified PG and other lipid molecules in the PS I, PS II, and LHCII complexes. However, it seems likely that these complexes bind larger numbers of lipid molecules than those identified in the crystal structures. It is possible that x-ray crystallographic analysis at higher resolution will reveal additional lipid molecules. The lipids in the complexes might play important roles in the folding of protein subunits and in the assembly of the complexes. Further analyses are necessary if we are to develop a full understanding of the contribution of lipids to the structures, functions, and assembly of these complexes in thylakoid membranes.

Almost all of the enzymes and corresponding genes required for the biosynthesis of PG in chloroplasts and cyanobacterial cells have been identified, and the basic pathway for the biosynthesis of PG has been characterized. However, it is very likely that the biosynthesis of PG and the desaturation of fatty acids bound to PG are regulated to maintain the correct levels of PG relative to levels of total lipids and, also, the specific extent of unsaturation of PG in thylakoid membranes. Such regulatory mechanisms remain to be identified.

The extent of unsaturation of PG is important for the development, growth and survival of plants at low temperatures. It is also important for the protection of the photosynthetic machinery against strong-light stress, in particular, at low temperatures. The repair of PS II after photodamage is a target of regulation and it is accelerated by unsaturated molecular species of PG. However, the repair of PS II involves many steps, such as synthesis of D1 de novo, processing of the carboxy-terminal extension of D1, and assembly of the manganese cluster. The specific steps in repair that are regulated by the unsaturation of PG, which is reflected by the physical state of PG in thylakoid membranes, remain to be characterized.

Since its discovery by Benson and Maruo, PG has been shown to be important in photosynthesis and in the tolerance to various forms of stress, such as strong light and chilling temperatures, of higher plants and cyanobacteria. Many interesting and important questions remain to be answered before we have a full understanding of all the roles, including the regulatory roles of PG in oxygenic photosynthesis.

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Chapter 12

The Role of Glycolipids in Photosynthesis

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Summary

A variety of sugars with different epimeric and anomeric configurations and linkages are incorporated into head groups of glycolipids. The occurrence of glycoglycerolipids, glycolipids containing sugar residues directly linked to diacylglycerol, is restricted to photosynthetic organisms and some bacteria. Thylakoid membranes of chloroplasts and cyanobacteria are characterized by a unique set of three glycoglycerolipids, that is, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG), and one phosphoglycerolipid, phosphatidylglycerol (PG). While glycoglycerolipids are the predominant lipids in thylakoids, DGDG and SQDG accumulate to even higher levels during phosphate deprivation. The strict correlation of galactolipids with oxygenic photosynthesis was previously taken as evidence for a specific role in photosynthetic light reactions. This was later confirmed by numerous studies on cyanobacteria, *Chlamydomonas* and plants. Mutants with decreased content of galactolipids are characterized by growth retardation, decreased chlorophyll content and reduced photosynthetic activity. DGDG is crucial for the structural integrity of the photosystem II (PS II) donor site, assembly of light harvesting complex II (LHCII) trimers and stability of PS I. MGDG plays an important role in the xanthophyll cycle activity, and the two galactolipids are integral constituents of different photosynthetic pigment–protein complexes. The analysis of transgenic plants accumulating alternative glycoglycerolipids showed that galactose is the preferred sugar in thylakoid lipids for photosynthesis. Analysis of mutants disrupted in SQDG biosynthesis revealed that SQDG is dispensable for photosynthesis in anoxygenic bacteria and in the cyanobacterium *Synechococcus*. In contrast, photoautotrophic growth of *Synechocystis*, another cyanobacterium, and of *Chlamydomonas*, depends on SQDG. SQDG is not essential for photosynthesis in *Arabidopsis* under optimal conditions. Under phosphate limitation, SQDG is important for photosynthetic activity because SQDG as an anionic lipid partially replaces PG. Anoxygenic photosynthetic bacteria contain a more diverse set of phospho- and glycoglycerolipids, but little information is available on the role of the glycoglycerolipids in photosynthesis. MGDG is an important constituent of the chlorosome monolayer, and a glucosylgalactosyldiacylglycerol is associ-

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ated with the *Rhodobacter* reaction center. Taken together, glycoglycerolipids in plants and bacteria not only establish the lipid matrix of thylakoids, but they also play an important role for the activity of photosynthetic pigment–protein complexes and replace phospholipids during phosphate deprivation.

I Introduction

Almost all life on earth depends on oxygenic photosynthesis of plants, eukaryotic algae and cyanobacteria. The characteristics of this type of photosynthesis are the use of H_2O as electron donor, the release of molecular oxygen and the involvement of two multimeric pigment–protein complexes designated photosystem I (PS I) and photosystem II (PS II). Anoxygenic photosynthesis is found in four different phyla of bacteria. The purple bacteria (*Proteobacteria*), which include the sulfur and the non-sulfur purple bacteria, are characterized by a simplified non-oxygen evolving reaction center of type II. A non-oxygen evolving type II reaction center is also found in the green filamentous bacteria (*Chloroflexi*), also known as green non-sulfur bacteria. The green sulfur bacteria (*Chlorobi*) contain a type I photosystem with a homodimeric reaction center. A similar homodimeric type I reaction center exists in the Gram-positive *Heliobacterium* (*Firmicutes*) (Ohoka, 2007). Anoxygenic bacteria use different sulfur compounds, H_2 and organic molecules as electron donors accompanied with the release of the corresponding oxidized compounds.

Photosynthesis is localized to intrinsic membrane systems that are known as thylakoids in plants and cyanobacteria. Although evolutionary not related, *Chlorobi* and most of the *Chloroflexi* bacteria form specialized compartments called chlorosomes, which harbor the main light-harvesting complex delimited by a unilayer membrane (Frigaard and Bryant, 2004; Hohmann-Marriott and Blankenship, 2007). The chlorosomes are attached to the cytoplasmic site of the plasma membrane where the reaction centers are located. In purple bacteria, the photosynthetic pigment–protein complexes are embedded into specialized domains of cytoplasmic membrane vesicles in a highly organized manner (Bahatyrova et al., 2004; Frese et al., 2004). The simple structure of the pigment–protein complex of *Heliobacterium* consisting of the reaction center and bacteriochlorophylls, which have antenna function, is integrated into the cytoplasmic membrane (Neerken and Amesz, 2001). The building blocks for the membranes are mainly glycerolipids, which provide the matrix for embedding the photosynthetic pigment–protein complexes. Phosphate-containing glycerolipids are abundant membrane lipids in all prokaryotic and eukaryotic organisms. The glycoglycerolipids (glycosyldiacylglycerols) are, in general, restricted to some bacteria (particularly Gram-positives) and to photosynthetic membranes of bacteria, plants and algae. Due to the widespread distribution of photosynthetic organisms in nature (including plants, photosynthetic bacteria, and algae), glycoglycerolipids represent the most abundant lipid class in the biosphere. Glycoglycerolipids, in general, contain one or two, rarely three or more sugar residues in their head group. The incorporation of sugars into lipids allows the formation of a great number of possible structures. The most common sugars found in glycoglycerolipids are glucose, galactose or mannose, or the charged sugar derivatives sulfoquinovose and glucuronic acid. The sugars are bound in α- or β-anomeric configuration, and in diglycosyldiacylglycerols or higher glycosylated glycerolipids, the glycosyl residues can form (1→2), (1→3), (1→4), or (1→6) linkages between each other. Despite the large variety of possible structures, only a small number of different glycoglycerolipids have been described in nature (for a review see: Hölzl and Dörmann, 2007).

II Glycoglycerolipids in Photosynthetic Membranes

Organisms carrying out oxygenic photosynthesis contain only a simple set of three glycoglycerolipids and one phosphoglycerolipid in their photosynthetic membranes, that is, two galactolipids, MGDG and DGDG, the sulfolipid SQDG, and the phosphoglycerolipid PG (Table 1). In thylakoids of plant chloroplasts, MGDG constitutes

Abbreviations: DAG – Diacylglycerol; DGDG – Digalactosyldiacylglycerol; GlcGDG – Glucosylgalactosyldiacylglycerol; LHCI – Light harvesting complex I; LHCII – Light harvesting complex II; MGDG – Monogalactosyldiacylglycerol; MGlcDG – Monoglucosyldiacylglycerol; NPQ – Non-photochemical quenching; PG – Phosphatidylglycerol; PS I – Photosystem I; PS II – Photosystem II; SQDG – Sulfoquinovosyldiacylglycerol; TGDG – Trigalactosyldiacylglycerol

about 50%, DGDG 30%, SQDG 5–10% and PG 10% of the membrane lipids. SQDG and PG are anionic at physiological pH. The lipid composition is more or less conserved between thylakoids of chloroplasts and cyanobacteria. The galactose residue in MGDG is always bound in β-anomeric configuration to the glycerol backbone (Fig. 1). The head group structure of DGDG consists of a terminal α-galactose (1→6)-linked to the inner β-galactose (Fig. 1). In contrast to this structure, some plants synthesize galactolipids containing two or more galactose moieties with all sugars bound in β-anomeric configuration, like trigalactosyldiacylglycerol (TGDG) in adzuki bean (Fig. 1) (Kojima et al., 1990). There are also reports on oligogalactolipids carrying three and more galactose residues with a mixture of α- and β-sugars (for a review see: Kelly and Dörmann, 2004). Oligogalactolipids are, in general, synthesized in only small proportions. TGDG was also described for some species of cyanobacteria (Zepke et al., 1978). The sulfoquinovose (6-deoxy-6-sulfoglucose) residue of SQDG represents a derivative of α-glucose (6-deoxy-α-glucose, α-quinovose) with a sulfonate group linked to the C6 carbon of the sugar (Fig. 1). While glycoglycerolipids accumulate in thylakoids, only a limited proportion of these lipids occur outside of photosynthetic membranes. During phosphate deprivation, synthesis of SQDG and DGDG is strongly increased (Table 1). Under phosphate starvation, DGDG is exported from plastids to extraplastidial membranes. Lipid export has not been observed for MGDG and SQDG whose occurrence in plant cells seems to be confined to plastidial membranes (Härtel et al., 2000; Gaude et al., 2004). However, MGDG and SQDG are found in few non-photosynthetic bacteria; DGDG with its typical structure described for plants and cyanobacteria was never reported to occur outside of organisms with oxygenic photosynthesis (for a review see: Hölzl and Dörmann, 2007).

The different species of anoxygenic bacteria show a broader spectrum of sugar headgroup structures compared to the simple set of glycoglycerolipids of chloroplasts and cyanobacteria, while in some species, glycoglycerolipids are completely absent (Hölzl and Dörmann, 2007). Reports on the quantitative distribution of the different glycoglycerolipids in anoxygenic bacteria are scarce (Table 1). Members of the green filamentous bacteria (*Chloroflexi*), such as *Chloroflexus aurantiacus*, contain MGDG. This organism synthesizes glucosylgalactosyldiacylglycerol (GlcGDG) with the outer glucose moiety in β-anomeric configuration (1→6)-linked to the inner β-galactose (Fig. 1) (Knudsen et al., 1982; Hölzl et al., 2005). MGDG was also described for some green sulfur bacteria (*Chlorobi*). The head group structures of further glycoglycerolipids occurring in *Chlorobi* were not resolved. SQDG was absent from all green bacteria investigated so far.

Purple bacteria vary considerably in their lipid composition. Some but not all species synthesize glycoglycerolipids. *Blastochloris viridis* (formerly *Rhodopseudomonas viridis*), a purple non-sulfur bacterium, contains MGDG in minor levels with identical head group structure as plant MGDG. It also synthesizes DGDG, however, with a terminal β-galactose (Fig. 1), which therefore differs from plant DGDG, and another charged glycoglycerolipid with α-glucuronic acid, but no SQDG (Linscheid et al., 1997). A further representative of the purple non-sulfur bacteria, *Rhodopseudomonas palustris,* has no glycoglycerolipids. Some members of *Rhodospirillales* and *Rhodobacter sphaeroides* (*Rhodobacterales*), which also belong to the purple non-sulfur bacteria, contain SQDG (Wood et al., 1965; Imhoff et al., 1982; Gage et al., 1992). Under phosphate starvation the accumulation of two further glycoglycerolipids was observed in *Rhodobacter*, which were described as a monoglycosyldiacylglycerol with unknown anomeric configuration and linkage, and a glucosylgalactosyldiacylglycerol, different from the *Chloroflexus* GlcGDG, with a terminal α-glucose (1→4)-linked to an inner β-galactose (Fig. 1, Table 1) (Benning et al., 1995). Members of *Chromatiaceae* (purple sulfur bacteria) contain SQDG and glycoglycerolipids with unknown structure, and members of *Ectothiorhodospiraceae* (purple sulfur bacteria) lack glycoglycerolipids (Steiner et al., 1969; Imhoff et al., 1982). Glycoglycerolipids are completely absent from the phototrophic Gram-positive *Heliobacterium* (Aase et al., 1994).

III Enzymes Involved in Glycoglycerolipid Biosynthesis

Although chloroplasts and cyanobacteria contain the same set of glycoglycerolipids they employ different pathways for the biosynthesis of MGDG

Table 1. Lipid compositions of different photosynthetic organisms grown under phosphate replete (+ P) or deficient (–P) conditions

Organism	Phosphate	MGDG	DGDG	GlcGDG	SQDG	PG	PE	PI	PC	DGTS[b]	OL
Arabidopsis wild type	+ P	48.9[a]	14.5	–[e]	1.6	8.6	6.7	–	19.7	–	–
(Kelly et al., 2003)	– P	45.5	26.3	–	4.4	8.9	4.0	–	10.8	–	–
Arabidopsis dgd1	+ P	45.8	1.3	–	1.9	10.4	13.4	–	27.2	–	–
(Kelly, et al., 2003)	– P	44.1	9.9	–	3.4	9.9	10.0	–	22.7	–	–
Chlamydomonas dw15.1	+ P	38.2	14.8	–	5.0	7.7	6.1	2.3	–	25.8[c]	–
(Riekhof et al., 2003)	– P	35.7	18.6	–	12.6	3.6	4.1	1.8	–	26.9[c]	–
Synechococcus	+ P	60.6	12.5	–	10.3	16.6	–	–	–	–	–
(Güler et al., 1996)	– P	42.5	28.0	–	22.3	7.2	–	–	–	–	–
Synechocystis	+ P	49.7	25.9	–	14.3	10.2	–	–	–	–	–
(Awai et al., 2007)	– P	38.8	30.2	–	20.2	10.8	–	–	–	–	–
Rhodobacter sphaeroides	+ P	–	–	1.2	2.2	22.8	39.6	–	27.7	1.1	5.5
(Benning et al., 1993;	– P	[d]	–	31.1	16.6	12.2	6.8	–	2.9	19.2	11.2
Benning et al., 1995)											

[a] Standard deviations ranged from less than 1 to ±10 %, depending on the study.
[b] DGTS, diacylglycerol *N*-trimethylhomoserine; GlcGDG, glucosylgalactosyldiacylglycerol; OL, ornithine lipid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine;
[c] DGTS and acylated SQDG were not separated in that study
[d] Monohexosyldiacylglycerol with unknown structure was detected under phosphate starvation (Benning et al., 1995)
[e] Dash indicates " not detectable " (i.e. below 1 % of lipids)

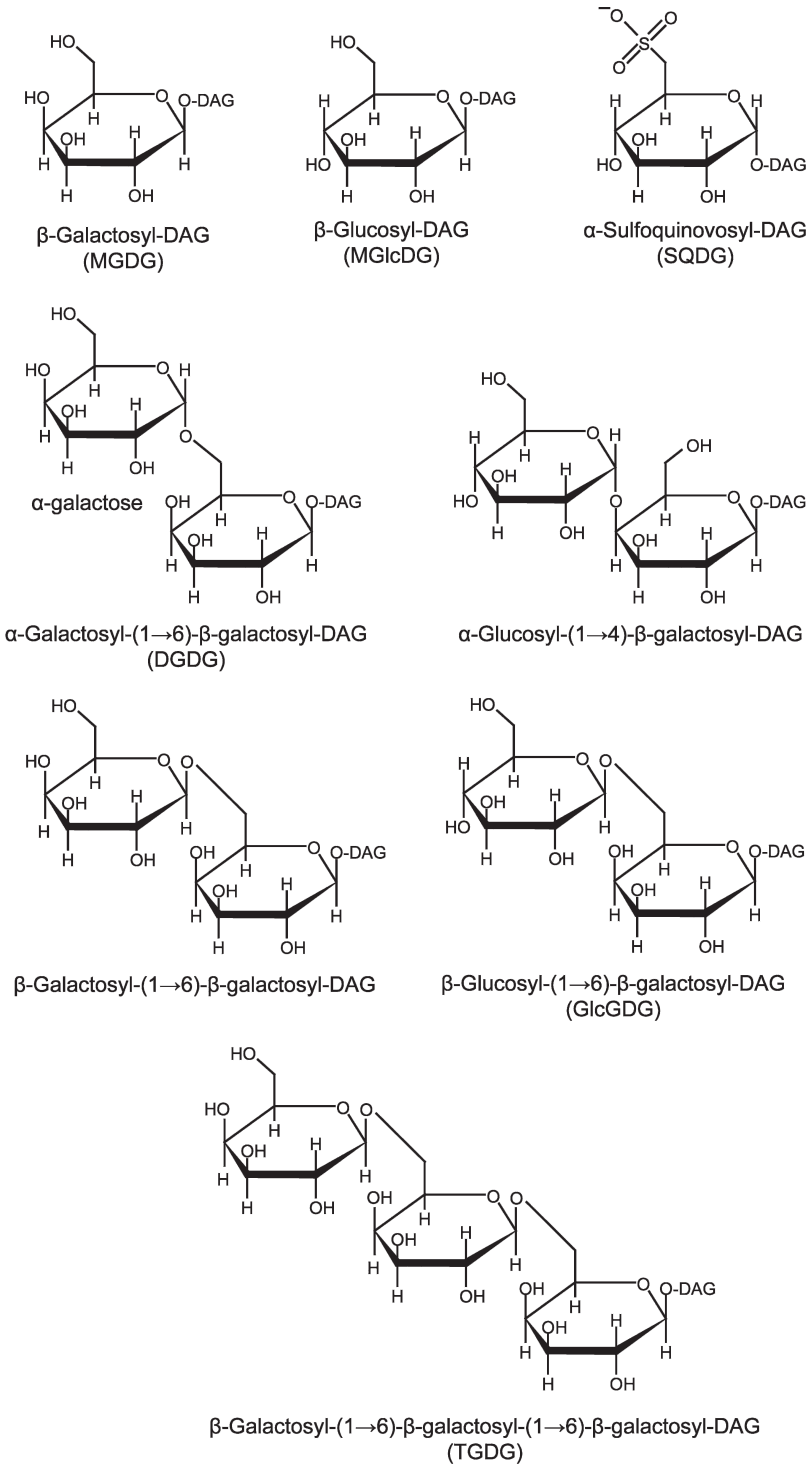

Fig. 1. Glycoglycerolipids in plants and photosynthetic bacteria. Plants, green algae and cyanobacteria contain a restricted set of three glycoglycerolipids, that is, monogalactosyldiacylglycerol (β-galactosyl-diacylglycerol, MGDG), digalactosyl-diacylglycerol (α-galactosyl-(1→6)-β-galactosyl-diacylglycerol, DGDG) and α-sulfoquinovosyldiacylglycerol (SQDG). β-Galactosyl-(1→6)-β-galactosyl-(1→6)-β-galactosyl-diacylglycerol (TGDG) is an oligogalactolipid found in some plant species in low levels. Additional glycoglycerolipids with different anomeric configurations and linkages occur in some bacterial species. DAG, diacylglycerol.

and DGDG. The organism investigated in greatest detail with respect to galactolipid biosynthesis is *Arabidopsis thaliana. Arabidopsis* contains three MGDG synthases with MGD1 as the most important activity (Miège et al., 1999; Awai et al., 2001). MGD2 and MGD3 play an important role under stress conditions, such as phosphate starvation, and they are expressed in specific tissues or at different developmental stages. All three enzymes are β-galactosyltransferases with UPD-galactose and diacylglycerol (DAG) as substrates. MGD1 is localized to the inner, MGD2 and MGD3 to the outer chloroplast envelope. *Arabidopsis* contains two DGDG synthases, DGD1 and DGD2 (Dörmann et al., 1999; Kelly et al., 2003). These enzymes are α-galactosyltransferases and transfer galactose from UDP-galactose onto MGDG. *DGD1* is expressed in all tissues, while expression of *DGD2* is usually very low. Expression of both genes is induced under phosphate starvation (Kelly and Dörmann, 2002; Kelly et al., 2003). The two enzymes are located in the outer envelope. The enzymes involved in the formation of oligogalactolipids, such as TGDG, have not been identified and the reaction mechanism remains obscure (van Besouw and Wintermans, 1978; Wintermans et al., 1981; Heemskerk et al., 1990; Xu et al., 2003). Oligogalactolipids are synthesized in a processive manner, that is, by sequential transfer of sugar residues by one enzyme. However, it is unclear whether the enzyme catalyzes a glycosyl hydrolyzation employing one MGDG molecule as donor and one as acceptor, or a glycosyl transfer reaction with UDP-galactose as sugar donor and DAG as acceptor.

Cyanobacteria harbor a different set of enzymes for galactolipid biosynthesis. In a first step, cyanobacteria synthesize monoglucosyldiacylglycerol (MGlcDG) in a UDP-glucose dependent manner with DAG as acceptor (Feige et al., 1980; Sato and Murata, 1982a, b; Awai et al., 2006). MGlcDG serves as an intermediate, which is subsequently converted to MGDG by an unknown epimerase. DGDG is formed by a cyanobacterial DGDG synthase (α-galactosyltransferase) with UDP-galactose and MGDG as substrates (Awai et al., 2007; Sakurai et al., 2007). As the corresponding genes have no orthologs in plants, their identification in cyanobacteria was difficult. The sequences for the MGlcDG and DGDG synthases were revealed by comparative genomic analysis.

In contrast to plants and cyanobacteria, only few studies have been devoted to the analysis of the synthesis of glycoglycerolipids in anoxygenic photosynthetic bacteria. The first bacterial MGDG synthase was cloned from *Chloroflexus aurantiacus* (Hölzl et al., 2005). This enzyme represents the bacterial counterpart to plant MGDG synthases sharing sequence homology and the same reaction mechanism. A second gene was isolated from *Chloroflexus*, which codes for a β-glucosyltransferase synthesizing GlcGDG by transfer of glucose from UDP-glucose onto MGDG (Hölzl et al., 2005). The sequence of this enzyme is homologous to MGDG synthases but not to DGDG synthases from plants. In *Blastochloris viridis*, MGDG is directly synthesized by galactosylation of DAG (Linscheid et al., 1997). Apart from *Chloroflexus*, the genes involved in glycoglycerolipid biosynthesis have not been identified in other anoxygenic photosynthetic bacteria.

The biosynthesis of the charged glycoglycerolipid SQDG requires at least two genes. In the first step, the donor UDP-sulfoquinovose is formed from sulfite and UDP-glucose (Sanda et al., 2001). The corresponding gene is conserved across the different kingdoms and is termed *sqdB* in bacteria or *SQD1* in plants (Benning and Somerville, 1992b; Essigmann et al., 1998; Weissenmayer et al., 2000; Aoki et al., 2004). The final step of sulfolipid synthesis is catalyzed by SQDG synthase, which is an α-glycosyltransferase transferring sulfoquinovose onto DAG. The genes in plants (*SQD2*) and cyanobacteria (*sqdX*) are homologous but show no similarity to other bacterial representatives (*sqdD*) (Rossak et al., 1995; Güler et al., 2000; Yu et al., 2002). Furthermore, bacteria contain additional genes (*sqdA* and *sqdC*), which are involved in SQDG biosynthesis, but their exact function remains unknown (Benning and Somerville, 1992a, b; Weissenmayer et al., 2000). SQDG synthase activity in plants is located to the inner chloroplast envelope (Tietje and Heinz, 1998).

IV The Role of MGDG, DGDG and SQDG in Oxygenic Photosynthesis

The galactolipids, MGDG and DGDG, are the predominant lipids in thylakoids of chloroplasts and cyanobacteria, but they are, in general, absent

from non-photosynthetic membranes. The sulfolipid SQDG represents a minor thylakoid lipid and its occurrence is not absolutely restricted to photosynthetic membranes. The strict correlation of galactolipids and, to a minor extent, of SQDG with oxygenic photosynthesis suggested that, besides of their function as bulk membrane lipids, these lipids provide more specific functions in photosynthesis. One of the bulk membrane functions of MGDG and DGDG is to maintain membrane fluidity in the thylakoid bilayer. These membranes are highly enriched in proteins, which are often organized into multi-subunit complexes. The membrane area occupied by proteins accounts for about 70%, the rest is constituted of lipids. Furthermore, it is assumed that 60% of the thylakoid lipids are bound to photosynthetic multi-subunit complexes where they build a shell around these structures. Moreover, lipids separate the complexes at their contact zones, and they fill gaps (Kirchhoff et al., 2002; Páli et al., 2003). Therefore, the characteristics of the thylakoid lipids are of high importance for the flexibility of the photosynthetic pigment–protein complexes. Consequently, a certain ratio of non-bilayer to bilayer forming lipids is crucial for a functional membrane. MGDG with its small head group forms non-bilayer or so-called Hex_{II} structures in aqueous environments, in contrast to DGDG (Israelachvili et al., 1980; Israelachvili, 1992). In this context, the anomeric configuration of the sugar head-group of the galactolipids may be of minor importance. Therefore, alternative glycoglycerolipids with similar physical characteristics can as well establish a functional membrane lipid bilayer. However, the existence of the additional epimerization step of converting MGlcDG to MGDG in cyanobacteria suggests that galactolipids with their typical head group structure are the preferred glycoglycerolipids in photosynthesis.

A Glycoglycerolipids in Photosynthetic Complexes

In the past, much effort has been devoted to the analysis of the role of glycerolipids in photosynthetic membranes. One of the difficulties encountered was the differentiation between lipid functions of establishing the lipid bilayer, and specific functions of lipids. Before different mutants depleted in galactolipids or SQDG, or high-resolution crystal structures of photosynthetic pigment–protein complexes were available, the investigations were restricted to in vitro experiments. These experiments comprised (i) reconstitution, crystallization and activity tests of pigment–protein complexes in the presence of different lipids, (ii) the removal of lipids or lipid classes from the thylakoid membranes by enzymatic digestion or with cyclodextrins, or (iii) the use of specific antibodies against lipids (see Siegenthaler and Murata, 1998). Data obtained from these experiments together with new insights derived from crystal structure analysis of photosynthetic pigment–protein complexes (see Chapter 10), suggest that thylakoid lipids play both structural (bulk membrane lipid) and functional (specific) roles.

Protection of the photosynthetic machinery against photo-damage caused by excess light is based on non-photochemical quenching (NPQ), which includes the activity of the xanthophyll cycle. The xanthophyll cycle describes a mechanism of de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin at high light to protect the photosynthetic machinery against reactive oxygen species. At low light this reaction is reversed. MGDG was shown to play an important role in the xanthophyll cycle activity. The enzyme involved, violaxanthin de-epoxidase, requires the presence of MGDG for its activity (Morosinotto et al., 2002; Latowski et al., 2004). The requirement for MGDG arises from its nature as a non-bilayer lipid rather than from the presence of a specific sugar head group. Therefore, MGDG can be replaced by the non-bilayer lipid phosphatidylethanolamine for stimulation of violaxanthin de-epoxidase activity.

Interactions between lipids and LHCII in plants are well documented. This complex constitutes about 30% of proteins in thylakoids and, therefore, is the most abundant integral membrane protein in nature. The main function of LHCII is to collect light and to transfer the excitation energy to PS II and PS I. The functional unity of LHCII is the trimer. It has been known for a long time that the two lipids PG and DGDG play an important role for the activity and stability of LHCII. PG is necessary for LHCII trimerization. DGDG is required for in vitro formation of two- and three-dimensional crystals of LHCII

(Nussberger et al., 1993). In contrast to PG, which is tightly bound to LHCII trimers, DGDG can be easily removed with mild detergents. The two lipids have different structural functions and it is concluded that several binding sites exist for DGDG at the periphery of LHCII. These conclusions are corroborated by insights obtained from the analysis of LHCII crystal structures from spinach and pea with 2.72 and 2.5 Å resolution, respectively (Liu et al., 2004; Standfuss et al., 2005). In spinach LHCII crystals, two molecules of DGDG mediate the contact between two adjacent trimers where the DGDG head groups are hydrogen-bonded to amino acids of the trimers. Based on these observations, a model for NPQ was suggested with DGDG mediating the escape of excitation energy from one to the adjacent trimer (Liu et al., 2004). Further data derived from LCHII reconstitution experiments in MGDG- and DGDG-containing liposomes revealed that these lipids are crucial for quenching of chlorophyll fluorescence (Moya et al., 2001). In contrast to spinach, three molecules of DGDG occupy a cave on the threefold axis at the luminal site of each trimer in pea LHCII (Standfuss et al., 2005). The differences in DGDG association with LCHII may be due to the crystallization characteristics because spinach LHCII is crystallized as icosahedral proteoliposome, and pea LHCII as stacked two-dimensional crystals with an alternating up-down orientation of the trimers. Here, the trimers have hydrophobic contact sites without binding of DGDG (Standfuss et al., 2005). In further in vitro experiments, the influence of thylakoid lipids on association kinetics and thermal stability of LHCII was investigated. DGDG, PG and MGDG show a positive effect on folding, assembly and stability of reconstituted recombinant LHCII towards thermal denaturation. The positive effect of lipids on the stability of LHCII may be due to physical differences caused by the presence or absence of lipids in detergent micelles rather than to specific lipid–protein interactions (Reinsberg et al., 2000).

In the last few years, a series of crystal structures of PS II has been published, one of the latest from the cyanobacterium *Thermosynechococcus elongatus* with a resolution of 3.0 Å, which was sufficient to detect lipids bound within this complex (Loll et al., 2005; Jones, 2007). The integral lipids comprise six molecules of MGDG, four DGDG, three SQDG and one PG per monomer. Eleven of these lipids are located around the D1/D2 reaction center separating it from the antenna and smaller subunits. One molecule of MGDG and two molecules of SQDG are found at the interface between two PS II monomers. This high number of lipids suggests an important structural role for assembly and flexibility of polypeptides and cofactors within PS II, which might be required for local mobility, especially of D1 proteins, during photodamage. MGDG and DGDG head group interactions were observed with chlorophylls and amino acids of PS II (Gabashvili et al., 1998); for more details see the review by Müh et al. (2008).

In analogy with the cyanobacterial PS II complexes, it is likely that lipids play structural and functional roles in PS II of plants as well. Previous biochemical studies support this assumption. Core fractions of PS II complexes isolated from maize are enriched in MGDG, and the individual protein components of PS II complexes are characterized by a heterogeneous lipid distribution (Trémolières et al., 1994). The entire complex contains all four thylakoid lipids. However, in highly purified antenna chlorophyll–protein complexes (CP43 and CP47), only MGDG with a remarkably high proportion of saturated fatty acids was present. This highly saturated MGDG supposedly forms a rigid lipid matrix involved in protein embedding into the membrane. Such an enrichment of saturated MGDG in the PS II core was also reported by Murata et al. (1990). DGDG and PG were largely lost during the purification process indicating a less tight association. A tenfold higher enrichment of lipids especially of highly unsaturated galactolipids compared to the other complexes was found in the chlorophyll–protein complexes CP24, CP26 and CP29. This lipid-rich environment may influence the binding properties between antenna and PS II core (Trémolières et al., 1994). Although SQDG was also detected in PS II, it was not further considered because of its low proportion. The ratio of lipid molecules per CP24, CP26 or CP29 polypeptide was estimated with 15–25 molecules of MGDG, 3–10 DGDG, 1 PG and 1 SQDG. The antenna complexes CP43 and CP47 contain 10–15 MGDG. The total ratio of lipid molecules per polypeptide of the PS II reaction center was estimated to be relatively low, but the whole PS II core may contain about ten galactolipid molecules

(Trémolières et al., 1994). Other PS II complexes isolated from the two cyanobacteria *Thermosynechococcus vulcanus* und *Synechocystis* and from the higher plants spinach and rice also showed a different lipid composition compared to thylakoid membranes (Sakurai et al., 2006). The PS II dimers from each organism contained 27, 20, eight, and seven lipid molecules, respectively. The lower number of lipids in cyanobacteria may be due to the different structural organizations of PS II in plants compared to cyanobacteria, which use phycobilisomes instead of LHCII for light harvesting. The highly purified complexes from cyanobacteria were characterized by an enrichment of PG. The lower content of lipids in these complexes compared to preparations as published by Kruse et al. (2000) did not affect PS II activity, indicating that the subset of lipids bound to PS II may be sufficient (Sakurai et al., 2006).

A different structural arrangement of lipids was also observed for preparations of PS I from tobacco, spinach and two different species of cyanobacteria (Makewicz et al., 1996). The functional unit of cyanobacterial PS I is the trimer, whereas the plant PS I exists as a monomer. Employing polyclonal, monospecific antisera raised against glyco- and phosphoglycerolipids, MGDG and PG were detected in PS I core complexes of all species investigated. A specific immunoreaction against MGDG was also observed for light-harvesting complex I (LHCI) of plants, but not of cyanobacteria, because cyanobacteria lack LHCI. More details on the functional role of lipids were obtained from the cyanobacterial PS I crystal structure resolved at 2.5 Å (Jordan et al., 2001). The complex contains four integral lipids, three molecules of PG and one MGDG. One PG and one MGDG are arranged close to the core peptides in the vicinity of the electron transfer cofactors. PG is found near the phylloquinone of the A-branch of the electron transfer chain, and MGDG near the phylloquinone of the B-branch. The two lipids may be involved in determining the different electron transfer rates of the two symmetric branches (Fyfe and Jones, 2005). The plant PS I crystal structure with 4.4 Å resolution does not allow modeling of lipids into specific binding sites (Ben-Shem et al., 2003). As plants use LHCI and LHCII for light capturing, the two complexes can interact with PS I in contrast to cyanobacteria, which lack LHCI and LHCII. Therefore, structural differences can be expected between PS I complexes from plants and cyanobacteria.

The sulfolipid SQDG and two molecules of a glycoglycerolipid (probably MGDG) were found in the 3.1 Å crystal structure of the cytochrome b_6f complex from *Chlamydomonas* (Stroebel et al., 2003). The lipid head groups may undergo specific interactions with amino acids of the complex. For reconstitution experiments of spinach cytochrome b_6f activity, DGDG and phospholipids, but not MGDG or SQDG are required (Chain, 1985). MGDG seems to be important for ATPase activity, as deduced from reconstitution experiments of ATPase in liposomes with different lipid environments (van Walraven et al., 1984). MGDG also shows a stimulatory effect on the incorporation of cytochrome *c* into liposomes prepared of thylakoid lipids, and on its redox chemistry (Kruk et al., 2003). In both cases, the requirement for MGDG is not based on its specific headgroup, but on its non-bilayer forming characteristics.

B Galactolipid Mutants

Identification and characterization of galactolipid-deficient mutants of plants and cyanobacteria provided new tools to investigate the in vivo roles of galactolipids in photosynthesis. For a long time, the only mutant available with reduction in MGDG content was an *Arabidopsis mgd1* mutant allele carrying a T-DNA insertion 5′ of the transcription initiation site (Jarvis et al., 2000). The *mgd1* plants are characterized by a 42% reduction of MGDG, by reduced chlorophyll content accompanied with a pale green leaf color, and by an altered chloroplast ultrastructure. It was concluded that the remaining MGDG-synthesis activity of this plant is derived from a “leaky” inactivation of the *MGD1* gene. The capacity for thermal dissipation of excess light (qE) was impaired in leaves of *mgd1*, due to an inefficient operation of the xanthophyll cycle. This is attributable to a reduced proton motive force caused by decreased conductivity of the thylakoids. Therefore, the thylakoid lumen is less acidic and the pH-dependent activation of the violaxanthin de-epoxidase and of the PsbS protein is impaired (Aronsson et al., 2008). The recently discovered *mgd1* mutant allele harboring a T-DNA insertion in the first exon of the *MGD1* gene is characterized

by a complete loss of MGDG and DGDG (Kobayashi et al., 2007). This plant is described as small albino with disrupted photosynthetic membranes. It lacks photosynthetic proteins, such as D1, D2 or LHCII, has no photosynthetic activity and is not capable of photoautotrophic growth. Mutants of MGDG biosynthesis in cyanobacteria have not yet been described.

The galactolipid mutant studied in greatest detail is *dgd1* (Fig. 2), which is completely devoid of DGD1 activity accompanied by a 90% reduction of DGDG (Table 1; Dörmann et al., 1995, 1999). Mutant plants of *dgd1* show a dwarf growth phenotype, an alteration of chloroplast ultrastructure, pale green leaf color caused by reduced chlorophyll content, and impairment in photosynthesis (a decreased stability of LCHII trimers, a decreased competence to adjust the photosynthetic apparatus to high-light stress, a reduced photosynthetic PS II quantum yield and changes in PS I stability and activity). Another *Arabidopsis* mutant, *dgd2* (Fig. 2), carrying a T-DNA insertion in the *DGD2* gene, contains similar levels of DGDG as wild type and thus shows no growth defect. The *dgd1 dgd2* double mutant (Fig. 2) is completely devoid of DGDG and shows more severe growth retardation as compared to *dgd1* with a strongly impaired capability for photoautotrophic growth (Kelly et al., 2003). Further investigations of the *dgd1* and *dgd1 dgd2* mutants revealed more details on the structural and functional relevance of DGDG in photosynthesis. The results indicate a specific role of this lipid for PS II. The loss of DGDG led to a changed stoichiometry of the photosynthetic pigment–protein complexes with a 60% reduction in the PS II to PS I ratio and to increased levels of LCHII relative to PS II and the inner antenna complexes (Härtel et al., 1997). DGDG is important for the structural and functional integrity of the PS II donor site, especially for the water-oxidizing complex, but it has no influence on the acceptor (Q_B binding) site (Reifarth et al., 1997; Steffen et al., 2005). A major fraction of DGDG was shown to serve as a bulk membrane lipid, but a certain level of DGDG, which presumably binds to PS II and LHCII (see above), is of functional importance during photosynthesis. DGDG deficiency also affects activity and stability of PS I. A subcomplex of PS I was detected in *dgd1* that was devoid of several subunits (Guo et al., 2005). The in vitro data indicated that DGDG is indispensable for the stability of PS I. Further in vivo analyses revealed an impairment of the intersystem electron-transport chain. The limitation is located to the acceptor site of PS I and caused by a reduced abundance of PS I or associated subunits (Ivanov et al., 2006). The altered ratio of MGDG to DGDG presumably impairs the bilayer membranes especially at the grana margins, which compromises assembly and insertion of PS I at these sites.

A recent study of *dgd1* acclimation to low temperatures revealed surprising results (Hendrickson et al., 2006). The *dgd1* plants showed a recovery of photosynthetic CO_2 uptake, of PS II and PS I photochemistry, and an increase of pigments to levels similar to that of wild-type cells after exposure to low temperature. The

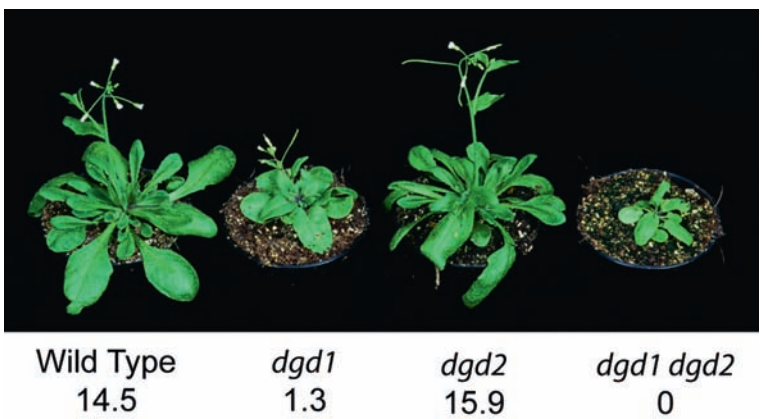

Fig. 2. Galactolipid deficient mutants of *Arabidopsis thaliana*. The lines *dgd1* and *dgd2* and the double mutant *dgd1 dgd2* carrying mutations in the *DGD1* and/or *DGD2* genes show different degrees of galactolipid reduction and growth retardation. The numbers represent the levels of DGDG in mole percent of total leaf lipids (See Color Fig. 14 on Color Plate 13).

cold treatment had no influence on the DGDG content in *dgd1* mutant cells. A shift to normal temperatures resulted in the re-establishment of the photosynthesis-deficient phenotype and in the decrease in CO_2 uptake. As the bilayer characteristics of membrane lipids are not only based on their chemical properties but also depend on temperature, a shift to lower temperature reduces the curvature stress particularly at the grana margins with a positive effect on PS I insertion into the membrane. These results suggest that the alterations of photosynthesis caused by DGDG deficiency result to a large extent from an impairment of global physical membrane properties. The cold treatment experiments were done with *dgd1* mutant plants still containing a residual content of DGDG. These experiments suggest that a major proportion of DGDG plays a role in maintaining the physical properties of photosynthetic membranes. As already indicated above, only a small number of DGDG molecules specifically interact with photosynthetic complexes. The residual content of DGDG in *dgd1* might be sufficient to sustain photosynthesis in the cold. This active role of a minor proportion of DGDG in photosynthesis cannot be observed under elevated temperatures due to the impairment of global physical membrane functions which affect photosynthesis in *dgd1*.

The recent isolation of the cyanobacterial gene for DGDG synthase allowed the generation of the DGDG-free mutant Δ*dgdA* of *Synechocystis*. Growth under optimal conditions was similar to that of wild type but photosynthesis was impaired (Awai et al., 2007; Sakurai et al., 2007). DGDG is important for the donor site of PS II in cyanobacteria similarly as observed for plants (Sakurai et al., 2007). The presence of DGDG facilitates the binding of extrinsic proteins, which are involved in stabilization of the oxygen evolving complex. In the Δ*dgdA* mutant, the loss of DGDG (20%) is mostly compensated with an accumulation of MGDG. Therefore, stability and activity of PS II in the Δ*dgdA* mutant might not only be affected by loss of direct interactions between DGDG and PS II, but could also originate from strongly altered membrane properties in the cells.

Galactolipid deficiency in mutants results in the impairment of global membrane functions. Therefore, it is difficult to distinguish between bulk membrane and specific functions of galactolipids in such a mutant background. An alternative approach is the replacement of the galactolipids removed in the mutant with surrogate lipids. This approach was followed by expression of a bacterial glucosyltransferase in the *Arabidopsis dgd1* mutant leading to the replacement of a major proportion of DGDG by GlcGDG in the transformed plants (Hölzl et al., 2006). The transformants grew like wild type indicating that a large proportion of DGDG serves as bulk membrane lipid. However, the results further showed that chlorophyll deficiency and the decrease in PS II quantum yield were only partially restored. Therefore galactolipids represent the preferred lipid class and play a specific role in oxygenic photosynthesis.

C Sulfolipid Mutants

A number of SQDG-deficient mutants of cyanobacteria and higher plants are available. Comprehensive studies were done with the alga *Chlamydomonas reinhardtii*. An SQDG-deficient mutant (*hf-2)* was generated by ultraviolet radiation (Sato et al., 1995). The cells show reduced growth, altered chlorophyll fluorescence and a 30–40% reduction in PS II activity monitored by oxygen evolution. Chloroplast ultrastructure is changed with highly curved thylakoid membranes. Another mutant of *Chlamydomonas* designated Δ*sqd1* was generated by insertional mutagenesis of the *sqd1* gene, an ortholog of *SQD1* from *Arabidopsis*. However, a second gene adjacent to *sqd1* was also affected in Δ*sqd1,* rendering the interpretation of the mutant phenotype difficult (Riekhof et al., 2003). The two mutants, *hf-2* and Δ*sqd1* are devoid of SQDG. In addition to SQDG deficiency, Δ*sqd1* also lacks an SQDG derivative with an acyl chain esterified to the C2 position of the sugar. This acylated sulfolipid was for the first time described in this work and also occurs in wild-type cells of *Chlamydomonas* in minor levels (Riekhof et al., 2003). As SQDG accounts for about 7% of total lipids (Sato et al., 1995) it is much less abundant than DGDG and, therefore, the effects observed in SQDG-deficient mutants may be rather specific in contrast to DGDG-deficient mutants. SQDG seems to be an important lipid under phosphate-limited conditions. As shown for many other organisms, *Chlamydomonas* replaces

a fraction of its phospholipids with SQDG and DGDG (Table 1). SQDG deficiency in the mutants leads to growth retardation (Riekhof et al., 2003). *Chlamydomonas* is also able to use SQDG as internal sulfur source under sulfur starvation (Sugimoto et al., 2007). SQDG was shown to be of structural and functional importance for PS II in *Chlamydomonas*. The treatment with various inhibitors of the acceptor site in PS II (herbicides) revealed an important structural role of SQDG for the Q_B binding site (Riekhof et al., 2003; Sato et al., 2003). The varying sensitivity of the mutants to different herbicides indicated that the Q_B binding site of PS II undergoes a conformational change. Measurements of PS II activity employing different benzoquinones as electron acceptors also revealed structural changes in the Q_B site of SQDG-deficient mutants. PS II activities ranged from wild type levels to a reduction to 50–60% in the mutant (Minoda et al., 2003). Detergent solubilization of photosynthetic complexes revealed that PS II from the SQDG-deficient *hf-2* mutant is less stable indicating an important structural role of SQDG for PS II (Sato et al., 2003). Electron flow from Q_A to Q_B was not disturbed in *hf-2*, and PS II quantum yield was similar to wild type demonstrating that PS II itself was not affected (Minoda et al., 2003). SQDG also plays a role in maintaining a correct conformation at the PS II donor site (Minoda et al., 2003). SQDG mutants show a decreased oxygen evolution rate that can be explained by a defect in the oxygen-evolving complex leading to a reduced electron flow from the manganese cluster to tyrosine Z. Supplementation of *hf-2* with sulfolipid in the growth medium restored the oxygen-evolving capacity.

A further physiological function of SQDG might be to confer heat tolerance to PS II (Sato et al., 2003). PS II complexes are more stable and active at high temperatures in the presence of SQDG in wild-type cells of *Chlamydomonas* than the *hf-2* mutant cells. Activity of PS II complexes that were heat-inactivated in darkness can be recovered by light exposure, even at elevated temperatures. This light-dependent recovery of PS II is strongly affected in the *hf-2* mutant indicating that SQDG supports the repair of PS II at high temperatures.

Complementation experiments were done with different SQDG derivatives modified at their head groups (Minoda et al., 2002). Authentic SQDG was most active in complementing PS II activity while methylated or oxidized SQDG forms were less efficient. These results imply a specific interaction of SQDG with PS II with the requirement of the authentic SQDG head group structure to maintain maximal PS II activity.

Sulfolipid does not provide an essential function for the photosynthesis of higher plants as shown in experiments with the two *Arabidopsis* mutants *sqd1* and *sqd2*. The down-regulation of *SQD1* activity in *sqd1* was achieved by antisense expression leading to a 30% reduction of SQDG (Essigmann et al., 1998). The complete elimination of SQDG biosynthesis was achieved by isolation of the *sqd2* mutant carrying a T-DNA insertion in the *SQD2* gene (Yu et al., 2002). This mutant with its complete loss of SQDG represents an excellent tool to study SQDG functions in photosynthesis of higher plants. Under optimal conditions SQDG is not important for growth, and the pigment contents of *sqd1* and *sqd2* plants are not affected. The 30% reduction of sulfolipid in *sqd1* has no influence on photosynthetic performance (Essigmann et al., 1998). The complete loss of SQDG in *sqd2* led to a slight reduction in effective PS II quantum yield (Yu et al., 2002).

Nevertheless, SQDG plays an important role in the adaptive mechanism of higher plants to phosphate deficiency. Under such conditions expression of *SQD1* and *SQD2* is upregulated and SQDG content is increased (Table 1; Essigmann et al., 1998; Yu et al., 2002). The level of PG in wild-type plants is reduced but remains constant in the *SQD2* mutant during phosphate deprivation. It is difficult to assess the role of SQDG in photosynthesis under phosphate limitation because phosphate deficiency causes pleiotropic effects on plant growth and physiology (including photosynthesis) that might not be directly attributable to membrane lipid changes. Furthermore, SQDG was also shown to be necessary to maintain a certain content of charged lipids in the thylakoid membranes, which is essential for growth and photosynthesis (Yu and Benning, 2003).

The importance of SQDG in cyanobacteria is species-dependent, and the proportion of this lipid varies considerably between different species. Proportions of SQDG with up to 66% are found in *Prochlorococcus*; on the other hand *Gloeobacter* completely lacks sulfolipids (van

Mooy et al., 2006). SQDG is essential for growth and photosynthetic activity in *Synechocystis*, whereas in *Synechococcus* it seems to play no discernible role. The mutants investigated so far were created by insertional mutagenesis of the respective *sqdB* gene (Güler et al., 1996; Aoki et al., 2004). The *Synechocystis* mutant designated *SD1* lost its ability to synthesize SQDG and requires supplementation of sulfolipid in the medium, which is incorporated into the thylakoid membranes. Transfer to SQDG-free medium leads to a reduction of internal SQDG content accompanied with a changed lipid composition, a strong impairment of growth and severely altered photosynthetic characteristics. SQDG, which constitutes 30% in wild type thylakoids, is decreased to 2% in *SD1*, whereas PG is increased from 8% to 48%. Therefore, the increase in PG cannot complement for the loss of the sulfolipid. The reduction of SQDG particularly affects the Q_B binding site and the oxygen-evolving capacity of PS II (Aoki et al., 2004). Transfer to SQDG-replete medium shows the reverse effect, demonstrating the specific involvement of SQDG in photosynthesis of *Synechocystis*. The loss of SQDG in the *Synechococcus sqdB* mutant has no influence on growth under optimal conditions (Güler et al., 1996; Aoki et al., 2004). Furthermore, photosynthetic activity and oxygen evolving capacity are nearly identical to those of wild-type cells. The SQDG content in wild-type cells of *Synechococcus* is about 10%. In the mutant, SQDG is replaced by the accumulation of similar levels of PG. Under phosphate starvation, PG is replaced with SQDG, which increases up to 22% (Table 1). The mutant is not able to respond to phosphate starvation in a similar manner and keeps the level of PG at a high level. It enters into a phosphate-depleted stage sooner and thus reaches the stationary phase earlier than the wild type. Therefore, PG and SQDG are mutually replaceable in *Synechococcus*.

V The Role of Glycoglycerolipids in Anoxygenic Photosynthesis

The bacteria investigated in greatest detail, with respect to the function of lipids in anoxygenic photosynthesis, are *Rhodobacter sphaeroides* and *Blastochloris viridis*. Most of the investigations are targeted to crystallization and analysis of bacterial reaction centers. No intrinsic lipids were found in crystal structures of the reaction center from *Blastochloris* (Jones, 2007). Several lipid structures were resolved in crystallized reaction centers of *Rhodobacter*, that is, two phospholipids (cardiolipin and phosphatidylcholine) and glucosylgalactosyldiacylglycerol (McAuley et al., 1999; Camara-Artigas et al., 2002). Different roles were ascribed to the three lipids. The reaction center of *Rhodobacter* contains bacteriochlorophylls, bacteriopheophytins, and ubiquinones as cofactors which are arranged in two membrane-spanning electron transfer branches (Jones et al., 2002). It is typical for the *Rhodobacter* reaction center that one branch is active and one is inactive. The glycoglycerolipid was modeled near the active branch with the disaccharide moiety near the bacteriochlorophyll monomer; phosphatidylcholine was found close to the inactive branch. The asymmetric distribution of the two lipids suggested that they contribute to the differences in energetics of the two branches. Furthermore, there are indications for the existence of binding sites for these two lipids as revealed by investigations of crystallized reaction centers from mutant proteins with altered binding sites for cardiolipin (Fyfe et al., 2004; McAuley et al., 1999). The less stable mutant reaction centers lacked phosphatidylcholine and the glucosylgalactosyldiacylglycerol due to a conformational change.

Apart from the comprehensive data derived from crystal structures, only few reports on the investigation of glycoglycerolipid function in anoxygenic photosynthesis exist. MGDG seems to be important for chlorosome biogenesis in green bacteria. In vitro experiments showed that this galactolipid is required for bacteriochlorophyll c association to photosynthetic complexes by hydrophobic interactions. Bacteriochlorophyll c is an important constituent of chlorosomes. The presence of MGDG in reconstitution experiments in vitro has an influence on the shape and size of the complexes, and the absorption spectra of MGDG-containing complexes are similar to those in chlorosomes. The following model was proposed for chlorosome biogenesis (Hohmann-Marriott and Blankenship, 2007). In this model chlorosomes are regarded as specialized lipid bodies with the unilayer membrane derived from the cytoplasmic leaflet of the cytoplasm membrane.

Galactolipids show a certain affinity to the other compounds of the chlorosome leading to an enrichment of these lipids in the chlorosome membrane, which, therefore, mainly consists of MGDG (Frigaard and Bryant, 2004).

Mutants of genes involved in glycoglycerolipid synthesis in anoxygenic bacteria were only described for *Rhodobacter*. Disruption of the *sqdB* gene resulted in the complete loss of SQDG (Benning et al., 1993). Under optimal growth conditions this lipid accounts for only about 2%. The mutation had no further effect on growth and photosynthetic activity. But SQDG is important under phosphate starvation when SQDG accumulates to levels of up to 16% in the wild type (Table 1). The mutant lost the capacity to accumulate SQDG under phosphate deficiency and was characterized by reduced growth.

VI Conclusions

Photosynthesis requires an extensive protein-rich membrane system. Non-photosynthetic membranes are predominantly composed of phosphoglycerolipids. Many photosynthetic organisms, particularly plants and cyanobacteria, use glycoglycerolipids to cope with the high demand of lipids required for establishing their photosynthetic membranes. A reason why plants and photosynthetic bacteria prefer glycoglycerolipids might be based on the fact that sugars are metabolically less expensive compared to phosphate, which has to be taken up from the soil. This renders photosynthetic organisms less dependent on phosphate availability. Only a restricted set of glycoglycerolipids, (MGDG, DGDG, SQDG, and a few other glycoglycerolipids in anoxygenic bacteria) are found in photosynthetic organisms. Two of the lipids, DGDG and SQDG, accumulate to even higher levels and replace phospholipids under conditions of phosphate deprivation. A predominant number of the glycoglycerolipids in photosynthetic membranes are required to establish the matrix of the bilayer, but a small proportion is of structural and functional importance for photosynthesis. *Arabidopsis* mutant analysis revealed that MGDG and DGDG are important for the stability and activity of various photosynthetic pigment protein complexes. One of the main functions of MGDG refers to its non-bilayer forming properties, for example, for activation of xanthophyll cycle activity. Replacement of galactolipids in *Arabidopsis* mutants with alternative glycoglycerolipids revealed that galactose is the preferred sugar in lipids of thylakoid membranes. The role of SQDG is species-dependent. In *Chlamydomonas,* the sulfolipid is involved in maintaining structural stability of PS II donor and acceptor sites and of the cytochrome b_6f complex. SQDG is also essential for *Synechocystis*. For *Synechococcus*, *Rhodobacter* and higher plants, this lipid seems to play a minor role in photosynthesis. Some photosynthetic bacteria lack sulfolipids. Glycoglycerolipids also seem to be important for anoxygenic bacteria for chlorosome assembly and specific interactions with photosynthetic reaction centers.

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Chapter 13

Role of Lipids in the Dynamics of Thylakoid Membranes

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Summary

Thylakoid membranes are dynamic systems in which the lateral mobility of proteins and lipids plays a key role in physiological processes including electron transport, regulation of light-harvesting, membrane biogenesis and turnover and repair of proteins. This chapter gives an overview of the importance of lateral mobility in thylakoid membranes, followed by a description of the various methods that can be used to measure diffusion of proteins and lipids in the thylakoid membranes of green plants and cyanobacteria. These methods include Fluorescence Recovery after Photobleaching (FRAP) and Single-Particle Tracking (SPT). We discuss the advantages and limitations of the various methods. We then summarize the information available on lateral diffusion coefficients of proteins and lipids in the thylakoid membranes of plants and cyanobacteria. Finally we discuss two key ways in which the lipid content of thylakoid membranes influences the lateral mobility of membrane components. Lipid composition, in particular the desaturation of the fatty acyl tails, has a strong influence on membrane fluidity and the phase transition temperature of the membrane. It is also becoming clear that the lipid:protein ratio has a strong influence on membrane properties. Recent work in which isolated granal membranes were "diluted" by fusion with liposomes shows that as the lipid:protein ratio increases, there is a pronounced increase in the mobility of protein complexes in the membrane. However, excessive lipid dilution destabilizes protein supercomplexes, reducing the efficiency of light-harvesting. Clearly there is an optimum density of packing of protein complexes into the membrane, which allows some fluidity in the membrane combined with a high density of photosynthetic complexes and efficient interaction of reaction centers and light-harvesting complexes.

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I Introduction

Many of the key processes occurring in thylakoid membranes must be dependent on the lateral diffusion of membrane components. Such processes include:

1. Electron transport. Here, plastoquinone molecules have to diffuse between Photosystem II (PS II) and the cytochrome b_6f complex (Dekker and Boekema, 2005).
2. Regulation of light-harvesting. In green plants, LHCII mobility plays a key role in state transitions (Allen and Forsberg, 2001; Dekker and Boekema, 2005), and it seems increasingly likely that a large-scale re-organization of PS II-LHCII supercomplexes is involved in non-photochemical quenching of chlorophyll fluorescence (Horton et al., 2008; Kiss et al., 2008).
3. Membrane biogenesis. For example, the formation of the complex three-dimensional structure of mature thylakoid membranes in green plants is likely to be a highly dynamic process involving the formation of supramolecular aggregates, accompanied by folding and perforation of the membrane (Mustárdy and Garab, 2003; see also Chapter 14). In cyanobacteria it appears that the initial stages of PS II biosynthesis take place in the cytoplasmic membrane (Zak et al., 2001). It is not clear how these complexes are transported to the thylakoids, but their incorporation into the mature photosynthetic apparatus must surely involve lateral diffusion within the thylakoid membrane system.
4. The turnover and repair of thylakoid membrane proteins. The repair cycle of PS II has been intensively studied, since it plays a key role in the maintenance of photosynthetic function (Barber and Andersson, 1992). In green plants it appears that photodamaged PS II centers must migrate from the grana to the stroma lamellae for repair, followed by migration back into the grana (Aro et al., 2005).

Abbreviations: LHCII – Chlorophyll *a*/*b*-binding light-harvesting complex of photosystem II; BODIPY FL – C_{12} 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-5-indacene-3-dodecanoic acid; FCS – Fluorescence correlation spectroscopy; FRAP – Fluorescence recovery after photobleaching; FTIR – Fourier transform infra-red; GFP – Green fluorescent protein; PCC – Pasteur Culture Collection; P700 – Photochemical electron donor chlorophylls of photosystem I; PS II – Photosystem II; SPT – Single-particle tracking; TIRF – Total internal reflection fluorescence

Like most other biological membranes, thylakoid membranes are densely crowded with proteins. The granal thylakoid membranes of green plants may be an extreme example; here atomic force microscopy shows that about 80% of the membrane area is occupied by protein (Kirchhoff, 2008; Kirchhoff et al., 2008a). Freeze-fracture electron micrographs show that cyanobacterial thylakoid membranes are also quite densely packed with protein (Nilsson et al., 1992). However, in terms of protein:lipid ratio, cyanobacterial thylakoid membranes are not unusually crowded; they fall within the normal range for prokaryotic membranes. For example, the protein:lipid ratio (weight:weight) is about 2.3 in thylakoid membranes of the cyanobacterium *Synechocystis* 6803 (Szalontai et al., 2000), compared to about 2.5 for the cytoplasmic membrane of *Escherichia coli* (Arechaga et al., 2000). However, a distinctive feature of thylakoid membranes is the presence of large-scale supramolecular assemblages of protein, such as semi-crystalline arrays of PS II-LHCII supercomplexes sometimes observed in granal membranes of green plants (Albertsson, 2001; Dekker and Boekema, 2005; Kirchhoff et al., 2008a). Cyanobacterial thylakoid membranes often exhibit rows of PS II dimers (Nilsson et al., 1992) and sometimes two-dimensional PS II arrays (Folea et al., 2008). Does this large-scale ordering affect the mobility of proteins and lipids in the membrane? And what is the influence of other factors, including membrane lipid composition and the protein:lipid ratio? In this chapter we discuss methods for the direct experimental measurement of protein and lipid mobility in thylakoid membranes, and we summarize what we know about the mobility of thylakoid membrane components. There is an emphasis on the role of thylakoid membrane lipids in controlling membrane fluidity, and importance of lipids for creating diffusion space for proteins and plastoquinone.

II Methods for Measuring Thylakoid Membrane Dynamics

How can we probe the mobility of lipids and proteins within thylakoid membranes? One strategy is to use spectroscopic techniques to monitor in real time some aspect of photosynthesis. For example, rates of electron transport can be monitored by flash

photolysis or oxygen electrode; modulated chlorophyll fluorescence can reveal regulation of light-harvesting. The mobility of some components of the photosynthetic apparatus can then be inferred, though only with the aid of a set of assumptions about the system. For example, a lower limit for the plastoquinol diffusion coefficient in higher plant thylakoid membranes was estimated from a lag time of electron arrival at P_{700}^{+} generated by PS II after a single-turnover flash (Mitchell et al., 1990). A complementary approach has been applied to green plant thylakoids, where the redistribution of thylakoid components between the grana and the stroma lamellae can be monitored by biochemical fractionation (Drepper et al., 1993) or immuno-electron microscopy (Haehnel et al., 1989). These latter methods of course do not monitor mobility within individual chloroplasts or cells in real time.

As a general rule, both the biochemical and the spectroscopic approaches probably underestimate the dynamics of the system, since what is observed is only the net redistribution of a component between two parts of a membrane following some change in conditions. The underlying kinetics of diffusion might well be faster. To give a well-characterized example, state transitions in cyanobacteria involve the redistribution of light-harvesting phycobilisomes between PS II and Photosystem I (PS I) (van Thor et al., 1998). State transitions take place on a timescale of a few seconds to a few minutes, which suggest a rather slow movement of phycobilisomes, given that the photosystems are in close proximity in the thylakoid membrane (Mullineaux, 1999). However, direct measurements of phycobilisome diffusion give a mean diffusion coefficient of about 0.03 $\mu m^2 s^{-1}$ (Mullineaux et al., 1997; Sarcina et al., 2001), which suggests that during the 1 min time-course of a state transition, a phycobilisome should diffuse on average about 2.7 μm from its starting position. This contrasts very sharply with the spatial separation between PS II and PS I, which might be only 20–30 nm (Mullineaux, 1999). The discrepancy highlights an unexpected but fundamental feature of the system: the phycobilisomes are only weakly bound to the reaction centers and are in rapid and continuous random motion. The kinetics of state transitions probably reflect the slow kinetics of a signal transduction pathway that alters phycobilisome-reaction center affinity, rather than the fast kinetics of diffusion. A similar argument may apply to state transitions in green plants, where phosphorylation of a pool of LHCII light-harvesting complexes leads to redistribution of LHCII between the grana and the stroma lamellae (Allen and Forsberg, 2001). The kinetics of the state transition are slow (taking place over several minutes), but they match the slow kinetics of LHCII phosphorylation (Saito et al., 1983). Thus the kinetics of state transitions in green plants could be limited by the rate of LHCII phosphorylation. The actual diffusion of LHCII between the grana and stroma lamellae may occur on a faster timescale. However, in apparent contradiction to this idea, the kinetics of phospho-LHCII redistribution between the grana and stroma lamellae appear relatively slow in the "temperature-jump" experiments of Drepper et al. (1993).

Some spectroscopic techniques give rather more direct indications about the mobility of thylakoid membrane components. For example a pyrene fluorescence quenching technique has been used to estimate the lateral diffusion coefficient for plastoquinone, data of considerable importance for understanding the kinetics of electron transport (Blackwell et al., 1994). Fourier transform infrared (FTIR) spectroscopy can be used to monitor the disorder of lipid fatty acyl chains. Although this does not measure the lateral diffusion of lipids, it is an excellent indicator of the fluidity of the membrane (Szalontai et al., 2000).

Perhaps the most powerful techniques for monitoring the lateral diffusion of membrane components are those that use fluorescence microscopy for direct observation of lateral movement in individual cells, chloroplasts or membrane preparations. One such method is Fluorescence Recovery after Photobleaching (FRAP). In the usual form of the technique, the sample is imaged with a laser scanning confocal microscope (Kubitscheck et al., 1994). In the case of thylakoid membranes, this can be used to image fluorescence from the native photosynthetic pigments as well as artificial fluorescent tags (Mullineaux et al., 1997; Mullineaux and Sarcina, 2002). To measure diffusion, the laser power is increased and the confocal laser spot is used to bleach fluorescence in a small region of the sample. The laser power is then reduced again and the sample is repeatedly imaged. Diffusion of the fluorophore results in a characteristic spread or blurring of the bleached

area, with fluorescence recovery at the centre of the bleached zone (Kubitscheck et al., 1994; Mullineaux and Sarcina, 2002). In favorable cases (when the membrane conformation is simple and predictable, and the membrane is homogeneous on the scale of several microns), a quantitative estimate of the diffusion coefficient can be obtained from FRAP measurements. One example is the approximately cylindrical thylakoid membranes of some elongated cyanobacteria, such as *Synechococcus* PCC 7942 (Mullineaux et al., 1997; Sarcina et al., 2001; see also Chapter 14). The geometry of a FRAP measurement on such a cell is illustrated in Fig. 1, and an example of such a measurement is shown in Fig. 2. Another thylakoid membrane system that has proved suitable for quantitative FRAP is isolated granal

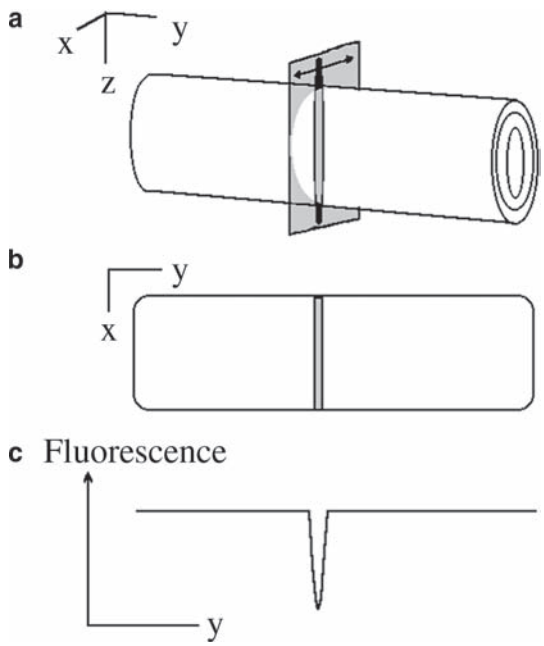

Fig. 1. Geometry of a one-dimensional confocal FRAP (fluorescence recovery after photobleaching) experiment to measure the mobility of a fluorophore in the cylindrical thylakoid membranes of an elongated cyanobacterial cell. (**a**) The confocal laser spot (elongated in the Z-direction) is scanned at high power across the center of the cell in the X-direction to bleach a line across the cell. (**b**) The laser power is reduced and the cell is imaged repeatedly in two dimensions by scanning the confocal spot in the XY plane. (**c**) One-dimensional fluorescence profiles are extracted from the image by integrating pixel values in the X-direction. Changes in the profile with time are quantified and used to estimate the diffusion coefficient of the fluorophore (reprinted from Mullineaux et al. (1997) with permission from Nature Publishing Group).

membranes from plant chloroplasts (Kirchhoff et al., 2008b). The membranes were adsorbed onto a fluid support consisting of an artificial lipid bilayer. Under these conditions the membrane fragments aggregate in two dimensions to form larger, flat membrane patches, which are suitable for FRAP measurements (Fig. 3). However, the majority of photosynthetic membrane systems are too convoluted and heterogeneous for fully-quantitative FRAP measurements. Nevertheless, FRAP can be used in these more complex systems as a qualitative method for observing the mobility of membrane components.

FRAP has been used to probe the mobility of photosynthetic pigment–protein complexes by visualizing the native fluorescence from fluorescent pigments. Examples include the phycobilisomes, PS II and IsiA in cyanobacteria, and PS II and LHCII in grana membranes. FRAP could potentially be used to probe the mobility in vivo of specific thylakoid membrane proteins genetically tagged with Green Fluorescent Protein (GFP). GFP-tagged thylakoid membrane proteins can sometimes be quite easily visualized, and it is straightforward to arrange combinations of excitation and emission wavelengths that distinguish GFP from the photosynthetic pigments (Komenda et al., 2006). There is also potential to use FRAP in vitro to probe the mobility of proteins tagged with fluorescent antibodies. FRAP can also be used to measure lipid mobility and membrane fluidity in general, by staining membranes with lipophilic fluorophores. The lipophilic green fluorophore BODIPY FL-C_{12} has been used as a fluorescent probe for FRAP measurements of membrane fluidity in the cyanobacterium *Synechococcus* PCC7942 (Sarcina et al., 2003; Fig. 2). FRAP measurements of BODIPY FL-C_{12} diffusion in this system clearly show the temperature-dependent phase transition of the membrane from liquid crystal to crystalline gel, and show the very pronounced effect of manipulating the desaturation of lipid fatty acyl groups (Sarcina et al., 2003; Fig. 4; see also Chapter 17).

A limitation of FRAP is spatial resolution. It is only capable of resolving mobility at relatively large scales of a micron or more. This limits its usefulness in photosynthetic systems, where some membrane components may well be diffusing only within small, tightly confined domains. FRAP also has the disadvantage that it measures

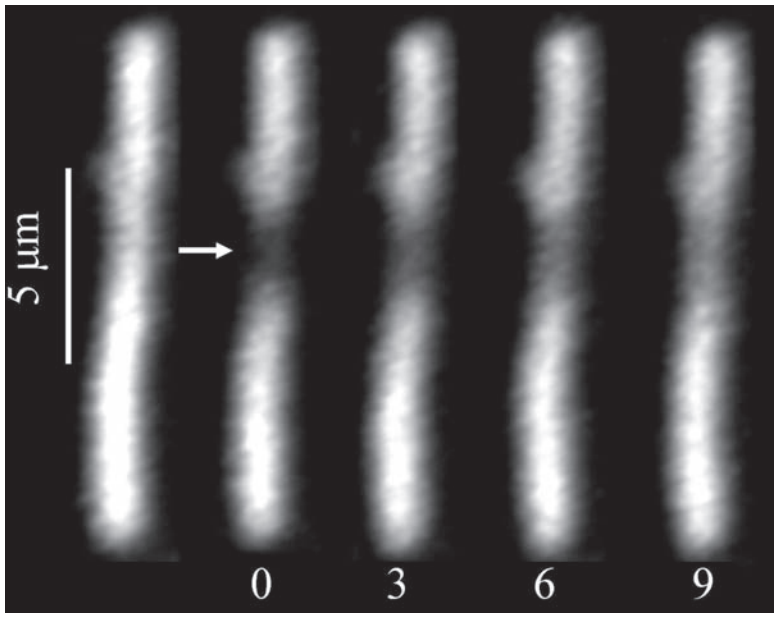

Fig. 2. A confocal FRAP (fluorescence recovery after photobleaching) experiment to measure the diffusion of the green lipophilic fluorophore BODIPY FL-C_{12} in thylakoid membranes of a cell of the cyanobacterium *Synechococcus* PCC 7942. The experiment uses the one-dimensional FRAP technique illustrated in Fig. 1. A line is bleached across the cell (position indicated by the *arrow*), and subsequent spread and recovery of the bleach is observed by imaging the cell at 3 s intervals (reprinted from Sarcina et al. (2003) with permission from Elsevier).

only the mean behavior of large numbers of fluorophores. Furthermore the FRAP bleach is somewhat disruptive, and may perturb the behavior of the membrane being examined. There are other techniques based on fluorescence microscopy that address some of these problems. Fluorescence Correlation Spectroscopy (FCS) uses a stationary laser spot, observing the diffusion of fluorophores in and out of the spot (Haustein and Schwille, 2007). Single-particle tracking (SPT) uses video-rate epifluorescence or Total Internal Reflection of Fluorescence (TIRF) microscopy to observe the diffusion of individual fluorescently-tagged molecules (Levi and Gratton, 2007; Wang et al., 2008). Both FCS and SPT require a "sparse" fluorescent tag, that is, the density of fluorescent tags in the membrane must be relatively low. This particularly applies to SPT, where individual fluorophores must be tracked. This means that FCS and SPT generally cannot employ the native fluorescence from photosynthetic pigments, since these are far too densely packed in the membrane. GFP-tagging is also unlikely to be suitable for SPT or FCS in photosynthetic membranes. However, there is potential for the use of FCS and SPT in vitro, using fluorescent antibodies to label specific membrane proteins at low density. SPT has been used in this way to visualize the movement of individual LHCII complexes in spinach thylakoid membranes (Consoli et al., 2005). This work shows the potential of the technique. SPT has much higher spatial resolution than FRAP, because, when a single fluorophore is imaged, its position can be determined very accurately by deconvoluting the blurred optical image. Thus SPT is particularly promising for resolving the movement of membrane components within the confined domains that are likely to be a feature of many photosynthetic membrane systems.

III Lipid and Protein Mobility in Thylakoid Membranes

Table 1 summarizes the lateral diffusion coefficients that have been measured by direct means (FRAP, SPT etc.) in the thylakoid membranes of green plants and cyanobacteria. It must be stressed

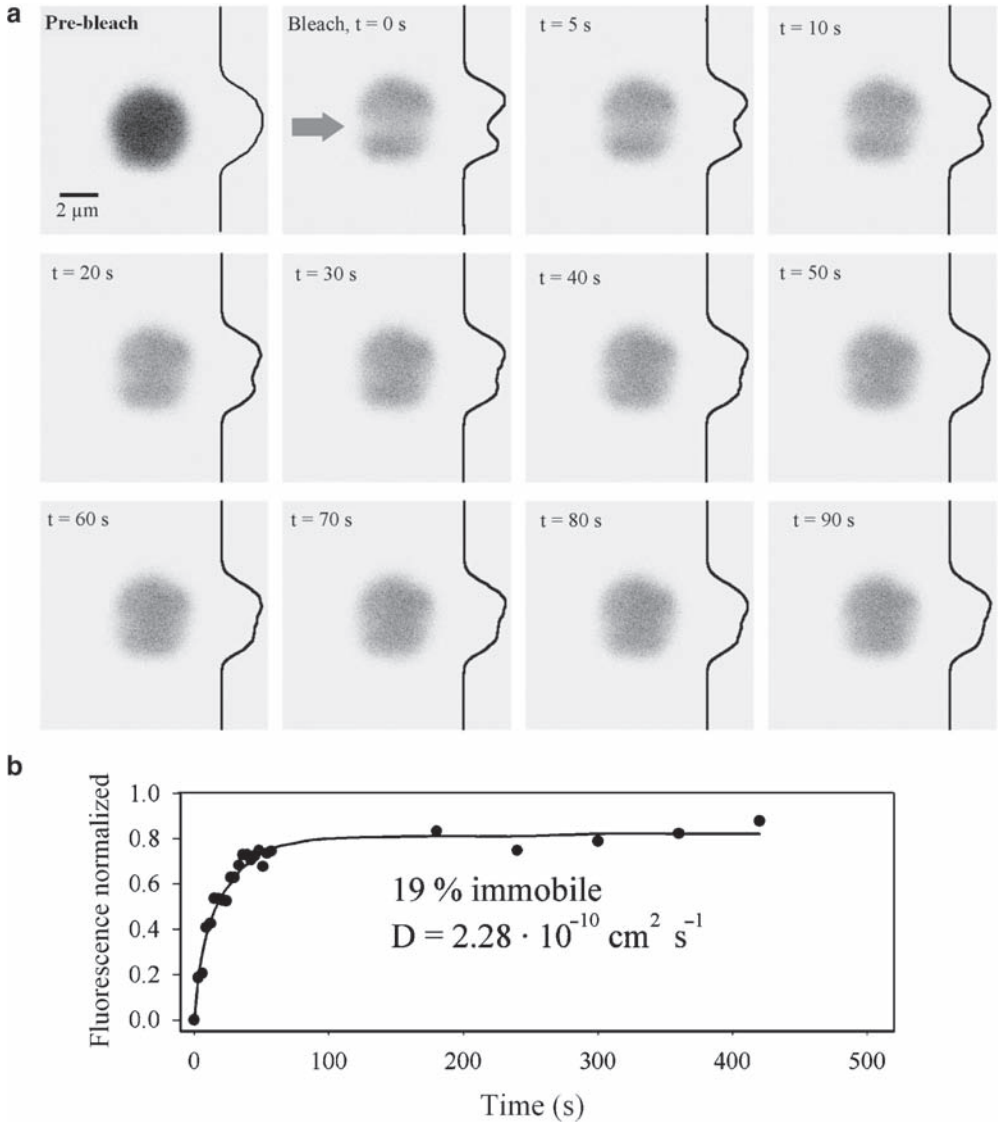

Fig. 3. FRAP (fluorescence recovery after photobleaching) measurement of the diffusion of chlorophyll–protein complexes in a patch of isolated spinach grana membranes, in this case diluted by fusion with liposomes. (**a**) Chlorophyll fluorescence images are shown in negative gray-scale. The membranes are adsorbed onto an artificial lipid bilayer as a fluid support. A line is bleached across the patch in the X-direction (position indicated by the *arrow*). The subsequent images show redistribution of fluorescence after the bleach. (**b**) Fluorescence recovery at the centre of the bleach is shown (reproduced from Kirchhoff et al. (2008b). Copyright American Society of Plant Biologists (http://www.plantphysiol.org/)).

that our picture of membrane mobility is still fragmentary: we have no information about the mobility of many important protein complexes, and the effects of many different physiological conditions remain to be explored. A common feature of both membrane systems is a high proportion of chlorophyll–protein complexes that are essentially immobile in the membrane. In cyanobacteria, PS II is virtually immobile under normal conditions (Mullineaux et al., 1997; Sarcina and Mullineaux, 2004). In spinach grana, about 75% of chlorophyll fluorescence is also immobile (Kirchhoff et al., 2008b). However, neither membrane system is completely static. In cyanobacteria, the IsiA chlorophyll–protein complex is capable of rather slow diffusion (Sarcina and Mullineaux, 2004), and in

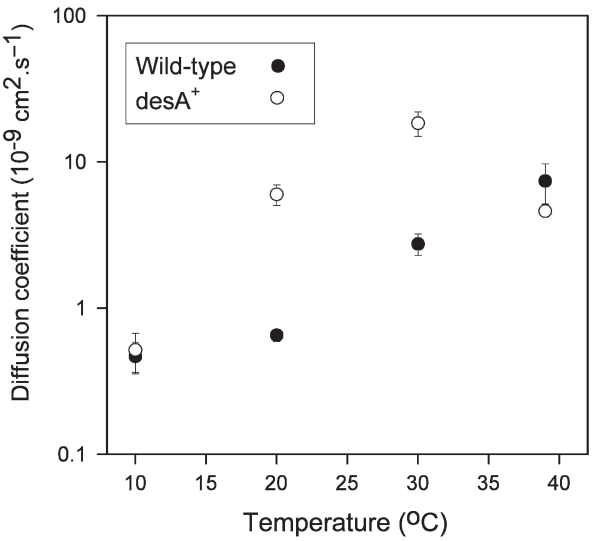

Fig. 4. Temperature dependence of the lateral diffusion coefficient of the lipophilic fluorophore BODIPY FL-C_{12} in thylakoid membranes of the cyanobacterium *Synechococcus* PCC 7942 (wild type and *desA*$^+$ transformant, which has an increased level of fatty acid desaturation) (reprinted from Sarcina et al. (2003) with permission from Elsevier).

spinach grana there is population of chlorophyll–proteins that diffuse at a similar rate, surprisingly fast considering the crowding of the membrane (Kirchhoff et al., 2008b). This mobile population of chlorophyll–proteins is responsible for about 25% of chlorophyll fluorescence from grana thylakoids. Its identity is not yet certain but it is possible that it represents a mobile sub-population of LHCII (Kirchhoff et al., 2008b). Preliminary work on intact chloroplasts from several plant species suggests that a similar population of chlorophyll–protein complexes can exchange between grana on a timescale of a few minutes (T.K. Goral and C.W. Mullineaux, 2009 unpublished).

Our current picture of the mobility of plant and cyanobacterial thylakoid proteins suggests that the two membrane systems have many features in common. In both systems, there is a substantial population of protein complexes that are essentially immobile, at least under "normal" conditions. This immobility is likely to be due to a combination of macromolecular crowding and assembly into large supercomplexes. Another significant factor may be the presence of domains protruding into the thylakoid lumen, since there are indications that protein mobility in the thylakoid lumen may be extremely restricted (Soriano et al., 1998; Kirchhoff et al., 2004a; Kana et al., 2009). However, in both membrane systems there are protein complexes that are mobile over long distances. Presumably these mobile complexes percolate through the spaces between the large, immobile supercomplexes.

Membrane lipids must also percolate through the gaps between the protein complexes. The lateral mobility of lipids has been probed in cyanobacteria by staining the thylakoid membranes with BODIPY FL-C_{12}, a green fluorophore anchored to the membrane by 12-carbon acyl chain (Sarcina et al., 2003). Although BODIPY is not a natural component of the membrane, FRAP measurements of BODIPY diffusion (Fig. 2) clearly give information on membrane fluidity, revealing the effects of temperature and the degree of unsaturation of the native lipids (Sarcina et al., 2003; Fig. 4). In wild-type *Synechococcus* PCC7942 at its growth temperature, the BODIPY diffusion coefficient is about 0.3 $\mu m^2\ s^{-1}$. The diffusion of comparable fluorescent probes in eukaryotic plasma membranes is faster by a factor of about 3–14 (Fulbright et al., 1997). BODIPY also diffuses faster in the plasma membrane of *E. coli* at growth temperature, by a factor of about 4 (A. Nenninger and C.W. Mullineaux, 2009 unpublished). The relatively slow diffusion in *Synechococcus* may be due to various factors, including the density and configuration of the protein complexes and the lipid composition of the membrane. Clearly there are several factors that control lipid mobility. Crowding can have an effect: measurement of plastoquinone diffusion coefficients in artificial proteoliposomes shows a strong retardation when membrane proteins are added (Blackwell and Whitmarsh, 1990). However, the comparison of *Synechococcus* with *E. coli* indicates that the slow lipid diffusion in *Synechococcus* is not simply a result of packing the membrane with protein. The *E. coli* plasma membrane has similar protein density to the *Synechococcus* thylakoid membrane (Arechaga et al., 2000; Szalontai et al., 2000) yet BODIPY diffusion is about four times faster in *E. coli*. Lipid composition is likely to be major factor. Increasing the desaturation of fatty acyl tails has a very marked effect in *Synechococcus*, increasing the BODIPY diffusion coefficient at growth temperature by a factor of about 6 (Sarcina et al., 2003; Fig. 4; Table 1; see also Chapter 17).

Table 1. Lateral diffusion of thylakoid membrane components. All the diffusion coefficients shown are at growth temperature (room temperature for plants, 30°C for cyanobacteria).

Component	Membrane system	Measuring technique	Mean diffusion coefficient ($\mu m^2\ s^{-1}$) ± standard deviation	Reference
Photosystem II	Cyanobacterial thylakoids	FRAP	<0.00002	Sarcina and Mullineaux, 2004
Photosystem II	Cyanobacterial thylakoids, after treatment with intense red light	FRAP	0.023 ± 0.004	Sarcina et al., 2006
IsiA	Cyanobacterial thylakoids	FRAP	0.0034 ± 0.0008	Sarcina and Mullineaux, 2004
Phycobilisomes	Cyanobacterial thylakoids	FRAP	0.03 ± 0.01	Sarcina et al., 2001
Chl–protein complexes (probably PS II supercomplexes)	Granal membranes from spinach	FRAP	<0.0001	Kirchhoff et al., 2008b
Chl-protein complexes (probably an LHCII sub-population)	Granal membranes from spinach	FRAP	0.0046 ± 0.0004	Kirchhoff et al., 2008b
LHCII	Spinach thylakoids	SPT	0.0084	Consoli et al., 2005
Phospho-LHCII	Spinach thylakoids	SPT	0.027	Consoli et al., 2005
BODIPY FL-C_{12} (lipophilic fluorophore)	Cyanobacterial thylakoids (wild-type *Synechococcus* 7942)	FRAP	0.28 ± 0.05	Sarcina et al., 2003
BODIPY FL-C_{12} (lipophilic fluorophore)	Cyanobacterial thylakoids (*Synechococcus* 7942 *desA*$^+$)	FRAP	1.8 ± 0.3	Sarcina et al., 2003
Plastoquinone	Spinach thylakoids	Pyrene fluorescence quenching	0.01–0.3	Blackwell et al., 1994

When the mobility of specific, native lipids is considered, it is likely that specific interactions with proteins will also play a role in constraining the mobility of the lipid. It is interesting that the diffusion coefficient of plastoquinone in spinach thylakoids is considerably slower than the diffusion of BODIPY in *Synechococcus* thylakoids, by a factor of 10 or more (Table 1). This diffusion coefficient was measured by the pyrene fluorescence quenching technique, which yields short-range diffusion coefficients on a scale of 10–100 nm (Blackwell et al., 1994). The plastoquinone long-range diffusion coefficient (which would be comparable with the BODIPY FRAP measurements) will be even lower if plastoquinone is confined to restricted domains in the membrane. Such "microdomains" were postulated to exist in grana thylakoids from functional electron transport measurements (Lavergne et al., 1992; Kirchhoff et al., 2000). So the slow diffusion of plastoquinone illustrates the strong effect of specific interaction with proteins. Presumably, plastoquinone diffusion is slowed by transient binding to slow-moving or immobile quinone-binding proteins, such as PS II or the cytochrome b_6f complex, or by trapping in "leaky" microdomains.

IV Effects of Lipid Composition on Membrane Fluidity and Tolerance of Low Temperatures

In most biomembranes fluidity is strongly dependent on temperature. Usually, a phase transition from liquid crystal to crystalline gel states occurs when the membrane is cooled to about 10–15°C below growth temperature. The phenomenon has been studied thoroughly in cyanobacteria (Chapter 17), although, to our knowledge, phase transitions have yet to be clearly demonstrated in higher plant chloroplasts. In cyanobacteria, the phase transition results in a marked decrease in lipid fluidity as measured (for example) by FTIR spectroscopy (Szalontai et al., 2000) or FRAP (Sarcina et al., 2003; Fig. 4). It is clear that the phase transition has physiological consequences, since phase transition correlates with reduced photosynthetic activity, various kinds of reversible and irreversible cell damage, and increased susceptibility to photodamage (Murata, 1989). Cyanobacteria respond to prolonged growth at lower temperatures by changing the lipid composition of the thylakoid membrane, mainly by increasing the desaturation of fatty acyl tails (Murata, 1989). This results in a lowering of the phase transition temperature. Studies on mutants in which lipid desaturation is altered show strong effects on lipid mobility, phase transition temperature (Fig. 4) and susceptibility to photodamage at low temperatures (Wada et al., 1994; Kanervo et al., 1995; Gombos et al., 1997).

The deleterious effects of the lipid phase transition probably result from a combination of increased permeability of the membrane with reduced lateral mobility. The increase in membrane permeability could result in leakage of ions and small molecules, and loss of proton motive force for ATP synthesis and membrane transport (Murata, 1989). The possible effects of reduced lateral mobility are less well-characterized. However, it seems rather likely that a reduced lateral mobility of plastoquinone could result in slower electron transport, accompanied by increased susceptibility to photodamage as result of a build-up of electrons at the acceptor side of PS II. An area that needs further exploration is the possible effect of slower protein diffusion below phase transition temperature. Generally the mobility of peripheral membrane proteins (those that interact with lipid head-groups at the membrane surface) is not much affected by phase transition (O'Toole et al., 1999). A well-characterized example in cyanobacteria is the phycobilisomes, whose lateral mobility decreases gradually with decreasing temperature, but with no sharp change at phase transition temperature (Sarcina et al., 2001). However, the lateral mobility of membrane-integral proteins must be strongly affected by phase transition. A possible consequence of this effect can be seen in the thylakoids of green plants, where the redistribution of phospho-LHCII from the grana to the stroma lamellae is prevented at low temperatures (Drepper et al., 1993). Potentially, the reduced lateral mobility of proteins below phase transition could have a number of deleterious effects, including:

1. The inhibition of any signal transduction pathways, which depend on the dynamic interactions of integral thylakoid membrane proteins. This probably includes, for example, state transitions

in cyanobacteria, which are clearly inhibited below phase transition (El Bissati et al., 2000). The inhibition of such feedback responses is in turn likely to result in decreased photosynthetic efficiency and increased photodamage.

2. Inhibition of the PS II repair cycle. Under low light-conditions, PS II appears immobile in the membrane. However, a partial mobilization and large-scale redistribution can be observed after exposure of cyanobacterial cells to intense light, and this may well be required for rapid PS II repair (Sarcina et al., 2006). In green plants, it is clear that PS II complexes must migrate between the grana and the stroma lamellae for the repair cycle to operate (Aro et al., 2005). Reduced lateral mobility of PS II could make a major contribution to the lower efficiency of PS II repair cycle at low temperatures and in desaturase mutants (Kanervo et al., 1995).

We still have little direct information on the effects of temperature and lipid composition on the lateral mobility of proteins in thylakoid membranes. This will be an important area for future study.

V Importance of Lipids for Creating Diffusion Space for Proteins and Plastoquinone

Biological membranes are a complex mixture of macromolecular complexes of various sizes, with smaller molecules (lipids). For obvious reasons, the smaller molecules tend to be much more mobile, percolating rapidly through the spaces between the larger molecules. Monte Carlo simulations suggest that as the density of macromolecules in the membrane increases, the fluidity of the membrane decreases. Beyond a critical level of crowding with macromolecules, the membrane becomes almost immobile (Saxton, 1994; Kirchhoff et al., 2004b). This suggests that a key role for lipids in thylakoid membranes (as in biological membranes in general) is to provide diffusion space, maintaining fluidity in the membrane.

Granal membranes in vitro provide a nice model system to test the effects of changing the protein:lipid ratio, since the membranes can be "diluted" by fusing them with liposomes made from native lipids (Haferkamp and Kirchhoff, 2008; Kirchhoff et al., 2008b). As would be expected, increased lipid dilution results in greater protein mobility (Kirchhoff et al., 2008b; Fig. 5). In undiluted grana there is a large population of immobile chlorophyll–proteins and a smaller, mobile population, as judged from chlorophyll FRAP measurements. As the granal membranes are diluted with additional lipid, the mobile population becomes larger and its mean diffusion coefficient increases (Kirchhoff et al., 2008b; Fig. 5). This might suggest that a lower protein:lipid ratio is favorable, since it would allow greater membrane mobility, perhaps leading to more rapid and efficient electron transport, signal transduction,

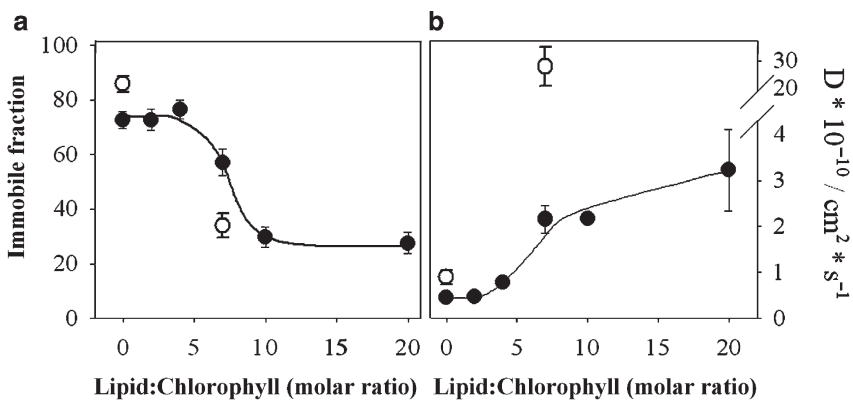

Fig. 5. Effects of dilution with additional lipid on the mobility of chlorophyll–protein complexes in isolated spinach grana membranes. Data estimated from FRAP measurements as illustrated in Fig. 3. *Filled circles*: membranes in standard buffer containing 7 mM $MgCl_2$. *Open circles*: membranes in "low-salt" buffer. The comparison demonstrates the influence of protein–protein interactions, which become weaker in "low-salt" buffer. (**a**) Mobile/immobile fractions of chlorophyll fluorescence. (**b**) Diffusion coefficient for the mobile fraction (reproduced from Kirchhoff et al. (2008b). Copyright American Society of Plant Biologists (http://www.plantphysiol.org/)).

and turnover and repair of complexes. However, the same experimental system also reveals that macromolecular crowding plays a key role in the function of photosynthetic supercomplexes. When granal membranes are diluted with additional lipid, energy transfer from LHCII to PS II becomes less efficient (Haferkamp and Kirchhoff, 2008). So a dense packing of complexes into the membrane is a requirement for efficient light-harvesting in this system. Clearly photosynthetic membranes maintain a delicate balance in the protein:lipid ratio. If the protein:lipid ratio were too high, lateral movement within the membrane would become impossible. But if the ratio were too low, photosynthetic supercomplexes would be destabilized and light-harvesting would become inefficient. The mechanisms that control the protein:lipid ratio in thylakoid membranes are a fascinating topic for future study.

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Chapter 14

Architecture of Thylakoid Membrane Networks

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Summary

The primary events of oxygenic photosynthesis are carried out within intricate membrane lamellar systems called thylakoid networks. These networks, which are present in cyanobacteria, algae, and higher plants, accommodate all of the molecular complexes necessary for the light-driven reactions of photosynthesis and provide a medium for energy transduction. Here, we describe the ultrastructure of thylakoid membranes and their three-dimensional organization in various organisms along the evolutionary tree. Along the way we discuss issues pertaining to the formation and maintenance of these membranes, the means by which they enable molecular traffic within and across them, and the manner by which they respond to short- and long-term variations in light conditions.

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I Introduction

The ability to perform oxygenic photosynthesis evolved, several billion years ago, in an ancestor of present-day cyanobacteria. Subsequently, it was inherited by algae and higher plants through a primary endosymbiotic event that eventually led to the development of the chloroplast. In both cyanobacteria and chloroplasts, photosynthetic electron transport and the consequent generation of proton-motive force and reducing power are carried out within flattened vesicles called thylakoids. These sac-(*thylakos* in Greek)-like membranes harbor the protein complexes that conduct the light-driven reactions of photosynthesis and provide a medium for energy transduction. Thylakoids are organized in lamellar networks of varying degrees of complexity that occupy a significant fraction of the cell or chloroplast volume.

To fulfill their function, thylakoid networks need to be structured in a specific manner. Contained within the cell or chloroplast, they need to be packed into small volumes, yet retain a large surface area to maximize light capture. They also have to be completely sealed in order to maintain chemiosmotic coupling, and highly connected, to ensure rapid equilibration of proton and electron (carrier) gradients and to allow for efficient trafficking of proteins, organic compounds, and lipids. Their structure should also provide for sufficient mechanical strength, to avoid breakages and collapse, and for ready delivery of proteins and lipids required for network formation and maintenance and for the repair of photodamaged protein components. In their constitution, the membranes need to have a specific lipid composition (which is discussed at length in other chapters), such that they can support vast amounts of pigment–protein complexes, as well as small organic molecules that serve as mobile electron carriers. In the broader scope of cell or organelle homeostasis, thylakoid networks have to be organized such that they do not impose a burden too severe for trafficking of molecules or inclusions inside the cell or chloroplast.

Superimposed on the above is a perpetual need to cope with alterations in light conditions, which can be vast and rapid or moderate and slow. This everlasting requirement necessitates a high degree of functional flexibility not only of the photosynthetic apparatus but also of the thylakoid membranes within which it is contained. This is particularly true for sessile land plants where acclimation by means of movement towards a more hospitable environment is impossible, dictating a sole reliance on internal adaptations. Indeed, as shall be discussed later, acclimation of land plants to rapid alterations in light quality and/or intensity is accompanied by massive thylakoid membrane rearrangements that are most likely of high energetic cost. Less appreciated and studied are changes in other environmental conditions, such as temperature and humidity, which are probably also accompanied by local or long-range reorganizations in the thylakoid membranes.

Photosynthesis is famed for its fast photochemistry. However, even the light-driven reactions of photosynthesis are rate-limited and regulated by the diffusion of small molecules and proteins within the complex construction of the thylakoid networks. To understand these processes, as well as processes related to the biogenesis, maintenance, and chromatic adaptation of the photosynthetic apparatus, detailed knowledge of the structure and the composition of the thylakoid membranes is essential. Such information includes network geometry, dimensions, and connectivity, as well as the way the various photosynthetic components, both macromolecular and molecular, are distributed and move about in the membranes. Also required is knowledge about the manner by which thylakoids gain access to the protein synthesis machinery and to vesicular transport highways, and about the factors and pathways involved in thylakoid network remodeling during development and light acclimation. As will be elaborated, the latter processes, at least in higher plants, may require the participation of dedicated machinery, perhaps similar to the ones operating in the mitochondria or in the Golgi apparatus.

In this chapter, we attempt to provide an overview of the organization of thylakoid membranes,

Abbreviations: CF – Chemical fixation; Chl – Chlorophyll; CEMOVIS – Cryo-electron microscopy of vitreous sections; EMT – Electron microscope tomography; EM – Electron microscopy; FS – Freeze substitution; HPF – High pressure freezing; LHC – Light-harvesting protein complexes; PS I – Photosystem I; PS II – Photosystem II; PBS – Phycobilisomes; PQ – Plastoquinone; UVB – Ultraviolet radiation B (280–315 nm); UVC – Ultraviolet radiation C (100–280 nm)

beginning with the most ancient known and quite simple systems, as in cyanobacteria and red algae, and culminating with the evolutionary younger and topologically complex networks of higher-plant chloroplasts. Along the way, we discuss key events in the evolution of thylakoid networks and issues pertaining to thylakoid network formation, maintenance, and acclimation to varying light conditions. The story of modern studies of thylakoid network organization, however, cannot be told without discussing electron microscopy (EM) techniques, which enabled researchers in the field to visualize thylakoids with a resolution two orders of magnitude higher than that attainable by the light microscope used by botanists in their explorations since the mid nineteenth century. We therefore begin this chapter with a brief description of specimen preparation methods used in cross-sectional EM analyses, emphasizing the importance of cryo-fixation methods, which are gradually replacing conventional chemical fixation, followed by a brief introduction to electron microscope tomography, which allows one to visualize cellular structures in three dimensions, at molecular or near-molecular resolution. Due to space limitations, we do not discuss freeze-fracture and freeze-etch EM methods, nor do we elaborate on the immense contributions they made, together with immuno-EM, to our understanding of the distribution and organization of the major photosynthetic protein complexes within the thylakoid membranes. For comprehensive discussions of these topics, the reader is referred to the following excellent reviews (Staehelin, 1986, 2003; Dekker and Boekema, 2005).

II Techniques in Electron Microscopy of Plant Samples

In 1851, a few years after the integration of the achromatic lens into the light microscope, the German botanist Hugo von Mohl wrote in his treatise on the vegetable cell:

> "If a tissue composed of young cells be left some time in alcohol, or treated with nitric or muriatic acid, a very thin, finely granular membrane becomes detached from the inside of the walls of the cells, in the form of a closed vesicle, which becomes more or less contracted, and consequently removes all the contents of the cell which are enclosed in this vesicle from the wall of the cell. Reasons hereafter to be discussed have led me to call this inner cell the 'primordial utricle' (primordialschlauch). In the centre of the young cell, with rare exceptions, lies the so-called nucleus cellulae of Robert Brown ('Zellenkern'; 'Cytoblast' of Schleiden)…. The remainder of the cell is more or less densely filled with an opaque, viscid fluid of a white color, having granules intermingled in it, which fluid I call 'protoplasm'". (translation in Goodale, 1889).

The above exemplifies that the cellular images we obtain, aside from being determined by the resolution power of the microscope used, also depend, to a large extent, on the specimen preparation methods used, as the latter may result in numerous cellular artifacts, such as the shrinkage described by von Mohl.

Chemical fixation (CF) is the conventional method applied for preservation of samples for EM. CF involves a reaction of the sample with chemical agents, which diffuse into the sample, arrest vital processes and cross-link various moieties in the cell. Fixation is followed by dehydration in an organic solvent, embedding in a plastic resin, sectioning and contrasting with heavy metal salts. Though most of our knowledge regarding cellular structures at high resolution is based on EM of chemically fixed cells, it is known that CF causes various artifacts. The slow diffusion time of the chemical reagents reduce the time resolution of the fixation process, and hence only relatively slow cellular processes may be trapped and revealed in the final image (Plattner, 1989). Native local gradients of pH, osmolarity and ion concentrations that occur in cells and between cellular compartments are perturbed by the buffers that are used during CF (Penttila et al., 1975; Collins et al., 1977; Lee et al., 1982). In addition, a substantial fraction of the lipids, often up to 70% of the total, is extracted (Maneta-Peyret et al., 1999), organelles deform and shrink, sometimes anisotropically (Murk et al., 2003), and cellular components may even be completely washed away during CF. Collapse artifacts, especially during the dehydration step, are also apparent (Kellenberger et al., 1992; Dubochet and Blanc, 2001). The extent of these and other artifacts may differ between cell types and organelles and it seems that the cells most prone to artifacts are those which were under various stress conditions prior to fixation (Szczesny et al., 1996;

Hess, 2007; and references therein). CF will undoubtedly remain an indispensable tool for structural research, as well as for immuno-localization of proteins (van Donselaar et al., 2007). However, high fidelity of structural details requires alternative preparation methods, ones that can immobilize structural elements in dimensions ranging from the size of whole tissue down to the level of macromolecules in their native state and site.

Cryo-immobilization techniques offer a superior alternative to CF. Freezing is a purely physical process, which entails merely the extraction of kinetic energy from the specimen and, depending on the dimensions of the sample, may take only milliseconds to tens of milliseconds. Ideally, upon freezing, water in aqueous biological samples would solidify in an amorphous, vitreous state rather than change phase into deleterious ice crystals. However, the vitrification of aqueous samples occurs only when the viscosity of water increases at a faster rate than the rate of ice crystal formation. At ambient pressure this requires ultra-high cooling rates ($>10^4$°C/s; Knoll et al., 1987), which can only be achieved when the samples are very thin (<10–20 μm). Plunge freezing in liquid ethane is, indeed, the method of choice for vitrification of thin aqueous layers (Dubochet and McDowall, 1981). In contrast, the cooling rate of bulky samples (yet ≤200 μm) is relatively slow due to the low thermal conductivity of water. The only method currently available for the vitrification of such thick samples without the addition of anti-freeze agents (cryo-protectants) is high-pressure freezing (HPF) (Moor and Hoechli, 1970; Dubochet and McDowall, 1981; Moor, 1987). Applying a pressure of 2,100 bar, at which the melting temperature of water is about −22°C, affects the hydrogen bond network of the water (Stillinger and Rahman, 1974; Jonas, 1982; Lüde-mann, 1996) in a way that slows down the rate of ice crystal formation in the sample. As a result, the chance of obtaining a vitrified sample increases even at relatively slow cooling rates. During HPF, intercellular gases in plant tissue are quickly compressed or even may implode and as a result the surrounding cells may collapse. It is therefore necessary to replace intercellular gases with an uncompressible liquid, or "space filler" under light vacuum prior to HPF. Usually hexadecane is used (Michel et al., 1991), but up to 20% dextran (Al-Amoudi et al., 2004) or 8% methanol in water (Pfeiffer and Krupinska, 2005) can also be utilized (see also review by McDonald, 2007).

The most compelling possibility for processing high-pressure-frozen samples is to cryo-section them, and to directly observe the frozen hydrated sections in the cryo-transmission electron microscope. Cryo-electron microscopy of vitreous sections (CEMOVIS; Al-Amoudi et al., 2004) is the closest possible thing to observing a sample in vivo, as its components remain embedded in their native medium, namely, water. This means that, unlike dehydrated samples, aggregation artifacts should not be anticipated in frozen sections. Moreover, electrons passing through the frozen section interact directly with constituents of the sample rather than with external heavy atoms of stains bound to them. Thus, contrast variations in the final image can be directly correlated to the real positions of macromolecular complexes, membranes, fibers, etc. Despite of these advantages, CEMOVIS is still not a common practice in most EM laboratories, mainly due to the technical difficulties and artifacts related to the mechanical forces applied to the sample during sectioning. Frozen samples can be well sectioned only when they are completely vitrified (Michel et al., 1991; Hsieh et al., 2006), which is rarely the case even when HPF is applied, and hence, the yield of good sections is relatively low. Section crevasses and knife marks, as well as a compression of about 30–60% along the sectioning axis, are common results of cryo-sectioning, which limit ultrastructural analysis (Al-Amoudi et al., 2005; Hsieh et al., 2006). Often, contrast is increased only after the, already beam-sensitive, section is "ironed" under the beam, which also results in loss of fine details, as well as in bubbling (Hsieh et al., 2002; Dubochet et al., 2007). Also, the fact that contrast emanates from the sample itself may be disadvantageous as some cellular components, which are apparent after staining of plastic sections, may completely lack contrast in frozen sections. Nevertheless, when good sections are obtained they may reveal structural elements that cannot be observed by any other method (Al-Amoudi et al., 2004; Bouchet-Marquis et al., 2008). Future holds promise for turning CEMOVIS into a more reproducible and, hopefully, artifact-free process. The sectioning process may be improved by

refining conventional sectioning techniques (Han et al., 2008), as well as by utilizing new instruments and techniques, such as oscillating diamond knives (Al-Amoudi et al., 2003) or focused ion beam milling (Marko et al., 2007). It is also hoped that the inherently poor contrast of frozen sections will be enhanced by the utilization of phase plates (Nagayama and Danev, 2008), which would eliminate the need to work at large underfocus values in the transmission electron microscope in order to obtain reasonable contrast.

Currently, the most common and practical method that follows HPF is freeze substitution (FS). During FS the sample is dehydrated in an organic solvent at low temperature in the presence of chemical fixatives (van Harreveld and Crowell, 1964; Humbel and Müller, 1986). Once the sample is dehydrated, the temperature can be raised safely (no danger of crystallization), and the sample is finally infiltrated with a plastic resin, sectioned and contrasted. Unlike the pure physical processing used in CEMOVIS, FS is a hybrid method, combining the benefits of cryo-immobilization (good and fast sample preservation) with the benefits of CF, namely, working at room temperature, easy sectioning, the ability to add contrasting agents and to probe the sections with antibodies for immuno-localization. Freeze-substituted samples, when compared to chemically fixed ones, lack some of the artifacts attributed to CF and are considerably more well-preserved – their membranes appear smoother and less undulated, organelles, especially in plant tissues, do not tend to detach from their surroundings (Studer et al., 1992), distortions are absent (Szczesny et al., 1996; Murk et al., 2003) and lipid extraction is limited (Pfeiffer et al., 2000), as opposed to CF (Maneta-Peyret et al., 1999). FS is also an optimal method for preserving the antigenicity of a sample without a large sacrifice of structural fidelity (Steinbrecht and Müller, 1987; Monaghan et al., 1998). However, to some extent, samples processed by HPF-FS may suffer from aggregation artifacts during water removal (Dubochet and Blanc, 2001).

Finally, after extensive sample preparation, the electron micrographs we look at are merely two-dimensional projections of three-dimensional objects confined within a section. Hence, even if a specimen is perfectly preserved and sectioned, information about the spatial relationships between cellular constituents is inaccessible due to superposition of densities along the axis of the electron beam. The traditional method for revealing the third dimension is obtaining a series of sequential thin sections of the embedded specimen and aligning the recorded 2D images of these sections. Then, the features of interest are delineated in each of the sequential images and a surface is interpolated across the resulting contours. Serial sectioning has been very useful in revealing the 3D structure of various specimens (Sjostran, 1974; Macagno et al., 1979; Hall, 1995); however, the resolution of this method in the depth dimension (z-axis) cannot exceed twice the thickness of the section (McEwen and Marko, 1999, 2001). Thus, the resolution obtainable in serial sections is limited to tens of nanometers.

Electron microscope tomography (EMT) provides 3D information down to a resolution of about 4–6 nm (Lučić et al., 2005; Robinson et al., 2007). EMT is based on reconstruction of a 3D object from a series of projected images collected from an object rotated around an axis perpendicular to the electron beam. The projections are taken at small angular intervals of 1–2° over an angular range of ±70–80° in a computer-controlled process. This step may be followed by the acquisition of another tilt series, perpendicular to the first one (dual-axis EMT), which reduces the loss of information that results from the limited tilt range in a single direction (Penczek et al., 1995; Mastronarde et al., 1997). The individual projections are then aligned in a common frame of reference in silico and the 3D object is reconstructed, usually by weighted back-projection algorithms.

Combining EMT with cryo-immobilization preparation methods allows one to visualize highly preserved cellular structures in three dimensions, at macromolecular or near macromolecular resolution. The structure of many cellular objects that had previously been observed by means of conventional EM has been reinterpreted, new structural motifs have been found, and prevailing paradigms are being challenged. Applied to large molecular assemblies, EMT has provided invaluable insights into the structure of the ribosome (Zhao et al., 2004a, b), proteosome (Böhm et al., 2000; Medalia et al., 2002), flagellar motors (Nicastro et al., 2005; Murphy et al., 2006), nuclear pore complexes (Stoffler et al., 2003; Beck et al., 2007) and viruses (Förster et al., 2005; Cyrklaff et al., 2007;

Subramaniam et al., 2007; Dai et al., 2008). It has likewise been instrumental in revealing the intricate organization of complex, pleiomorphic structures, which impede high resolution 3D analysis by other methods, such as the cytoskeleton, in eukaryotes (Kurner et al., 2004; Hoog and Antony, 2007; O'Toole et al., 2007; Koning et al., 2008; Perkins et al., 2008) and in prokaryotes (Scheffel et al., 2006; Jensen and Briegel, 2007), and the spindle pole body of yeast (O'Toole et al., 1999; Melloy et al., 2007). In addition, EMT has revolutionized our understanding of the architecture and connectivity of complex membrane systems, such as mitochondria (Frey and Mannella, 2000; Nicastro et al., 2000; Mannella et al., 2001), the endoplasmic reticulum (Puhka et al., 2007), the Golgi apparatus (Marsh et al., 2001; Mironov et al., 2001; Mogelsvang et al., 2003), the photosynthetic networks of cyanobacteria (van de Meene et al., 2006; Nevo et al., 2007; Ting et al., 2007), and higher-plant chloroplasts (Shimoni et al., 2005; Austin et al., 2006). Indeed, the vision of obtaining highly resolved 3D information of whole cells (Konorty et al., 2008; Noske et al., 2008) or even tissues (Segui-Simarro et al., 2004; Hsieh et al., 2006), ultimately locating specific molecular complexes within the cell (sometimes referred to as 'visual proteomics' (Baumeister, 2002)) seems to be within reach.

III Thylakoid Network Organization in Cyanobacteria and Algae

A Cyanobacterial Thylakoid Networks

Cyanobacteria, introducers of oxygenic photosynthesis to Earth and the progenitors of chloroplasts in algae and plants (Gray, 1992; Des Marais, 2000; Xiong et al., 2000; Falkowski, 2006), are a diverse group of organisms. They inhabit a wide array of habitats, such as freshwater, marine, rock and soil, as well as a variety of quite extreme environments, such as hot springs and frigid deserts. Cyanobacteria include various unicellular species, as well as different colonial forms, such as filaments, sheets, balls, and heterocyst-forming cells. They exhibit a diversified set of thylakoid membrane morphologies, which along with their cellular structure and complexity reflect specific environments and physiologies, in addition to species specificities. For additional information of cyanobacterial membrane systems and dynamics refer to these excellent reviews (Liberton and Pakrasi, 2008; Mullineaux, 2008; Also see Chapter 13).

In essence, only two things distinguish the photosynthetic apparatus of cyanobacteria from that of green algae and higher plants. The first is the lack of segregation between the cell cytoplasm and the photosynthetic machinery. The second, which has a direct impact on the organization of the thylakoid membranes and the distribution of photosynthetic protein complexes within the membranes, is the molecular device they utilize for light harvesting. Unlike the small, membrane-integral, chlorophyll-containing light-harvesting protein complexes (LHC) of higher plants and green algae, cyanobacteria, as well as red algae, depend on large, water-soluble protein complexes, called phycobilisomes (PBS), as their light-harvesting antenna. The PBS contain non-chlorophyll pigments (phycoerythrin, phycocyanin, and allophycocyanin) and reside on the cytoplasmic face of the thylakoid membranes, alternately arranged on adjacent layers (Fig. 1a; Edwards and Gantt, 1971; Stanier and Cohen-Bazire, 1977). PBS are made of three major elements, core and rod proteins, both comprised of pigment-phycobilin proteins, and linker proteins, which attach the formers to the thylakoid membranes. Depending on the species and physiological or environmental conditions, the phycobilin composition and/or the size or shape of the rods may vary significantly (see reviews by Grossman et al., 1993; Adir, 2005). Protruding up to 25–45 nm from the membranes (Grossman et al., 1993), these massive, hemispherically or hemidiscoidally shaped, complexes do not allow for close packing of the thylakoids, hence preventing the segregation in lamellar structure and protein composition observed in higher plants and some algae.

In most cyanobacteria, the thylakoid network is generally arranged in multiple (3–10), onion-like concentric shells that follow the contour of the cell, surrounding the cytoplasm (Fig. 1a and b). These shells are sealed double bilayers (15–20 nm) that enclose the luminal compartment within them. This concentric arrangement poses a dilemma to how communication and intracellular transport are achieved between the compartments

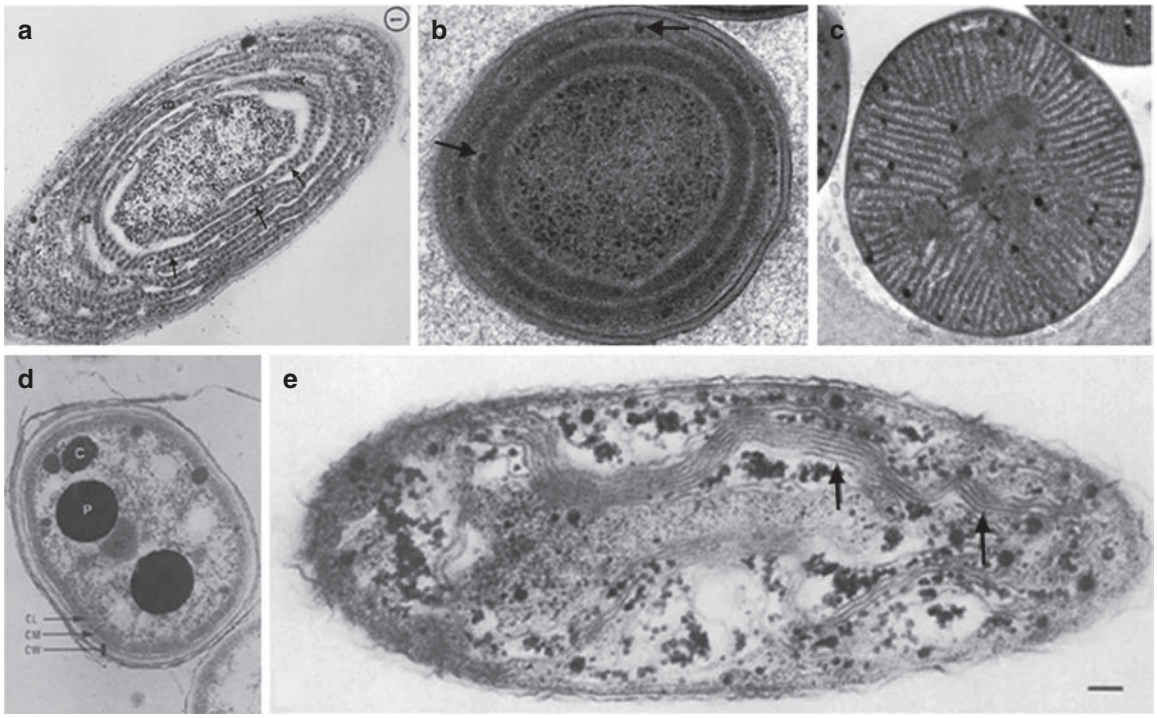

Fig. 1. Distinct thylakoid network organizations in cyanobacteria. A concentric thylakoid arrangement is common to many cyanobacteria, such as *Synechococcus lividus* (**a**) and *Synechococcus* sp. PCC 7942 (**b**). The large phycobilisome complexes, alternately arranged on the cytoplasmic faces of adjacent thylakoid layers, are apparent (*arrows*) in *S. lividus* (**a**). Often, particles, such as ribosomes and glycogen granules (*arrows*), are found between adjacent thylakoid layers (**b**). Less common morphologies have likewise been observed. One example is the radial thylakoid membrane arrangement in *Microcoleus chthonoplastes* (**c**). Other, more extreme examples include *Gloeobacter violaceus* (**d**), which is completely devoid of a thylakoid network, and *Prochlorothrix hollandica* (**e**), which lacks phycobilisomes and possesses appressed membranes (*arrows*) that are reminiscent of some green algal and higher-plant thylakoids (adapted from: (**a**) copyright Edwards and Gantt, 1971, originally published in The Journal of Cell Biology, 50: 896–900; (**d**) Rippka et al., 1974, reproduced with kind permission from Springer Science and Business Media; (**e**) Miller et al., 1988, reproduced with permission of the Company of Biologists. We thank Stolz for his kind contribution of the image in (**c**)).

created by the thylakoid layers. More specifically, routes accessible to molecular traffic between the plasma membrane and the thylakoids and between the thylakoid layers themselves must exist within the cell for proper membrane network maintenance. Quite often, various particles, such as ribosomes and glycogen granules, are observed between adjacent thylakoid layers (Fig. 1b). The cytoplasm enclosed in the center contains the site of carbon fixation – the carboxysome, numerous ribosomes, and occasionally glycogen granules and lipid bodies. This typical thylakoid organization has been observed in many cyanobacteria, such as various unicellular *Synechococcus* strains (Allen, 1968; Edwards et al., 1968; Edwards and Gantt, 1971; Nierzwicki-Bauer et al., 1983; Sherman et al., 1994), filamentous species, such as *Nostoc* (Wildon and Mercer, 1963), *Microcoleus* sp. (Nevo et al., 2007) and others (Stanier and Cohen-Bazire, 1977).

Accompanying the general architecture are various structural features that have been observed in different cyanobacterial cells. A common one, as seen in *Synechococcus* sp. PCC 7002 (Nierzwicki-Bauer et al., 1983), members of *Anabaena*, *Dermocarpa*, *Gleocapsa* and *Pleurocapsa* (Kunkel, 1982), as well as in *Synechocystis* sp. PCC 6803 (van de Meene et al., 2006) and others (Whitton et al., 1971; Stanier and Cohen-Bazire, 1977), is the convergence of thylakoid layers at peripheral sites near the plasma membrane. In some of the species, the thylakoids at these sites were observed to be associated with fibrous-coated cylinders termed ‘thylakoid centers’

(Fig. 2a; Kunkel, 1982; van de Meene et al., 2006). Detailed 3D models of the convergence of thylakoid sheets to thylakoid centers, including the precise organization of the sheets around the centers, were recently generated from tomograms of *Synechocystis* sp. PCC 6803 cells (van de Meene et al., 2006).

Even though the thylakoid layers converge quite closely to the plasma membrane, the existence of a direct connection between the two membrane systems has been under debate. Such a direct connection would have important implications on the biogenesis of the thylakoid network, as well as on the transport of material between the two membrane systems (Mullineaux, 1999). Some works report close, but rare and sometimes not conclusively direct, connections (Allen, 1968; Nierzwicki-Bauer et al., 1983; van de Meene et al., 2006), while others suggest that the thylakoid network is a separate entity from the plasma membrane (see review by Stanier and Cohen-Bazire, 1977; Liberton et al., 2006).

Another feature that has been observed in thylakoid networks of several members of the *Cyanothece* group is that, in addition to the typical arrangement of several parallel concentric shells, the two innermost layers protrude to form spherical enclosures. Serial section examination

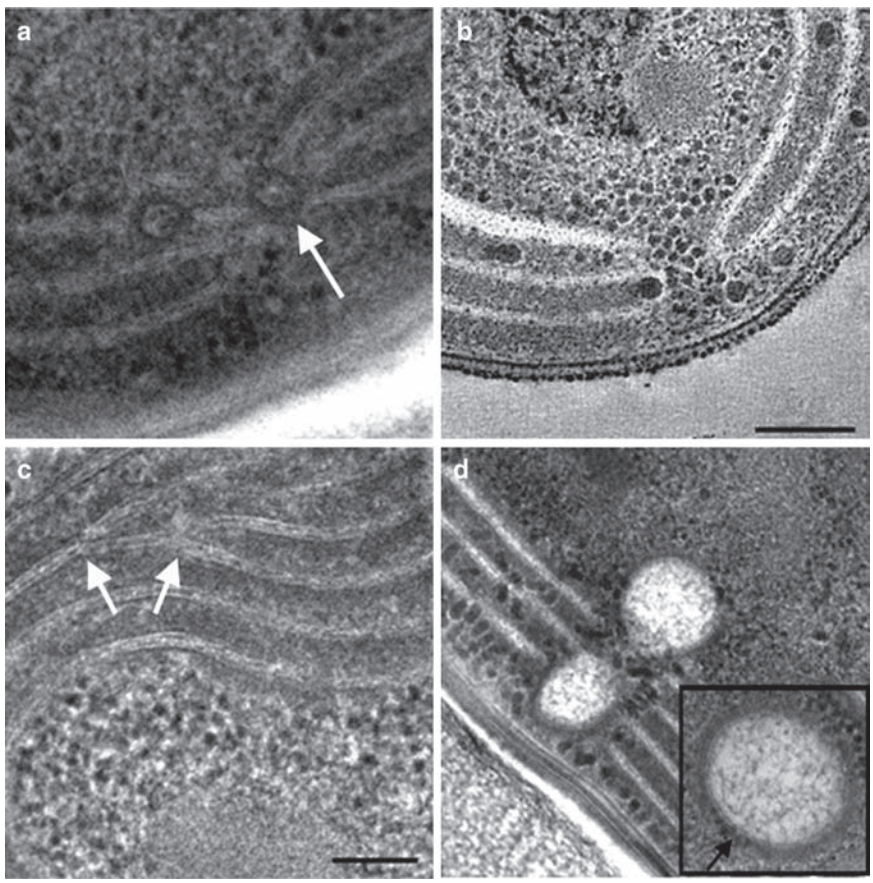

Fig. 2. Structural features of cyanobacterial thylakoid membranes. Convergence of thylakoid membrane to regions near the plasma membrane is quite common. In some species, such as *Synechocystis* PCC 6803, the membranes converge to cylindrical structures termed 'thylakoid centers' (**a**, *arrow*). Thylakoid membrane perforations or gaps (**b**) and interconnections (**c**) in different cyanobacteria facilitate molecular transport across and within the lamellar system. The abundance of ribosomes in the vicinity of the perforations (**b**) suggests that, in addition to allowing unperturbed transport, they serve as sites for co-translational insertion of proteins. Connectivity within the thylakoid network involves branching, fusion (**c**) and interconnections of adjacent layers. Large, membrane-bounded (**d** inset, *arrow*) vesicles observed in *Microcoleus* sp. often appear to pass through the perforations (**d**) (adapted from: (**a**) van de Meene et al., 2006, with kind permission from Springer Science and Business Media and the author; (**b**–**d**) Nevo et al., 2007).

revealed that the layers forming these enclosures create bottleneck connections with the concentric thylakoids from which they originate. The thylakoid enclosures contained ribosome-rich cytoplasm and spherical electron-translucent granules (Porta et al., 2000). Similar vesicule-shaped enclosures were also observed in other cyanobacteria, such as *Nostoc* (Blumwald and Tel-Or, 1982).

Thylakoid morphologies that differ somewhat from the canonical organization have also been observed. For example, the well-characterized *Synechocystis* sp. PCC 6803 possesses thylakoid sheets that vary in shape and size, and form large loops and whorls in the cell interior (Liberton et al., 2006). Somewhat similar are the thylakoids in *Synechocystis* sp. PCC 6711, which are convoluted and occupy a rather large portion of the cytoplasm (Stanier, 1988). Other, more unusual, thylakoid structures are found in some members that belong to *Cyanothece* (other than the ones described above), which have a thylakoid arrangement consisting of numerous, short and wavy thylakoids that are arranged radially and sometimes in bundles (Reddy et al., 1993; Komarek and Cepak, 1998). A radial thylakoid arrangement has also been observed in *Microcoleus chthonoplastes* (Fig. 1c; Stolz, 2007). In some other cyanobacteria, the thylakoid membranes are randomly arranged within the cell (Gantt and Conti, 1969).

A solution to the longstanding issue of how transport and communication are carried out within cells that are seemingly separated into isolated compartments by the thylakoid layers has recently been provided by EMT studies. Dual-axis tomography of two distantly related cyanobacteria, *Synechococcus* sp. PCC 7942 and the filamentous *Microcoleus* sp., revealed numerous common architectural designs (Nevo et al., 2007). The photosynthetic membranes of both species contain multiple perforations while their sac-like integrity is maintained. The perforations, which are filled with various particles, such as ribosomes, glycogen granules and lipid bodies (Fig. 2b), allow for molecular traffic throughout the cell and reveal that the central cytoplasm is actually not an isolated compartment. Notably, the abundance of ribosomes in and around the perforations suggests that, in addition to allowing feasible passage of particles, these are also sites for co-translational insertion of proteins into the thylakoids. Different thylakoid layers, which appear independent of each other in 2D sections, in fact, branch or split and fuse to other layers (Fig. 2c). This essentially results in an interconnected system that encloses a single lumen and thus provides a continuous media for trafficking of both water- or lipid-soluble substances throughout the entire network (Nevo et al., 2007).

Membrane gaps, similar in essence to the perforations observed in *Synechococcus* sp. PCC 7942 and *Microcoleus* sp., were also noted in the marine cyanobacterium *Prochlorococcus* in a cryo-electron tomography study (Ting et al., 2007). The membrane gaps appear in regions where the thylakoid layers 'terminate' or are interconnected with other layers. The passageways formed by the gaps allow for continuity of the central cytoplasmic space with that found at peripheral sites of the cell. Connections between adjacent thylakoid layers were also apparent in *Prochlorococcus* (Ting et al., 2007). It is noteworthy to mention that *Prochlorococcus* belongs to the Prochlorophytes, a group that belongs to the cyanobacteria yet differs from most members in some aspects (see below). However, the architectural motifs that allow for viable intracellular trafficking are probably conserved.

Notably, electron tomography of *Microcoleus* sp. cells revealed large vesicles delimited by a membrane bilayer enclosing a granular matrix (Nevo et al., 2007). These vesicles were dispersed throughout the cells, appearing in the cell center, periphery, and inside the perforations in the thylakoid membranes, where they appear to be caught in transit between the plasma membrane and the cell interior (Fig. 2d). Examination of the vesicles revealed that they are either isolated or quite often, fused or connected to the thylakoid membranes or to other vesicles. Based on these observations, we propose that they may serve in the transport of certain photosynthetic complexes from the cell membrane, where early steps of their biogenesis take place, to the thylakoids. This shuttling mechanism may be similar to the vesicular transport system of chloroplasts (Westphal et al., 2001, 2003).

Finally, some cyanobacteria exhibit highly unusual organizations. These, which range from complete lack of thylakoids to arrangements reminiscent of those found in green algae and higher-plant chloroplasts, reflect evolution of the thylakoid network from the most primitive cyanobacteria *en route* to present-day chloroplasts.

The former situation is encountered in the small unicellular cyanobacterium *Gloeobacter violaceus*, which lacks thylakoid membranes (Fig. 1d). Evolutionary studies show that *Gloeobacter* is one of the earliest branching cyanobacteria (Swingley et al., 2008 and references therein). Supporting this phylogenetic distance is the sequenced genome of *Gloeobacter*, which lacks many of the genes that encode for PS I, PS II and phycobilisome protein subunits that are found in most other cyanobacteria (Nakamura et al., 2003). Photosynthesis in *Gloeobacter* is carried out in its sole membrane unit, the plasma membrane. Light-harvesting is achieved by a reduced PBS complex made exclusively of rod-shaped phycobiliprotein bundles that are oriented perpendicularly to the plasma membrane and hosted within an unusual electron-dense layer that lies beneath the membrane (Rippka et al., 1974; Guglielmi et al., 1981).

At the other extreme are members that belong to the Prochlorophytes, whose classification has not always been straightforward. This group differs from other cyanobacteria in their photosynthetic pigment composition, lacking the characteristic phycobilins, while possessing both chlorophylls *a* and *b*, as in higher plants and green algae. Thus, and even though in other aspects the Prochlorophytes are quite similar to cyanobacteria, they have not always been regarded as cyanobacteria (reviewed in (Matthijs et al., 1994; Lewin, 2002). Several thin-section and freeze-fracture studies reveal that the thylakoids in some species that belong to this group are organized differently than those of typical cyanobacteria. Tight packing or appression of thylakoid layers as seen in *Prochloron* sp. (Giddings et al., 1980), *Prochlorothrix hollandica* (Fig. 1e; (Miller et al., 1988) and *Prochlorococcus marinus* (Chisholm et al., 1988), are possible due to their lack of phycobilisomes, as the latter oblige adjacent thylakoids to remain separated. Notably, in these species, lateral heterogeneity of the two photosystems, as in higher plants and green algae, has been observed (Giddings et al., 1980; Chisholm et al., 1988; Miller et al., 1988).

B Algal Thylakoid Networks

As discussed above, two major changes had accompanied the evolutionary transition from cyanobacteria to algae and higher plants. The first, the primary endosymbiotic event of an ancient cyanobacterium, likely similar to *Synechocystis* sp., by a non-photosynthetic eukaryotic host, is the source of all plastids (Palmer, 2000). The engulfed cyanobacterium, now surrounded by two membranes, eventually became a discrete cellular organelle, the chloroplast. The three distinct membrane units of the chloroplast, the inner and outer envelope membranes – relics of the endosymbiotic event (Falkowski et al., 2004) – and the thylakoid membrane network, form three unique soluble compartments within the organelle: the inter-membrane space (between the two envelope membranes), the stroma – a soluble matrix that surrounds the thylakoids and serves as the site of carbon fixation, and the lumen enclosed within the thylakoids. The chloroplast had undergone numerous genetic and morphological alterations during plastid evolution, which began with the primary endosymbiosis and continued along the different branches that gave rise to algae and higher plants. Among these changes, multiple genetic transfers, deletions and duplications involving both the plastid and nuclear genomes had taken place, necessitating tightly regulated communication between the two organelles. The second major change, loss of the bulky soluble phycobilisomes, whose function was replaced by the flat, membrane-integral light-harvesting complexes, had direct consequences on thylakoid network structure.

Ultimately, all algae are of monophyletic origin; their plastids evolved from the primary endosymbiont that marked the transition between prokaryotic and eukaryotic photosynthetic organisms. The phylogenetic relationships among algae, however, are quite complex as they are of both paraphyletic and polyphyletic origin. Algae are a diverse group of organisms prevalent in most habitats including various terrestrial ones, marine, freshwater, hot springs and ice. They vary from small, unicellular flagellate forms to non-motile and colonial forms and to highly complex multicellular organisms that possess full tissue differentiation. The latter include giant seaweeds, which consist of several groups of multicellular red, green and brown algae.

Red algae are the plastid-containing organisms that are evolutionary closest to the aforementioned endosymbiotic event. In these algae, the thylakoid network is enclosed within chloroplasts, yet, like cyanobacteria, they possess phycobilisomes as their primary light-harvesting complexes [although they also have Chl *a*-binding LHC proteins (Wolfe et al., 1994)]. Hence, the thylakoid

membrane organization in red algae is quite similar to that of cyanobacteria. One of the most studied red algae is *Porphyridium cruentum*, a unicellular, marine alga. *Porphyridium* cells contain a single stellar- or amoeboid-shaped chloroplast that occupies a major portion of the cell volume. The thylakoid membranes are arranged along the periphery of the chloroplast in a parallel fashion, with adjacent lamellae equidistant from one another (Fig. 3a). Some thylakoids are randomly

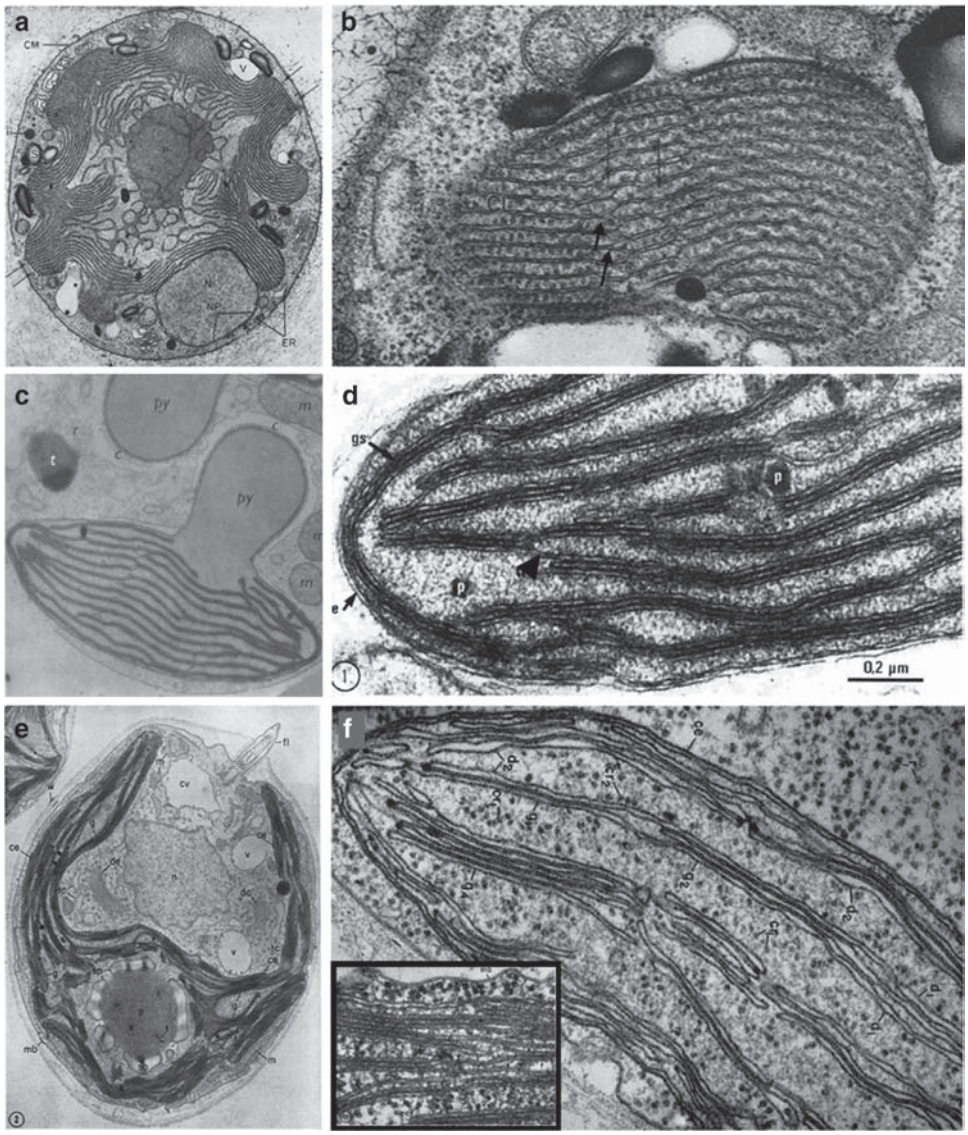

Fig. 3. Thylakoid network organization in red, brown and green algae. Possessing phycobilisomes as their light-harvesting antenna, the thylakoid network of the red alga *Porphyridium cruentum* is quite similar to that of cyanobacteria with unstacked, parallel layers arranged in the periphery of the chloroplast (**a**). Often, interconnections between thylakoid layers are observed (**b**, *arrows*). Brown algal thylakoids are organized into bands usually comprising three stacked layers, which run parallel to each other along the contour of the chloroplast membranes (**c**, **d**). Connectivity in the system is maintained by frequent crossings of layers from one band to another (**d**, *arrowhead*). The single chloroplast in the green alga *Chlamydomonas reinhardtii* is lobed and occupies most of the cell volume (**e**). The thylakoid membranes are mostly appressed with 2–20 layers in each stack (**f** inset). Following the greening of a mutant that is devoid of thylakoids in the dark provided valuable insight into the biogenesis of the network. After 9 h of illumination, the thylakoid network is very similar to mature, light-adapted one (**f**) (adapted from: (**a** and **b**) copyright Gantt and Conti, 1965, originally published in The Journal of Cell Biology, 26: 365–381; (**c**) Evans, 1966, reproduced with permission of the Company of Biologists; (**d**) Berkaloff et al., 1983, reproduced with permission of Blackwell; (**e** and **f**) copyright Ohad et al., 1967a, b originally published in The Journal of Cell Biology 35: 521–551 and 35: 553–584 with kind permission of the author).

arranged in and around the pyrenoid – a central opaque region, which is the site of carbon fixation in algae (Brody and Vatter, 1959; Gantt and Conti, 1965). As in cyanobacteria, the PBS in *Porphyridium* (phycobilisomes were, in fact, first observed in this alga) are situated on the outer thylakoid surface and alternately arranged on adjacent thylakoid layers (Gantt and Conti, 1965, 1966). Quite often, and also very similar to cyanobacteria, interconnections between thylakoid layers are observed. Fusion and branching of the lamellae occasionally form a honey-comb pattern (Fig. 3b; Gantt and Conti, 1965).

During evolution, loss of the light-harvesting phycobilisomes has occurred in two independent instances, as they are missing in organisms belonging to two different lineages that were derived from the primary endosymbiotic event. These organisms include some of the algal divisions that were derived from secondary endosymbiosis of red algae, and thus are accounted as part of the red algal lineage, and members of the green lineage, which includes green algae and land plants (Durnford et al., 1999; Simpson and Stern, 2002). Here we will consider two representatives of the above lineages, brown and green algae.

Being secondary endosymbionts of red algae (Cavalier-Smith, 2000), brown algae have chloroplasts surrounded by four membranes, with the outer two being continuous with the endoplasmic reticulum and the outer membrane of the nucleus. The thylakoid membrane arrangement in brown algae differs from that of cyanobacteria and red algae, partly because they lack PBS, which do not allow for membrane appressions. In different brown algae examined (*Chorda*, *Fucus* and *Giffordia* (Bouck, 1965; Berkaloff et al., 1983), *Dictyota* (Neushul, 1971) and several others (Evans, 1966), the thylakoid layers are organized into stacks or bands of three layers (Fig. 3c and d) often regarded as "three-associated thylakoids" (Gibbs, 1970; Berkaloff et al., 1983; Lichtle et al., 1992). The bands run parallel to each other along most of the chloroplast length and sometimes appear to join a peripheral or girdle thylakoid stack that follows the contour of the oval-shaped chloroplast (Fig. 3c). Similarly to cyanobacteria and red algae, thylakoids belonging to different layers (here bands) join to each other (as in *Fucus serratus* Fig. 3d, arrowhead; Bouck, 1965; Berkaloff et al., 1983). Unlike their red algae ancestors, thylakoids in brown algae rarely enter or cross the pyrenoid, which, when present, also appears as an out-pocketing of the chloroplast (e.g., in *Pylaiella littoralis*, see Fig. 3c; Bouck, 1965; Evans, 1966). At a first glance, the close proximity of the thylakoid layers in brown algae gives a sense of similarity to grana stacks of green algae and higher plants. Nevertheless, these bands are neither structurally nor functionally equivalent to grana. Support of this distinction comes from both freeze-etching studies of *Dictyota*, suggesting structural similarity of the thylakoids to those of the red alga *Porphyridium* (Neushul, 1971), and from immunolabeling studies, showing that the LHC of brown algae (*Laminaria* and *Fucus*) is distributed evenly along the entire length of the thylakoids, both on membranes facing the stroma matrix or on ones buried within the stacks (Grebvy et al., 1989; Lichtle et al., 1992).

Green algae are the algal group most closely related to land plants; in fact, some green algae share a more recent common ancestor with land plants than with other green algae (McCourt, 1995). The thylakoid organization of green algal chloroplasts is not uniform. In some, the thylakoid structure is rather simple, somewhat similar to that of brown algae. In other species, on the other hand, it is almost indistinguishable from that of higher plants.

A representative of the former group is the most studied green alga, *Chlamydomonas reinhardtii*. This unicellular alga possesses one lobed chloroplast, which occupies most of the cell volume (Fig. 3e). Situated at the posterior part of the chloroplast is the pyrenoid, which is traversed by a tubular system that is directly connected to the thylakoid membranes. The thylakoids in *C. reinhardtii* are stacked (with the number of layers ranging from 2 to 20; e.g., see Fig. 3f inset) along most of the chloroplast length, but not as uniformly as the three-layered thylakoid bands of brown algae. Quite often, unstacked lamellae, which are usually extensions of peripheral layers of the stacks, are observed to bifurcate and fuse with other stacks. These unstacked regions, however, do not extend over long distances like higher plant stroma lamellae (Ohad et al., 1967a, b; Goodenough and Levine, 1969). A similar thylakoid organization is also typical of other green algae, such as *Chlorella*, *Oocystis marssoni* and others (Pendland and Aldrich, 1973; Das, 1975; Meisch et al., 1980).

Work on the *C. reinhardtii* y-1 mutant (which does not synthesize chlorophyll in the dark, yet is indistinguishable from the wild type in the light) has provided valuable insight into the biogenesis of the thylakoid membranes. Dark grown y-1 cells (virtually thylakoid-less) were transferred to the light (greening process), and the thylakoids were observed after different times. Upon illumination, thylakoid remnants (from the dark), mostly circular and some elongated, were gradually replaced by considerably longer lamellae that transformed into granum discs by aligning in parallel and becoming closely paired to one another. These events were accompanied by bending, branching and fusion of discs to form grana with some of the constituent layers appearing to share a continuous lumen. After 9 h of illumination, the thylakoid morphology became quite similar to the light-adapted one (Fig. 3f; Ohad et al., 1967a, b).

The thylakoid organization in other species of green algae, e.g., *Carteria*, *Nitella*, and *Acetabularia* (Crawley, 1964; Lembi and Lang, 1965; Menke, 1966), is very much like that of higher plants. In these species, the thylakoid membranes exhibit a highly differentiated morphology with well-defined granal and stroma lamellar domains, which goes hand in hand with the lateral heterogeneity of the major photosynthetic protein complexes. These intricate morphologies, the most complex of thylakoid membranes and perhaps of any lamellar system existing in cells, are discussed in the next section.

IV Thylakoid Networks of Higher Plants

A Ultrastructure and Three-Dimensional Organization

The creation of an ozone layer, with its ability to filter UVC and some of the UVB radiation, allowed the occupation of terrestrial habitats ca. 500 million years ago during the Ordovician period (Rozema et al., 2002; Falkowski et al., 2004). Land plants are believed to have evolved from the *Charophyceae*, a small class of green algae that inhabit mostly freshwater. Consequently, higher plants have significant similarities to some of the green algae, including in chloroplast and thylakoid structure (Kenrick and Crane, 1997).

The most notable characteristic of higher-plant thylakoid membranes is their sharp segregation into two distinct morphological domains: grana – cylindrical stacked structures approximately 0.3–0.6 μm in diameter, containing a varying number (between 10 and 100, depending on species and light conditions) of tightly packed thylakoids, and stroma lamellae – unstacked paired-membranes, which interconnect the grana (Fig. 4). These two domains are organized in a complex 3D lamellar network that encloses a single lumen (Mustardy, 1996).

The differentiation of thylakoids into granum and stroma lamellar domains is thought to be a morphological reflection of the uneven distribution of the photosynthetic protein complexes within the membranes (Andersson and Anderson, 1980); for excellent reviews of this topic, refer to (Anderson, 1999; Albertsson, 2001; Staehelin, 2003). Having flat stromal-exposed surfaces, photosystem II (PS II) and its light-harvesting antenna complex, LHCII, can be accommodated in the appressed regions of granum stacks. By contrast, photosystem I (PS I) and the CF_0/CF_1 ATP synthase, which protrude significantly into the stroma, can only populate non-appressed membrane regions, which include the stroma lamellae and grana end membranes and margins (Fig. 5).

Of the above protein complexes, LHCII is unique in the sense that it acts as a major stabilizer of granal membrane stacking. This stabilization involves both specific and non-specific interactions (also see Section IV.B) and has been demonstrated in several ways. Thylakoids of a Chl *b*-less barley mutant, which contain little or no LHCII, were observed to have poorly stacked granal domains (Goodchild et al., 1966). Purified LHCII was shown to self-interact to generate appressed planar sheets. Likewise, when incorporated into vesicles, it mediated their stacking. Pretreatment of the antenna complex with a protease inhibited the formation of stacks, presumably due to a change in the overall charge of the complex (Goodchild et al., 1966). In addition, ordered lamellar structures have been observed to form upon mixing of LHCII with any of the principal lipids of the thylakoid membranes (McDonnel and Staehelin, 1980); notably, even with the non-bilayer forming lipid monogalactosyldiacylglycerol (Simidjiev et al., 2000).

As already mentioned in the Introduction, the study of higher-plant chloroplasts and, subsequently,

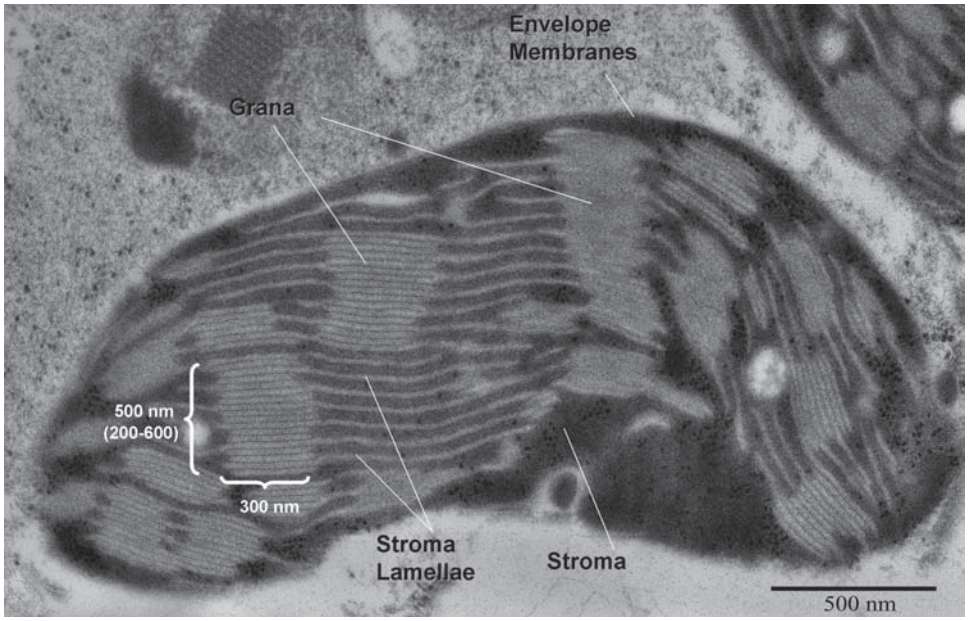

Fig. 4. Thylakoid membrane organization in higher-plant chloroplasts. The grana appear as cylindrical stacks measuring ~300 nm in diameter and 200–600 nm in height. Multiple strips of paired membranes, the stroma lamellae, which run roughly parallel to each other, connect these stacks. The average thickness of the thylakoids of both the grana and stroma is ~20 nm; within the grana, the surfaces of adjacent layers are separated by 3–4 nm. Ribosomes, which appear as heavily stained particles, are localized at the periphery of the thylakoids or in the empty spaces between neighboring stroma lamellae or near grana-end membranes (the top- and bottom-most layers of the grana).

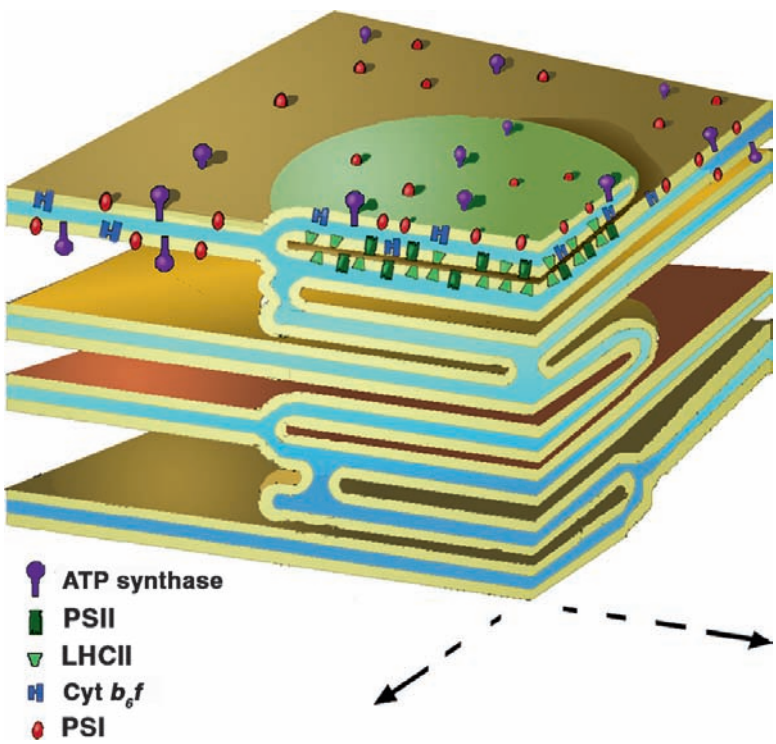

Fig. 5. Distribution of the major photosynthetic complexes within the thylakoid network of higher plants. PS II and its cognate light-harvesting complex, LHCII, are concentrated mainly in the appressed granal domains whereas PS I and ATP synthase populate non-appressed regions, which include the stroma lamellae and grana end membranes and margins. The other major photosynthetic complex, cytochrome b_6f, is distributed roughly evenly between the two membrane domains. (Note that, for clarity, only representatives of each type of protein complex are drawn; the actual protein density is much higher) (See Color Fig. 15 on Color Plate 13).

of their thylakoid membranes had begun in the mid nineteenth century, when only the light microscope with its ~0.2 μm resolution was available. Following the descriptions of chloroplasts by von Mohl (1837) and Unger (1848) as 'chlorophyll granules' (von Mohl, 1837) and 'pigment-bound structures' (reviewed in Vothknecht and Westhoff, 2001), the term 'plastid' was introduced by Schimper (1883) to remain, although now used in a broader sense to describe pigment-containing organelles in plant cells. Minuscule granulations – dots seen in the comparatively transparent surrounding of the chloroplast, led to the birth of 'granum (pl. grana)' and 'stroma' (Meyer, 1883; Schimper, 1885), thus achieving the fullest structural description of the chloroplast that can be obtained by visible-light microscopy (Vothknecht and Westhoff, 2001; Staehelin, 2003).

Going beyond 'grana' and 'stroma' necessitated shorter wavelengths, as well as higher magnification power. Evidence for morphological heterogeneity in higher-plant chloroplast thylakoid membranes came initially from UV micrographs, in which dark grana structures could be seen engulfed by lighter colored stroma lamellae (Doutreligne, 1935; Heitz, 1936; Menke, 1940a). This leap in information was soon followed by an even larger one, enabled by a new type of microscope, the electron microscope, built in 1931 by Ernst Ruska and Max Knoll and made commercially available by Siemens in 1939. A year later, Kausche and Ruska's brother, Helmut, who were hired by Siemens to work on applications of the microscope, published the first electron micrographs of chloroplasts (Kausche and Ruska, 1940). The same year, Wilhelm Menke used EM to visualize leaf preparations of *Aspridistra elatior*, *Nicotiana rustica* and *Spinacia oleracea* with a ×25,000 magnification (Menke, 1940b). Unfortunately, sample preparation methods in these groundbreaking works were quite aggressive, leaving chloroplasts in a fractured and distorted shape, which only hinted at the complexity of their structure. Advancements in fixation methods yielded much better images a few years later, with several publications clearly showing granal stacks (Algera et al., 1947; Granick and Porter, 1947) and others showing 2D renditions of lamellae bifurcations (Hodge et al., 1955; Steinmann and Sjostrand, 1955), although misinterpreting the staining to be a compound lipid-coated protein layer, about 125 Å in thickness (Hodge et al., 1955; Menke, 1962). Soon after Menke's term 'thylakoid' was introduced (Menke, 1962), the first evidence for a double membrane system enclosing an aqueous lumen was provided by Heslop-Harrison and co-workers using permanganate staining (Heslop-Harrison, 1963). In the mid 1960s, the ultrastructure of thylakoid membranes, namely, the structure of the thylakoid vesicle, the partition of the membranes into stroma and grana lamellae, and the spacing between neighboring layers in the latter, had been established.

Understanding the spatial organization of the thylakoid membranes and the manner by which the distinct, yet intimately linked, grana and stroma lamellar domains are connected to each other was the next matter. Although several models had been proposed in the 1950s (Hodge et al., 1955; Steinmann and Sjostrand, 1955), the model proposed by Menke (1960), in which ordered arrays of grana stacks are traversed by equally-spaced, perpendicularly oriented stroma lamellar sheets (Fig. 6a) is usually considered to be the first model that describes higher-plant thylakoid membrane organization. Subsequently, elucidation of the double membranal nature of the thylakoid lamellae with its enclosed luminal space (Heslop-Harrison, 1963) and the emerging notion that the lumen is likely to be continuous throughout the entire lamellar system gave rise to a renewed view of the spatial relationship between grana and stroma lamellae. In 1963, Weier et al. (1963) proposed a highly interconnected fretwork in which the grana are transversely intersected at multiple planes along their long axis by the stroma lamellae, which take the form of narrow bridges or tubes (Fig. 6b). The latter was subsequently proven to be a fixation artifact (Falk and Sitte, 1963). According to an alternative model, by Heslop-Harrison (1963), the stroma lamellae, here regarded as perforated sheets rather than tubes, intersect the grana at a small angle, enabling a single stroma lamella to adjoin several layers or discs along the granum cylinder (Fig. 6c). Loculi within each disc have been proposed to be connected to the stroma lamellae through narrow tubular openings, dubbed frets.

The notion that a single stroma lamellar sheet can be connected to multiple loculi present on

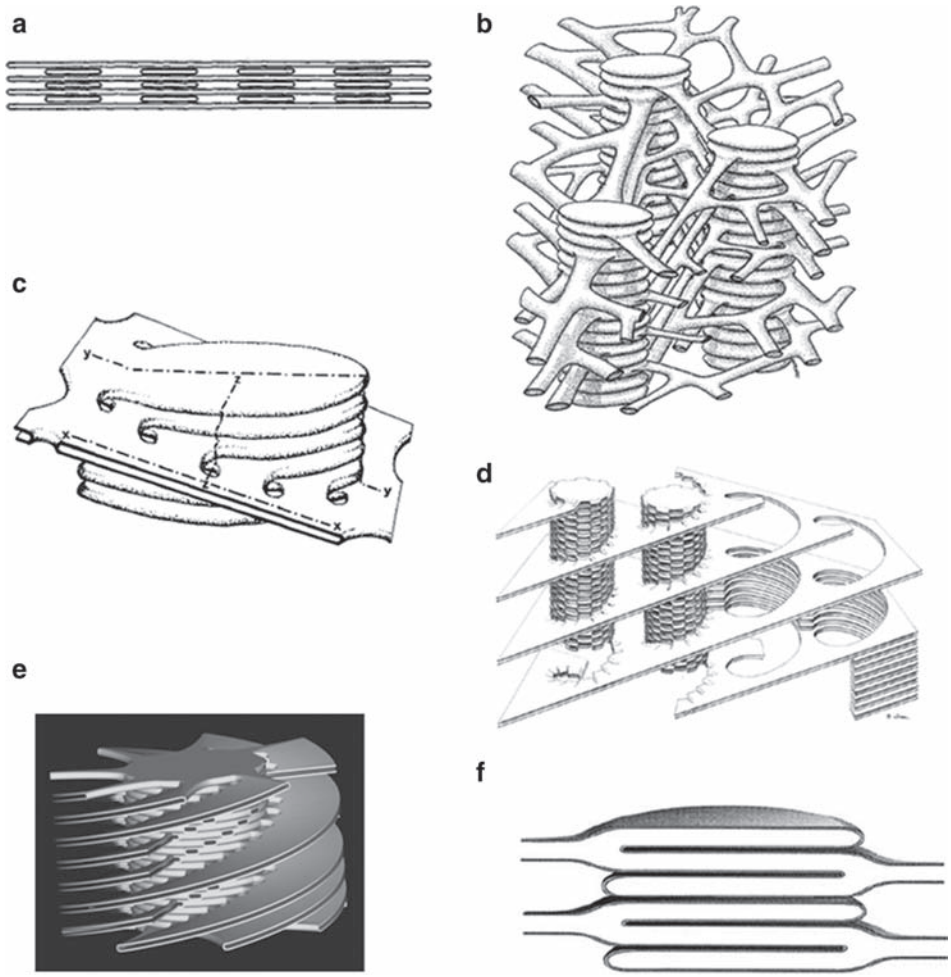

Fig. 6. Models of higher-plant thylakoid membrane organization. (**a**) A model proposed by Menke (1960) showing horizontal sheets, the stroma lamellae, traversing appressed membrane domains, the grana. (**b**) A tubular fretwork arrangement proposed by Weier (1963). (**c**) A model proposed by Heslop–Harrison in 1963 in which a single stroma lamellar sheet intersects a granum at an angle to its axis and, therefore, is able to connect to several layers within the granum body. (**d**) The helical fretwork model, introduced by Wehrmeyer (1964) and modified by Paolillo (1970), suggests that the stroma lamellae wind around the grana and join them at multiple sites. (**e**) A computer graphic model by Mustárdy and Garab (2003) based on full serial sectioning of a granum–stroma assembly showing the helical arrangement of the stroma membranes around the granum. The two membrane types are joined by numerous slits. (**f**) The folded-membrane model proposed by Arvidsson and Sundby (1999) to account for rapid grana stacking and unstacking. The network is constructed by of a single continuous membrane which folds on itself to form the granum stacks (adapted from: (**a**) Menke, 1960, (**b**) Weier, 1963 and (**c**) Heslop-Harrison, 1963, with kind permission from Springer Science and Business Media; (**d**) Paolillo, 1970), reproduced with permission of the Company of Biologists; (**e**) Mustárdy and Garab, 2003, reprinted with permission from Elsevier and the author; (F) Arvidsson and Sundby, 1999, reproduced with permission from The Australian Journal of Plant Physiology 27(7): 687–694, copyright CSIRO Publishing, Melbourne Australia and with kind permission of the authors).

different layers within a granum stack is appealing since such an arrangement greatly increases the connectivity of the system. The problem with the model proposed by Heslop-Harrison (1963) is that it requires the stroma lamellae to run obliquely to the grana that they intersect, whereas median sections of grana typically reveal horizontal intersections between the two. The natural solution to this problem was a helical arrangement in which the stroma lamellae wind around the granum bodies. The helical fretwork model was introduced by Wehrmeyer (1964) and extensively investigated and modified by Paolillo (Fig. 6d; Paolillo, 1970), who proposed that multiple

helical frets surround a single granum (Paolillo and Falk, 1966; Paolillo et al., 1969) and, following a detailed serial thin-section analysis, that the helices are right-handed (Paolillo, 1970). The helical fret model gained favor in lieu of evidence from other investigations, which showed that the apparent tilt of the stroma lamellae in serial thin-sections was inverted between sections in front and behind the granum, suggesting a pitch-rise of the fretwork connecting the stroma lamellae and the granum (Brangeon and Mustárdy, 1979). Further support came from scanning electron microscopy of sonicated chloroplasts showing spiraling lamellae surrounding isolated granum bodies (Mustárdy and Janossy, 1979), and from freeze-fracture EM showing stroma lamellae that are circularly arranged around a granum body (Staehelin, 1986). A detailed description of this model, including computerized graphic representations (Fig. 6e), can be found in an article by Mustárdy and Garab (2003), who also discuss the implications the structure has on the function and plasticity of the thylakoid network.

A common feature of the models proposed described so far is that they place strong geometrical constraints on the ability of the granum–stroma assembly and, consequently of the entire lamellar network, to undergo structural rearrangements. Nonetheless, as will be discussed in the next section, higher-plant thylakoid membranes have been observed to undergo massive rearrangements to the extent of complete unstacking of the appressed granal domains. This led Arvidsson and Sundby (1999) to propose a model in which the grana assume a significantly less constraining, highly flexible organization capable of rapid unwinding. This model assumes that the thylakoid network is constructed by folding of a single continuous membrane. Symmetrical bifurcations, made at different planes, lead to invaginations that fold on themselves to form granal stacks (Fig. 6f). The result is a regularly folded structure, which is stabilized solely by surface interactions between appressed thylakoid membranes and, thus, can be readily dismantled.

Combining dual-axis electron microscope tomography with high-pressure freezing and freeze substitution, we recently determined the 3D organization of thylakoid networks within dark-adapted lettuce leaves at ~5 nm resolution (Shimoni et al., 2005). The structures revealed that the granum–stroma assembly is formed by bifurcations (quite similar to those proposed by Arvidsson and Sundby) of the stroma lamellar membranes into multiple, roughly parallel, discs (Fig. 7). The stromal membranes form wide, slightly undulating, lamellar sheets that intersect the granum body perpendicular to the granum cylinder. Each sheet enters and exits the granum at approximately the same plane, which is at odds with the proposed helical arrangement of the stroma lamellae around the grana. Most notably, we found that the layers that constitute the granum stack are directly connected to each other. This is achieved by upward and downward bending of the granum discs at their edges, allowing them to join their upper and lower neighbors. Interestingly, adjacent granum layers are rotated counterclockwise relative to each other along the granum axis, giving rise to a cholesteric-like organization with right-handed asymmetry.

The combination of bifurcations of the stroma lamellae and the vertical connections between adjacent granal thylakoids leads to a highly interconnected structure that encloses a single, continuous lumen. This internal connectivity may facilitate the equilibration of reduced plastoquinone and/or proton gradients across or along the granum, as well as the trafficking of proteins and lipids between the granum layers and between granal and stroma lamellar domains. It is also expected to endow the network with substantial mechanical strength. As will be discussed in the next section, the interwoven nature of the assembly also has important implications on the stacking/unstacking response of grana to variations in light conditions.

How do higher-plant thylakoid networks form? Investigations of meristematic tissues, leaves at various developmental stages and leaf senescence during seasonal changes (Kutik, 1998), as well as greening of etioplasts in roots (Webb, 1982) and etiolated seedlings (Robertson and Laetsch, 1974) were carried out to address this question. Under natural developmental conditions, chloroplasts develop from undifferentiated plastids called proplastids, which are smaller, spherical or irregularly-shaped and contain few, small, single thylakoids and vesicular structures (reviewed in Lopez-Juez and Pyke, 2005). The membrane material that forms the thylakoids originates from the inner envelope in the form of invaginations or

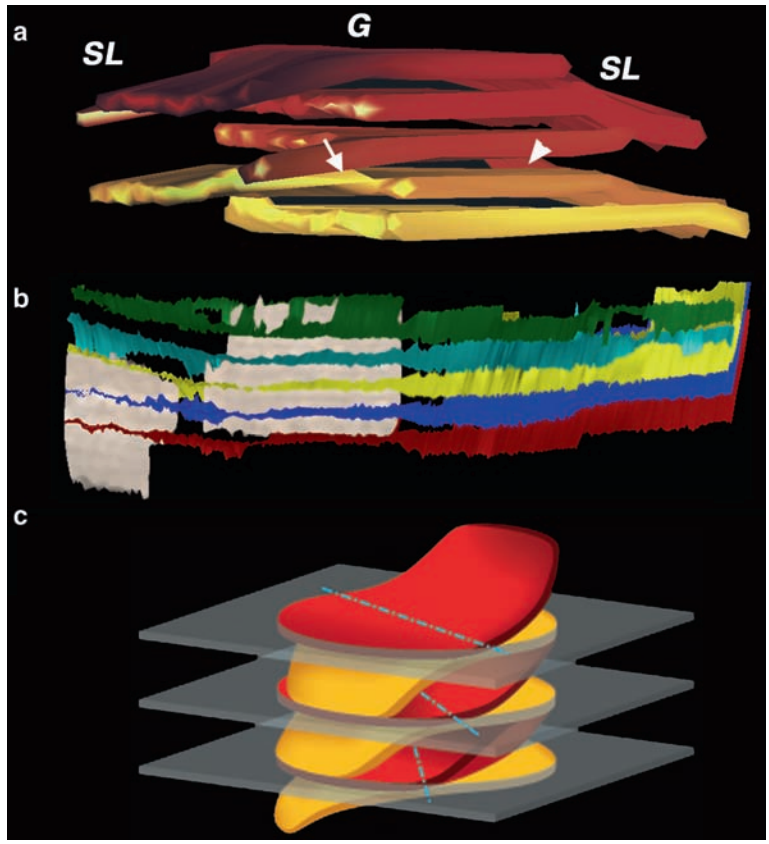

Fig. 7. Architecture of higher-plant thylakoid networks as derived from dual-axis tomographic data. (**a**) Three-dimensional structure of a granum–stroma assembly of a dark-adapted lettuce chloroplast. The granum layers (G) are contiguous with the stroma thylakoids (SL) that bifurcate at the granum–stroma interface. Adjacent layers in the granum are joined by internal connections (*arrows*). (**b**) An ensemble consisting of two grana interconnected by multiple stroma lamellae. The stroma lamellae (colored) form wide, slightly curved sheets that run parallel to each other and intersect the grana (white/gray objects) at an angle roughly perpendicular to the axis of the granum cylinder. The grana act as defined regions of sheet consolidation and increased connectivity. (**c**) A topological model of the granum–stroma assembly. The granum is made of repeating units that consist of two layers (red and yellow), which are formed by bifurcations of the stroma lamellae (gray). In each unit, part of the top layer (red) bends upwards and fuses with the layer above it whereas the other layer (yellow) bends downwards at the opposite side and fuses with the layer below. As indicated by the *blue dashed lines*, the units are rotated (counterclockwise) relative to each other (adapted from Shimoni et al., 2005, www.plantcell.org, copyright American Society of Plant Biologists) (See Color Fig. 16 on Color Plate 14).

vesicles. Accordingly, a continuum between the premature internal membrane system of *undeveloped* plastids and the inner envelope is often observed (Muhlethaler and Frey-Wyssling, 1959; von Wettstein, 1959; Carde et al., 1982; Hoober et al., 1991; Morre et al., 1991; Kroll et al., 2001). Studies on plastid differentiation in grasses suggested that the transition from proplastids to chloroplasts starts off with the emergence of long lamellae that are later joined by smaller, disc-shaped structures that form the grana (Sprey, 1973; for review, see Schnepf, 1980; Vothknecht and Westhoff, 2001). A detailed serial-section analysis performed by Brangeon and Mustárdy (1979) indicates that differentiation begins with one lamella of undefined form that expands and separates into parallel independent thylakoid sheets that are subsequently reconnected by interbridging to form the mature network. Grana were proposed to be formed at the margins of specific perforation sites within the parental lamellar sheets through a complex cascade involving membrane overgrowth, branching, and bridging via fusion. Unfortunately, only a few proteins implicated in thylakoid membrane formation and/or remodeling have thus far been identified

(see next section) and no further details are available at present on the mechanisms underlying these processes.

B Network Remodeling during Light Acclimation

Biological systems are subjected to constantly changing environmental conditions that, unless effectively coped with, can interfere with their homeostasis. For the photosynthetic apparatus, the most important external factor is light, which the system must carefully adjust to in order to maximize performance and minimize photo-induced damages. This is particularly challenging for land plants, which cannot adjust by moving towards a more suitable light environment. In these plants, acclimation to variations in light quality and/or intensity is accompanied by large-scale rearrangements of the thylakoid membranes.

In some instances, chromatic adaptation may be nearly static, almost fixed in time or varying very slowly, as in cases of adaptation to light climates or environmental niches. Figure 8 provides an example of such an adaptation, showing a huge granal stack that extends across a chloroplast of the extreme shade plant *Alocasia macrorrhiza* (Goodchild et al., 1972). This partiality towards massive grana containing a large number of thylakoid layers (over 100 in *Alocasia*) in dim light seems to be generic since it is also exhibited by sun plants grown at low light intensities (Bjorkman et al., 1972). Conversely, thylakoids of plants subjected to high irradiance during growth have smaller grana that contain fewer thylakoids (Bjorkman et al., 1972). Plants grown under PS I- or PS II-specific light display stacking profiles equivalent to those observed at low or high irradiance, respectively (Fig. 9; Chow et al., 1991). The aforementioned alterations in granum size and stacking are accompanied by changes in antennae size and in PS II/PS I stoichoimetries (Anderson et al., 1988).

Sometimes, however, adaptations need to be rapid. A passing cloud, a gale of dust, or sun flecks, during which the irradiance can change significantly in quality and by up to three orders of magnitude in intensity, all necessitate an appropriate response in order to sustain photosynthetic efficiency and minimize damage to the system. In oxygenic photosynthetic machineries, which rely on two photosystems, this requires more than just adjusting the total amount of absorbed light. This is because the two electron pumps, which are connected electrically in series, have different absorption characteristics and exciton trapping efficiencies. Prevention of potentially deleterious situations that result from imbalanced electron flow between the two photosystems thus necessitates a differential action that modulates the relative, rather than the overall, light trapping capacity of the two pumps. This is achieved through rapid-response regulatory processes called ‘state transitions’.

Ever since their first report by Murata (1969) and by Bonaventura and Myers (1969), state transitions have been studied extensively, mostly in green algae and higher plants (Bennett, 1977, 1979a, b, 1980; Allen et al., 1981; Hodges and Barber, 1983; for reviews refer to: Bennett, 1991; Allen, 1992b; Allen and Forsberg, 2001; Haldrup

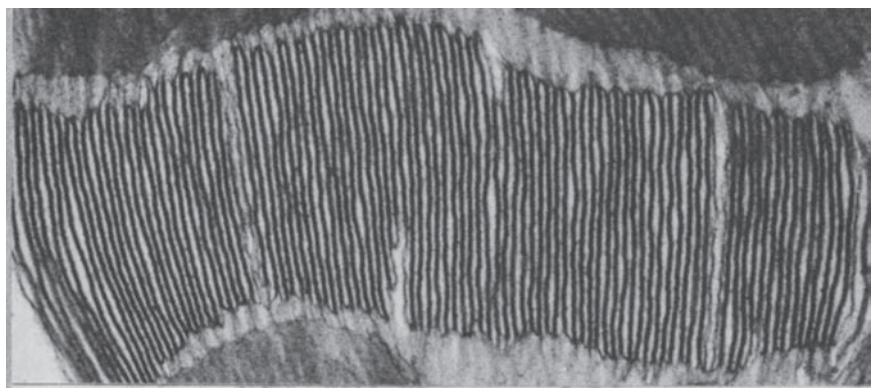

Fig. 8. Grana stacking in shade plants. A giant granum stack observed in a chloroplast of *Alocasia macrorrhiza*, which resides in extreme shade forests (adapted from Goodchild et al., 1972, with kind permission from Carnegie Institution).

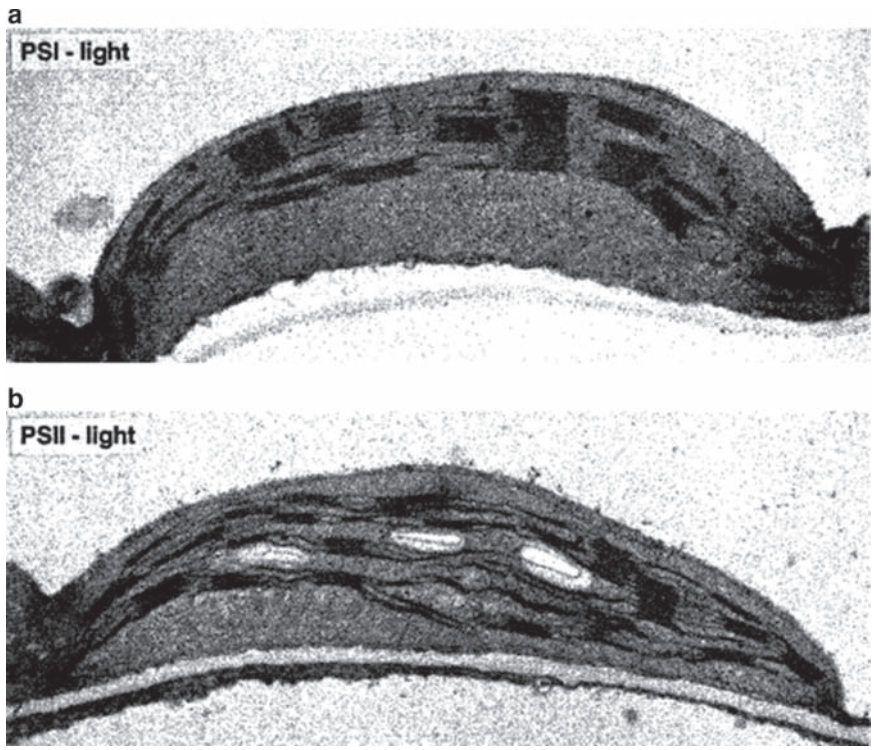

Fig. 9. Stacking profile of thylakoid networks adapted to different light conditions. Shown are palisade cell chloroplasts from spinach plants grown in PS I- (**a**) or PS II- (**b**) light (reprinted from Chow et al., 1991, with kind permission from Elsevier).

et al., 2001; Wollman, 2001; Kanervo et al., 2005). In these species, in the so-called state I, observed in darkness or under light conditions favorable for PS I, LHCII is associated primarily with PS II. Under light conditions that overexcite PS II, a fraction of LHCII becomes phosphorylated by dedicated kinases (Snyders and Kohorn, 1999, 2001; Bellafiore et al., 2005). Activation of the latter is driven by the intersystem electron mediator, cytochrome b_6f (Zito et al., 1999), and is governed by the redox state of the plastoquinone (PQ) pool, which becomes more reduced under conditions that favor state II. The phosphorylated fraction of LHCII then dissociates from PS II and binds to PS I, thus increasing the latter's ability to harvest light at the expense of the former. Reversal of the light conditions back to state I inactivates the kinases, and the LHCII antennae become dephosphorylated by constitutively active phosphatases (Aro and Ohad, 2003). Subsequently, the (dephosphorylated) LHCII complexes re-associate with PS II, restoring its original (prior to the transition) capacity to absorb light.

As was mentioned in the previous section, LHCII plays a key role in the formation and stabilization of appressed membrane domains. Its shuttling between PS II in the grana and PS I in the stroma lamellae during state transitions is therefore expected to lead to structural changes in the grana and, perhaps even in the entire thylakoid network. Elucidating the nature of these rearrangements and characterizing their topological consequences and underlying mechanics have been the subjects of an intensive study, often giving rise to intriguing ideas and hypotheses.

The first demonstration of externally induced structural rearrangements in higher plant thylakoids had, however, nothing to do with light. In 1966, 3 years before the discovery of state transitions, Izawa and Good (1966) presented electron micrographs of envelope-free spinach chloroplasts in which the thylakoid membranes were completely unstacked, appearing as concentric, well-separated sheets when viewed in transverse section (Fig. 10). The excessive unstacking of the membranes was achieved by incubating

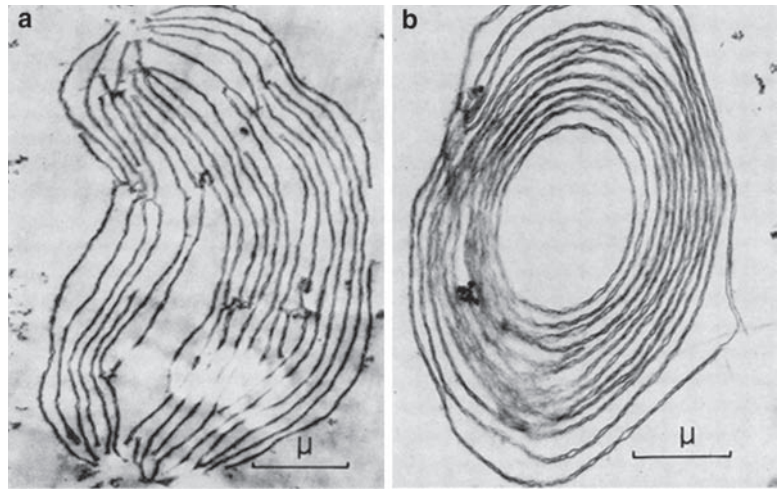

Fig. 10. Thylakoid membrane morphology in a low-salt, hypotonic buffer. Isolated spinach chloroplasts were suspended in 0.05 M Tricine-NaOH (pH 7.3). Shown are sections along the length (**a**) and width (**b**) of the chloroplasts. Note the complete absence of grana and the continuous sheets of double-membraned lamellae (adapted from Izawa and Good, 1966).

the de-enveloped chloroplasts in low-salt buffers and could be counteracted by the addition of mono- or di-valent cations, which restored stacks that appeared to be similar to granal stacks. The ability of cations to reform appressed membrane domains in unstacked thylakoids turned out to be non-specific but exhibited a strong dependency on their valency, with divalent cations being more effective, concentration wise, than monovalent cations (Murakami and Packer, 1971), as can be expected from polyelectrolyte condensation theories.

The observed sensitivity of granal domains to the presence (or absence) of cations and the apparent dependence on their valence led James Barber to suggest in the early 1980s that surface charges play a key role in grana stacking/unstacking (Barber, 1980, 1982). According to his model, which is an application of the DLVO theory, electrostatic repulsion prevents close appression of thylakoids unless their surfaces are effectively screened by counterions, whereupon van der Waals and other attractive forces take over and shift the equilibrium towards the stacked state. This model readily explained the unstacking of thylakoid membranes in low-salt buffer solutions. More importantly, it also suggested a means by which the addition of a negative charge to the stromal-exposed region of LHCII by (its just discovered) phosphorylation leads to its expulsion from the appressed granal domains to the stroma lamellae.

About a decade later, John Allen proposed an alternative model for the molecular basis that underlies the transfer of phospho-LHCII from the grana to the stroma lamellae (Allen, 1990, 1992a, b). According to this model, coined the 'molecular recognition' model, LHCII is in dynamic equilibrium between two bound states, with PS II or with PS I. Phosphorylation of LHCII shifts the equilibrium towards the latter, presumably due to an induced conformational change in LHCII. This model also emphasizes the importance of specific protein–protein interactions to grana stacking, as was proposed earlier by Mullet and Arntzen (1980). The molecular recognition model gained support from experiments with *Arabidopsis* plants that lack the H subunit of PS I, which serves as a docking site for mobile phospho-LHCII (Lunde et al., 2000). It appears that in these transformants LHCII remains attached to PS II even after its phosphorylation.

While undoubtedly important and influential, the two (not necessarily mutually exclusive) models described highlight only some of the interactions and forces that govern grana formation and disassembly. These are likely to include, in addition to double layer electrostatic repulsions and attractions and dipole–dipole interactions, specific and non-specific protein–protein interactions (mostly associated with LHCII), depletion interactions (crowding effect), hydration forces, steric exclusions, as well as short- and long-range

lipid-mediated interactions (Barber, 1982; Stys, 1995; Pali et al., 2003; Chow et al., 2005; Dekker and Boekema, 2005; Kim et al., 2005). Moreover, as discussed in Section IV.A, the granum–stroma assembly is stabilized, at least in state I, by multiple membrane connections. Significant alterations in the grana, therefore, require more than a mere perturbation in the balance of the aforementioned forces.

Two additional questions remained to be answered: what is the extent of the structural rearrangements that occur in thylakoid membranes in vivo, namely, during light-induced state transitions, and how do these rearrangements initiate and proceed. Since the early 1980s, there had been a growing notion that the massive alterations in membrane structure induced at low ionic strength conditions are perhaps too drastic and that they may not precisely imitate those that occur following photo-induced phosphorylation of LHCII during state I → state II transitions. This notion became stronger after experiments with isolated thylakoids, which revealed that the effects produced by phosphorylation of LHCII (induced by directly reducing the PQ pool) depend heavily on the level of Mg^{2+} in the media. At Mg^{2+} concentrations below 5 mM, LHCII phosphorylation resulted in extensive unstacking of the membranes and was accompanied by intermixing of the two PSs (a hint for such mixing was already provided in 1967 by studies showing that all the membrane fractions derived from low-salt induced unstacked thylakoids had both PS I and PS II activities (Anderson and Vernon, 1967); 9 years later, mixing was demonstrated directly by freeze-fracture and freeze-etch studies of unstacked thylakoid membranes [Staehelin, 1976]). When higher Mg^{2+} concentrations were used, unstacking was much more limited and the changes in chlorophyll fluorescence were attributed to changes in the absorption cross sections of the two PSs inflicted by grana–stroma lamellae migration of phospho-LHCII (Horton and Black, 1983; Telfer et al., 1983, 1984). It was also shown that excessive unstacking by means of cation depletion is associated with lesions and ruptures of the membranes (Brangeon, 1974; Briantais et al., 1984) and may lead to irreversible changes in the structure of the granum–stroma assembly or in the organization of PS II-LHCII arrays that lie within it (Brangeon, 1974; Briantais et al., 1984; Garab et al., 1991; Shimoni et al., 2005). In addition, massive reorganization of the granum–stroma assembly was incompatible with most of models of higher-plant thylakoid networks, with the notable exception of the model proposed by Arvidsson and Sundby (see Section IV.A). These observations and considerations led to the opposing view that structural alterations in thylakoid membranes during state transitions are of a highly limited nature, mostly confined to the grana margins (see e.g., Kyle et al., 1983; Drepper et al., 1993; Mustardy and Garab, 2003; Shimoni et al., 2005).

Recently, we reported a detailed analysis of the reorganizations that occur in the membranes of de-enveloped, photosynthetically-active *Arabidopsis* chloroplasts during state transitions (Chuartzman et al., 2008). To thoroughly characterize the structural events that accompany the transitions, which were induced by exposure to PS I- or PS II-specific light, we employed atomic force microscopy, scanning- and transmission electron microscopy, as well as confocal imaging. Surprisingly, the membrane rearrangements (which were largely reversible) that occurred during the transitions were large scale (Fig. 11). Specifically, the transition from state I to state II was accompanied by rather extensive alterations in granum structure including unstacking, dislocations of layers, sometimes outside of the appressed domains, rotational movements, and fragmentation of the grana into smaller bodies. These rearrangements in granum structure were accompanied by macroscopic changes in the thylakoid network, which became disordered, underwent a significant expansion and adopted a wavy appearance indicating a large increase in deformability.

The above observations indicate that, albeit not reaching the extent observed upon salt-induced membrane unstacking, the structural rearrangements in chloroplast thylakoid membranes during state transitions are nevertheless substantial. This raised (again) the question of how such extensive rearrangements can be accommodated by the lamellar network, in particular, by the granum–stroma assembly. As mentioned above and elaborated in the previous section, in state I, the granum layers are *physically* linked to the surrounding stroma lamellae, as well as to each other by multiple lateral and vertical connections. Topologically, some of these connections must break

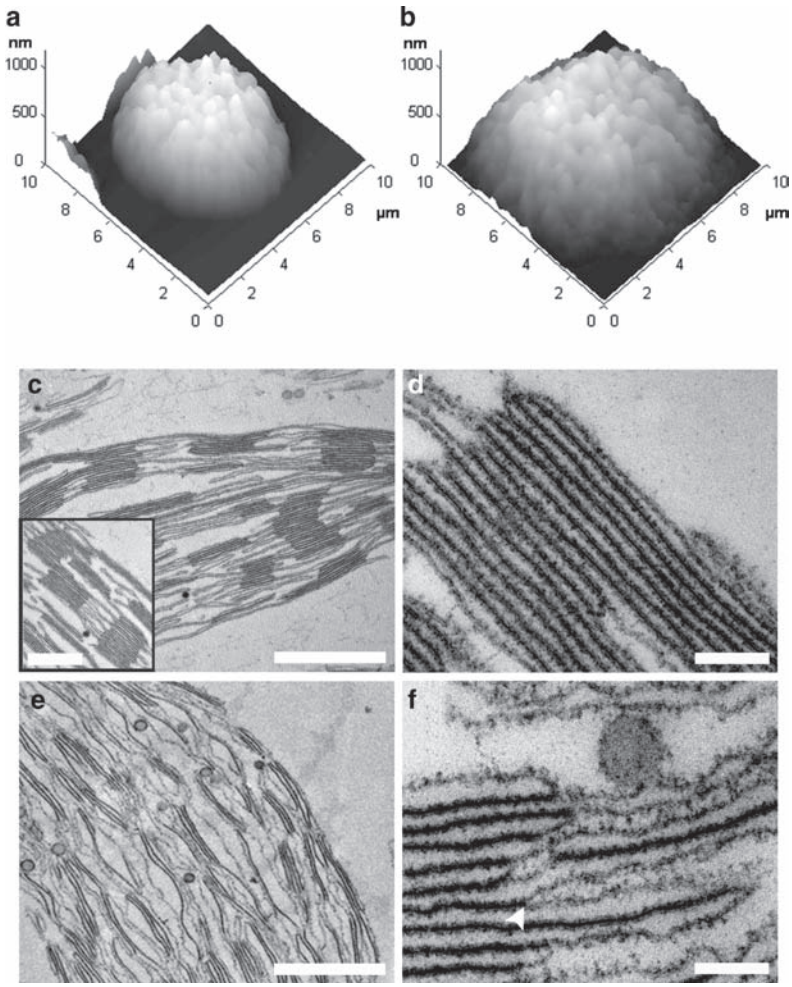

Fig. 11. Structural alterations of higher-plant thylakoid membranes in state transitions. Atomic force microscope images of de-enveloped chloroplasts showing that the transition from state I (**a**) to state II (**b**) is accompanied by changes in chloroplast height and diameter. During the transition, the grana which exhibit a tightly appressed, well-ordered morphology in state I (**c**, **d**) undergo massive structural rearrangements, giving rise to loosely stacked, irregular structures with rough boundaries (**e**, **f**) (adapted from Chuartzman et al., 2008, www.plantcell.org, copyright American Society of Plant Biologists).

for the aforementioned structural rearrangements in granum structure to take place.

A mechanistic model that we have proposed for the primary events that lead to reorganization of the granum–stroma assembly during state I → state II transition is shown in Fig. 12. We suggest that LHCII phosphorylation and subsequent migration to the stroma lamellae bring about two key events that occur at the grana margins: (i) disruption of attractive interactions between adjacent layers, leading to retraction of the latter in these regions and (ii) alteration of local lipid and/or protein composition in the margins, which further destabilizes these highly curved membrane domains. Together, these events inflict an increasing strain on the vertical and lateral connections of the assembly (Fig. 12b), eventually causing some of them to break down (Fig. 12c). Facilitated by the highly connected nature of the system, the localized alterations in granum structure subsequently propagate throughout the entire lamellar network, leading to the macroscopic changes observed in its organization and connectivity.

Our proposed model raises the question of whether breakage of the lateral and/or vertical connections of the layers in the grana, during state I → state II transition, and their reformation during the reverse transition, can take place

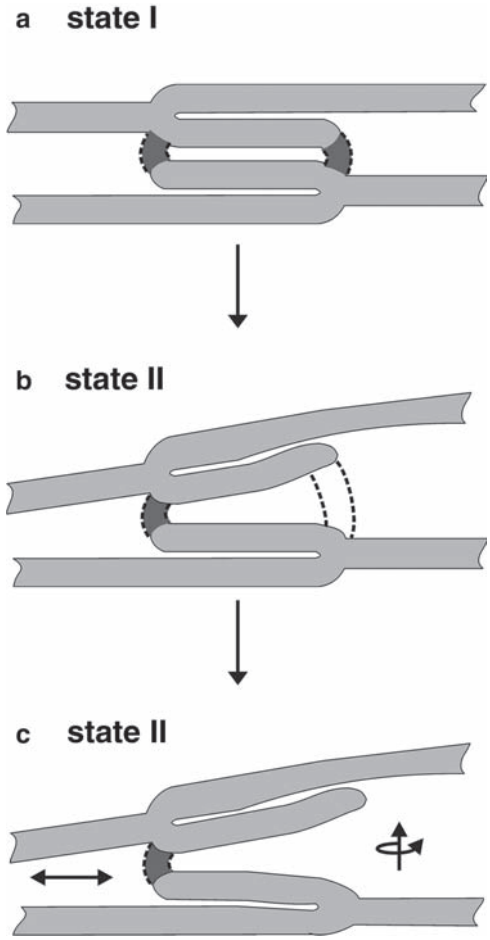

Fig. 12. A model for the structural rearrangements in the granum–stroma assembly during state I → state II transition. (**a**) In state I, the granum layers are linked to the stroma lamellae and to each other by the lateral and vertical (indicated as darker regions delimited by *dashed lines*) membrane connections described in the legend of Fig. 7. Consequent to LHCII migration from the grana to the stroma lamellae, induced upon transition to state II, the margins of the granum body become unstable and adjacent layers retract from each other at the edges of the granum body. This movement subjects the internal grana bridges to strain (**b**), which once reaching a critical threshold, causes some of the bridges to break (**c**). The subsequent events are less clear but probably involve rotation and vertical and lateral movements of the layers where breakage occurred. Restoration of the grana stacks during the reverse transition requires reformation of the membrane bridges that interconnect neighboring layers in the granum (adapted from Chuartzman et al., 2008, www.plantcell.org, copyright American Society of Plant Biologists).

spontaneously. While this possibility cannot be excluded, we note that such membrane fission/fusion processes, which are likely to be associated with significant energy barriers, often require dedicated protein machineries to stabilize transition states along the reaction pathways. Such machineries indeed operate in the mitochondria, the Golgi apparatus, and in the endoplasmic reticulum (Misteli and Warren, 1995; Dekker and Boekema, 2005; Mannella, 2006; Atilgan and Sun, 2007; Voeltz and Prinz, 2007). In chloroplasts, membrane-remodeling proteins, such as FZL, VIPP1 and Thf1, have been identified and found to be associated with thylakoids (Li et al., 1994; Kroll et al., 2001; Wang et al., 2004; Gao et al., 2006). These or hitherto unidentified proteins may participate in the breakage and restoration of the interlayer granal bridges during state transitions, as well as during long-term acclimation and network development.

C Why Do Higher-Plant and Some Green Algal Thylakoid Networks Have Grana?

We end our review of higher-plant thylakoid membranes with a brief summary of the hypotheses raised over the years about the physiological driving force that underlies grana formation in these systems. For a more extensive discussion of this question, the reader is referred to recent reviews by Chow et al. (2005) and Mullineaux and Emlyn-Jones (2005).

As discussed in Section IV.A, the formation of grana is strongly dependent on LHCII. The quest for 'why grana?' should thus begin by considering the benefits provided by the formation of stacked arrays enriched in LHCII molecules for light harvesting and funneling. An immediate consequence of this stacking is increase in dimensionality. Such an increase was perhaps needed to compensate for the loss of dimensionality associated with the substitution of phycobilisomes by LHCII as the primary accessory antenna. This could be particularly important for the ability of land plants to prosper in the newly established shade environments that necessitated efficient light harvesting, a need that was not encountered by their immediate progenitors – presumably a kind of algae that inhabited shallow waters (Mullineaux and Emlyn-Jones, 2005). The close appression of layers in granal stacks may also allow for vertical energy transfer between LHCII in one membrane layer and PS II in another (Trissl et al., 1987; Dekker and Boekema, 2005). This might have been necessary to relieve constraints

imposed on lateral diffusion of plastoquinones from PS II to the cytochrome by the presence of extremely large amounts of membrane-integral LHCII molecules around the reaction centers.

Another line of hypotheses focuses on the lateral segregation of the two photosystems into two morphological domains. Segregation of principal components, in space or time, often provides better means for control, in particular when the system is subjected to strong fluctuations. An obvious target for such regulation is the absorption of light and its subsequent funneling to the reaction centers of the two electron pumps. It was proposed that segregation of the light-harvesting components of the two photosystems serves to minimize spillover of excitation energy from PS II to the longer wavelengths absorbing PS I (Anderson, 1981) or to synchronize the activity of the comparatively sluggish PS II with the faster exciton trapping PS I (Trissl and Wilhelm, 1993). As discussed in the previous section, segregation of the two PSs also enables fine-tuning of their relative light-harvesting capacity by moving a relatively small fraction of LHCII molecules between the two photosystems over short distances via the grana margins. This, in turn, allows for efficient and rapid acclimation to variations in light quality and/or intensity.

In addition to modulations in the funneled excitation light energy, the two photosystems are subservient to manipulations in the extent of their coupling, which, in turn, dictates the mode of electron flow in the system. While the exact molecular basis is still under debate (Chow, 1984, 1999; Chow et al., 2005), it is quite clear that granal stacking promotes linear electron transport (which results in the production of both ATP and reducing power) whereas unstacking leads to enhanced cyclic electron transport (which generates only ATP). Alternating between these two modes may provide plants with another means by which to adjust to variations in light conditions. For example, it was proposed that enhancing cyclic electron transport is required after prolonged exposure to high light, to enable recovery (under low light), or during induction of photosynthesis after dark adaptation, at which time linear electron transport is too slow for efficient utilization of light energy (Anderson, 1989; Joliot and Joliot, 2002, 2005).

In addition to the above, the unique structural and compositional features of the grana have been proposed to play a role in several other processes. It was suggested that, by virtue of their organization and composition, grana might have a regulatory role in non-photochemical quenching processes in PS II (Horton, 1999). Grana were likewise proposed to protect inactivated D1 and D2 proteins against premature degradation during prolonged exposure to high light (Anderson and Aro, 1994) and to allow for dynamic flexibility in the light reactions via modulations in electron and proton transfer reactions (Kramer et al., 2004; Horton and Ruban, 2005; Horton et al., 2005).

In a broader perspective, the segregation of thylakoid membranes into granal and stromal lamellar domains could be looked upon as yet another example of cellular compartmentalization with its associated regulatory merits. The latter might have been required to better control pathways and to prevent undesired interactions, which became more complicated and more abundant, respectively, as the system became more complex (Anderson et al., 1988; Anderson, 1999). Clearly, distinguishing between all of the above hypotheses and ideas is not a trivial task, perhaps impossible altogether.

V Concluding Remarks

Researchers of thylakoid membranes have not been idle during the 17 decades following von Mohl's seminal description of 'chlorophyll granules' in green leaves. To date, we have a fairly detailed description of the lipid and protein composition of the thylakoid membranes, as well as of their ultrastructure. We also have a good picture of the way photosynthetic protein complexes are distributed within the membranes (Andersson and Anderson, 1980; Albertsson, 2001; Nelson and Ben-Shem, 2004; Dekker and Boekema, 2005; Merchant and Sawaya, 2005) and data concerning the exact manner by which thylakoid networks are organized in space are hastily accumulating (Shimoni et al., 2005; Liberton et al., 2006; van de Meene et al., 2006; Nevo et al., 2007; Ting et al., 2007). Nevertheless, there are many things we still do not know about these intricate membranes. The biogenesis of thylakoid

membranes, the evolution of their structure and composition, their maintenance, and their remodeling in response to variations in environmental conditions, are all far from being fully understood. Most likely, we have not yet identified all of the machineries that participate in these processes. In addition, the functions and mode of operation of the components known to us are only partially understood. Since most of our structural knowledge of thylakoid membranes comes from EM investigations, we know very little about the dynamics of these networks. Here, the introduction of scanning force microscopy (Kaftan et al., 2002; Scheuring et al., 2003, 2004; Bahatyrova et al., 2004; Scheuring and Sturgis, 2005), with its ability to visualize surface topographies at near molecular resolution, under aqueous buffered solutions, may bridge the gap between structural and dynamic information. The most challenging task, however, will be to obtain detailed information on the local arrangements and densities of all the major photosynthetic complexes within the membranes, the way lipid- and water-soluble electron carriers are distributed within their respective phases, and the diffusional properties of all these components. Combining this information, which is already beginning to accumulate (Consoli et al., 2005; Dekker and Boekema, 2005; Kirchhoff et al., 2008), with information about the spatial organization and connectivity of thylakoid membranes, the ultimate goal of understanding the working of the photosynthetic machinery at the system level may be approached.

Acknowledgements

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Chapter 15

Regulatory Role of Membrane Fluidity in Gene Expression

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Summary

Plants and other photosynthetic organisms experience frequent changes in environment. Their ability to survive depends on their capacity to acclimate to such changes. In particular, fluctuations in temperature and/or osmolarity affect the fluidity of cytoplasmic and thylakoid membranes. The molecular mechanisms responsible for the perception of changes in membrane fluidity have not been fully characterized. However, the analysis of genome-wide gene expression with DNA microarrays has provided a powerful new approach to studies of the contribution of membrane fluidity to gene expression and to the identification of environmental sensors. In this chapter, we summarize the knowledge on the mechanisms that regulate membrane fluidity, on putative sensors that perceive changes in membrane fluidity, and on the subsequent expression of genes that ensures acclimation to a new set of environmental conditions.

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I Introduction

Various kinds of environmental stress, such as temperature stress and osmotic stress, cause alterations in the physical properties of the membrane lipids in living cells. It seems likely that cells perceive these alterations via sensory proteins embedded in their membranes. These proteins transfer the signals from the environment to networks of signal-transduction pathways, with the resulting regulation of gene expression (Murata and Los, 1997; Los and Murata, 2000; 2004). Chemical and genetic modification of the physical properties of membrane lipids can have similar effects on the expression of genes that are involved in the acclimation of cells to various conditions (Vigh et al., 1993; Carratu et al., 1996; Horváth et al., 1998). The physical state of membrane lipids also acts directly to regulate the activity of membrane-bound proteins, such as the translocators of small molecules, ion channels (Sukharev, 1999), receptor-associated protein kinases (Wood, 1999; Hohmann, 2003), and sensor proteins (Tokishita and Mizuno, 1994; Aguilar and de Mendoza, 2006).

Until recently, studies of the influence of membrane fluidity on gene expression have been limited to studies of a small number of genes whose expression has been examined by Northern blotting, focusing on the feedback regulation of membrane fluidity and on genes that are responsible for the maintenance of the physical properties of membrane lipids (Murata and Los, 1997; Hohmann, 2003). However, since DNA microarrays that cover the entire genomes of various organisms have become available, it has been possible to analyze the genome-wide expression of genes, that are associated with acclimation (Murata and Los, 2006; Los et al., 2008). The use of DNA microarrays, in combination with the site-directed mutagenesis of genes that are responsible for the maintenance of membrane fluidity, has provided a very powerful method for studies of the contribution of membrane fluidity to gene expression and for identification of various sensors of environmental conditions.

In this chapter we shall focus on the mechanisms that regulate the fluidity of membrane lipids, on putative sensors that perceive changes in membrane fluidity, and on the subsequent expression of genes that ensure acclimation to a new set of environmental conditions. In particular, we shall discuss findings obtained by the analysis of genome-wide gene expression with DNA microarrays.

II Modulation of Membrane Fluidity

A Measurements of Membrane Fluidity

The extent of molecular disorder and molecular motion within a lipid bilayer is referred to as the fluidity of the membrane. Several methods can be used to monitor membrane fluidity. The most common method involves measurements of the anisotropy of the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) (Grunberger et al., 1982; Lentz, 1993; Laroche et al., 2001). DPH is incorporated into membranes in parallel to the acyl chains of membrane lipids and the fluorescence of DPH is only weakly depolarized when it interacts stably with rigidified membranes (Lentz, 1993). However, since DPH is not completely free to rotate within membranes, the extent of the interactions between DPH and membrane lipids is restricted. Thus, this method is unsuitable for quantitative measurements of membrane fluidity, although the comparisons certainly can be made.

Another method, Fourier transform infrared (FTIR) spectroscopy, can be used for quantitative analysis of the physical state of membrane lipids (Szalontai et al., 2000). FTIR spectroscopy allows us to monitor the disorder of the acyl chains of lipids and the interactions between lipids and membrane proteins in terms of the frequency of the symmetric CH_2 stretching mode near 2.81 cm^{-1}. This frequency decreases by approximately 2–5 cm^{-1} upon the transition of the membrane lipids from an ordered to a disordered state. Thus, low and high frequencies of the CH_2 stretching mode correspond to the rigidified and fluid states of membrane lipids, respectively.

Abbreviations: DPH – 1,6-Diphenyl-1,3,5-hexatriene; FTIR spectroscopy – Fourier transform infrared spectroscopy; PAS domain – Per-ARNT-Sim conservative motif; rbp – RNA-binding protein; SRE – Sterol responsive element; SREBP – Sterol responsive element binding protein

B Effects of Changes in Temperature

The effects of changes in temperature on membrane fluidity have been demonstrated by DPH fluorescence polarization in fish (Cossins, 1977; Pehowich et al., 1988), bacteria (Sinensky, 1974), and cyanobacteria (Horváth et al., 1998). The cited studies focused on the effects of low temperature and demonstrated clearly that membrane fluidity decreases with a decrease in temperature (Fig. 1). Recently, these changes have also been examined by FTIR spectroscopy (Szalontai et al., 2000; Inaba et al., 2003; Chapter 17). The frequency of the CH_2 stretching mode in cytoplasmic membranes and thylakoid membranes isolated from the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*) decreases with a decrease in temperature.

The effects of high temperatures on the physical state of membranes have also been studied, albeit less extensively (Carratu et al., 1996; Vigh et al., 1998). High temperatures cause the fluidization of membranes (Fig. 1), which at the extremes can lead to disintegration of the lipid bilayer.

C Effects of Osmotic Stress

The effects of hyperosmotic stress on membrane fluidity have been examined using DPH, although, as mentioned above, this method provides rather comparative information about membrane fluidity. When phospholipid vesicles were exposed to hyperosmotic stress that was due to the addition of polyethylene glycol to the medium, an increase in anisotropy was observed (Yamazaki et al., 1989). A similar effect was observed in yeast cells when glucose was added to the medium to raise the osmolarity (Laroche et al., 2001). These findings suggest that hyperosmotic stress might reduce membrane fluidity similarly to low-temperature stress. However, more studies are necessary to confirm this possibility in the biological membranes of living organisms.

The effects of hypoosmotic stress on membrane fluidity have not been studied in detail but it has been suggested that hypotonic stress might fluidize membranes similarly to heat stress (Los and Murata, 2004; Meyer et al., 2006).

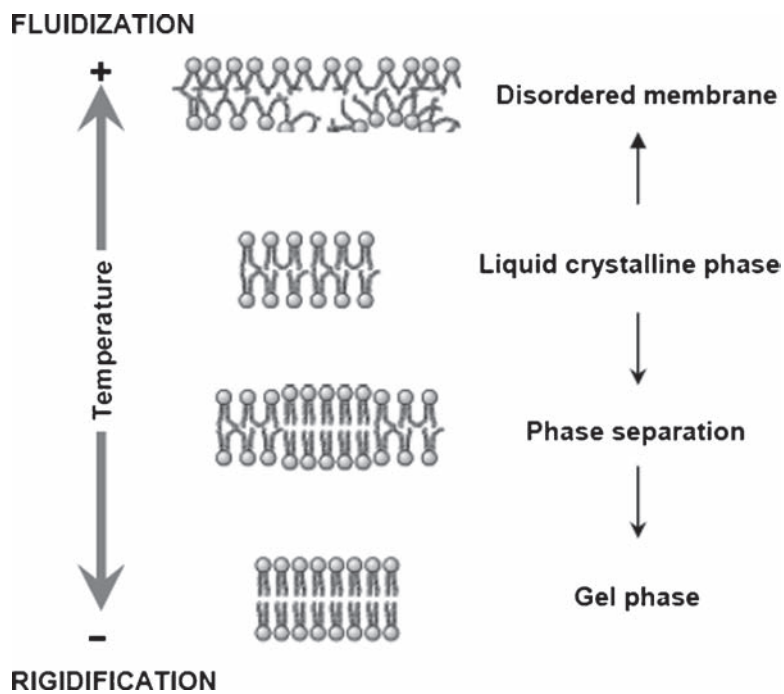

Fig. 1. Schematic representation of changes in membrane structure and the behavior of lipid bilayers at low and high temperatures. Low temperatures cause *rigidification* of membranes, whereas high temperatures cause *fluidization* of membranes (adapted from Hazel, 1995; Los and Murata, 2004).

Aliphatic alcohols cause the fluidization of membranes and they are often used to simulate heat and hypotonic stress (Gudi et al., 1998; Sugiura et al., 1994; Kabelitz et al., 2003). However, the relevance of such an approach is questionable, since heat stress and benzyl alcohol (both of which provoke the fluidization of membranes) induce different sets of genes in *Synechocystis* (Inaba et al., 2003). Thus, the physiological impact of heat stress and that of membrane-fluidizing reagents on cell membranes might be rather different.

D Effects of the Unsaturation of Fatty Acids

The dependence of membrane fluidity on the extent of unsaturation of fatty acids in membrane lipids is a well-characterized phenomenon, which has been demonstrated in animals (Thewke et al., 2003), fish (Macartney et al., 1996; Tiku et al., 1996), fungi (Maresca and Kobayashi, 1993), yeasts (Bossie and Martin, 1989; Rodríguez-Vargas et al., 2007), plants (Lyons and Raison, 1970; Nishida and Murata, 1996), bacteria (Sinensky, 1974; Cybulski et al., 2002), and cyanobacteria (Los and Murata, 1998; 1999; 2004). Cyanobacteria are particularly suitable for studies of such phenomena (Glatz et al., 1999) because the number of unsaturated bonds in their fatty acids can be altered by genetic manipulation of the genes that encode the fatty acid desaturases (Wada et al., 1990; Tasaka et al., 1996; Chapter 17). Two strains of cyanobacteria, namely, *Synechocystis* and *Synechococcus* sp. PCC 7942 (hereafter, *Synechococcus*), have been used to study the effects of the unsaturation of fatty acids on membrane fluidity. *Synechocystis* is characterized by the presence of four genes, designated *desA*, *desB*, *desC* and *desD*, for fatty acid desaturases and by its ability to synthesize fatty acids with four double bonds. Thus, its membrane lipids contain high levels of unsaturated fatty acids.

When the *desA* and *desD* genes were inactivated in *Synechocystis*, a dramatic decrease in membrane fluidity (Tasaka et al., 1996; Szalontai et al., 2000) was detectable in the resultant *desA*$^-$/*desD*$^-$ strain, which had in addition lost the ability to acclimate to low temperatures. It should be noted that the optimal temperature for the growth of these cyanobacterial strains is close to 35°C, and they experience cold stress when the temperature falls to 20–25°C.

Unlike *Synechocystis*, *Synechococcus* has only one gene for the Δ9 desaturase and it synthesizes mono-unsaturated fatty acids exclusively. When the *desA* gene of *Synechocystis*, which encodes the Δ12 desaturase, was introduced into the genome of *Synechococcus*, the resultant *desA*$^+$ strain produced considerable amounts of di-unsaturated fatty acids (Wada et al., 1990). This change resulted in a considerable increase in membrane fluidity (Sarcina et al., 2003), which was linked to the ability of cells to survive at a low temperature. Thus, an increase in desaturation of fatty acids increases membrane fluidity, and this is necessary for cyanobacterial cells and for plants to tolerate chilling and to survive at low temperatures (Ishizaki-Nishizawa et al., 1996; Orlova et al., 2003). Similar effects of engineered membrane fluidization on growth and survival have been recently observed in yeast cells transformed with plant Δ12 desaturase genes (Rodríguez-Vargas et al., 2007).

A clear effect of membrane rigidification on cold-induced gene expression was demonstrated by genome-wide analysis, using DNA microarrays, of wild-type and *desA*$^-$/*desD*$^-$ cells of *Synechocystis* (Inaba et al., 2003). Sixteen genes whose expression was not induced by cold in wild-type cells (for example, several heat-shock genes, such as *hspA*, *clpB1*, *dnaK2*) became strongly cold-inducible upon rigidification of membrane lipids. The level of cold-inducible expression of another group of 17 genes was increased from two- to threefold upon rigidification of the plasma membrane (Inaba et al., 2003). These results indicate that expression of a relatively large number of cold-inducible genes is influenced by membrane fluidity.

E Feedback Regulation of Fluidity by Desaturation of Fatty Acids

Three of the four genes for desaturases in *Synechocystis* (*desA*, *desB*, and *desD*) are cold-inducible (Los et al., 1993; 1997). The enhanced synthesis de novo of these three fatty-acid desaturases under cold stress and the subsequent introduction of additional double bonds into the fatty-acyl chains of membrane lipids are involved in the maintenance of membrane fluidity in the

liquid-crystalline phase and prevent the membranes from undergoing phase transition to the lethal gel phase (Hazel, 1995). The cold induced expression of desaturase genes in order to compensate for a decrease in membrane fluidity, is a widespread phenomenon that can be observed in almost all taxa of poikilothermic organisms, from bacteria to plants, fish, and animals (Wodtke and Cossins, 1991; Macartney et al., 1994; Tiku et al., 1996; Los and Murata, 2004; Aguilar and de Mendoza, 2006).

Synthesis of unsaturated fatty acids as a compensatory response to a decrease in membrane fluidity at low temperatures was demonstrated first in *Escherichia coli* (Sinensky, 1974), and the phenomenon was designated "*homeoviscous acclimation*". Later on, the existence of a feedback loop between membrane rigidification and the compensatory expression of genes for desaturases was demonstrated directly by the chemical hydrogenation of unsaturated fatty acids in the plasma-membrane lipids of *Synechocystis* (Vigh et al., 1993; Los and Murata, 2004). Palladium-catalyzed saturation of a small portion of plasma-membrane lipids at the optimal growth temperature caused the immediate induction in expression of the gene, which is responsible for desaturation of fatty acids in membrane lipids. This enzyme reversed the chemically induced rigidification of membrane lipids and returned them to their optimal fluid state (Vigh et al., 1993).

It is important to keep in mind that the cold-induced enhancement of the expression of the genes for desaturases depends on the extent of the shift in temperature but not on the absolute temperature (Los et al., 1993; Vigh et al., 1993). Cells pre-acclimated to 36°C induce transcription of genes for desaturases only 30°C, although cells pre-acclimated to 32°C induce the specific transcripts only below 26°C.

One might expect that the desaturation of fatty acids might also compensate for the rigidification of membrane lipids in cells exposed to hyperosmotic stress. However, genome-wide analysis of transcription in *Synechocystis*, using DNA microarrays, indicated that hyperosmotic stress does not activate the transcription of genes for desaturases (Kanesaki et al., 2002; Shoumskaya et al., 2005). Nevertheless, in *Bacillus subtilis*, hyperosmotic stress caused a decrease in the fluidity of cell membranes and subsequently increased the levels of unsaturated fatty acids in membrane lipids (Lopez et al., 2000). This phenomenon might be due to the enhanced expression of the genes for desaturases, or to the activation of preexisting desaturases. This problem requires further careful analysis.

In some bacteria, *cis–trans* isomerization of unsaturated fatty acids plays an important role in the regulation of membrane fluidity (Okuyama et al., 1990; 1991; Heipieper et al., 2003). The conversion of *cis*-unsaturated fatty acids to *trans*-unsaturated fatty acids apparently occurs with considerable efficiency during the adaptation of membrane fluidity to changes in the cellular environment.

Another mechanism to adjust membrane fluidity has been characterized in the systematic study of fish, which live at different latitudes (Logue et al., 2000). Fluorescence anisotropy measurements with DPH and detailed determination of fatty acid composition in individual molecular species of lipids revealed cold-adaptive increase in membrane disorder. In phosphatidylcholine, the cold-adaptive increase in unsaturation was mostly associated with increased proportions of polyunsaturated fatty acids in the *sn*-2 position. In phosphatidylethanolamine compositional adaptation involved exchanges between saturates and monounsaturates exclusively at the *sn*-1 position. Thus, temperature-dependent adjustment of membrane physical properties relies on the balance between bilayer-stabilizing and destabilizing tendencies. In fish it depends on molecular shapes of the two phosphoglycerides that balance *sn*-1 and *sn*-2 fatty acids to compensate for difference in habitat temperature (Logue et al., 2000).

The transcriptional control of homeostasis in the unsaturation of fatty acids and the fluidity of membrane lipids of warm-blooded animals and humans has been also proposed (Thewke et al., 2003). It has been suggested that membrane fluidity might be regulated by changes in levels of oleate and polyunsaturated fatty acids relative to the total level of fatty acids, as well as by changes in levels of cholesterol relative to the total level of lipids.

The expression of all the genes for the enzymes involved in the biosynthesis of cholesterol is regulated at the transcriptional level by a common mechanism. The promoters of these genes contain sterol-responsive elements (SREs) that bind transcription factors known as SRE-binding

proteins (SREBPs), as described in detail elsewhere (Thewke et al., 2003). The promoters of several genes for enzymes involved in fatty acid biosynthesis, such as acetyl-CoA-carboxylase, fatty acid synthase, and stearoyl-CoA-desaturase, also contain SRE-like elements and some SREBPs specifically activate the biosynthesis of fatty acids (Thewke et al., 2003). Thus, the biosynthetic pathways for the synthesis of cholesterol (a membrane rigidifier) and oleate (a membrane fluidizer) are interrelated. In mammalian cells, chemical fluidizers of membranes induce the synthesis of cholesterol (Brown and Goldstein, 1999; Edwards and Ericsson, 1999), as predicted by the hypothesis of "homeoviscous acclimation" that was proposed initially for bacteria and plants. The existence of a sensor of the fluidity of plasma membranes in animals has been suggested (Macartney et al., 1996), although such a sensor has not yet been identified. Nevertheless, in animals, changes in membrane fluidity do affect the activities of G proteins, which are components of signal-transduction systems, such as those involved in photoreception, taste, and olfaction (Gudi et al., 1998), and are linked to transmembrane receptors. It has been demonstrated that the fluidization of membrane lipids can activate heterotrimeric G proteins directly on the cytosolic face of the plasma membrane.

III Perception of Membrane Rigidification

A Cold-Inducible Genes in Cyanobacteria

There are several comprehensive reviews of the responses of cyanobacteria to cold stress (Murata and Wada, 1995; Los and Murata, 1999; 2004). Cold-inducible genes have been grouped into several categories, as follows: (1) genes for fatty acid desaturases that are responsible for adjustments in membrane fluidity; (2) genes for RNA-binding proteins (Rbps) that, probably, serve as RNA chaperones similarly to the Csp proteins of *E. coli* and *B. subtilis*; (3) genes for RNA helicases that destabilize the secondary structures of mRNAs, thereby overcoming inhibition of the initiation of translation at low temperatures; (4) genes for ribosomal proteins, an excess of which is necessary for acclimation of the translational machinery to cold; (5) genes for caseinolytic proteases that participate in the renewal of photosystem II; and (6) various other genes that do not fall in any of the other five categories. The availability of genome-wide DNA microarrays of *Synechocystis* has provided new opportunities for studies of responses of the entire genome to cold stress (Suzuki et al., 2001). The expression of close to 50 genes is strongly induced in *Synechocystis* under cold stress (Table 1). In addition to the above-mentioned groups, some other important genes appear to be induced at low temperatures, namely, the *rpoA* gene for RNA polymerase; the *sigD* gene for sigma factor D; the *fus* gene for elongation factor EF-G; the *hliA*, *hliB*, and *hliC* genes that encode high light-inducible proteins, which are involved in the regulation of photosynthesis; the *ndhD2* gene for subunit 4 of NADH dehydrogenase; the *cytM* gene for an alternative form of cytochrome *c*; several genes that are expressed in response to oxidative stress; and several genes for proteins of as yet unknown function (Table 1). Thus, it is evident that cold stress enhances the expression of many genes whose products control membrane fluidity, transcription, translation, and the energy status of the cell.

B A Sensor of Cold Stress in Cyanobacteria

The cold sensor Hik33 (histidine kinase 33) was originally identified in *Synechocystis* as a regulator of the cold-inducible expression of the *desB* gene, which encodes the ω3 desaturase, after random mutagenesis of a strain that carried a reporter gene, in which the *desB* promoter had been fused to the gene for a bacterial luciferase (Suzuki et al., 2000). Subsequent analysis of the genome-wide pattern of gene expression using DNA microarrays demonstrated that Hik33 regulates the expression of 28 of the 45 cold-inducible genes (Suzuki et al., 2001).

The amino acid sequence of the Hik33 transmembrane sensory histidine kinase contains several conserved domains, namely, a type-P linker, a leucine zipper and a PAS domain (Fig. 2). The type-P might be responsible for intermolecular dimerization of the protein under cold stress, when rigidification of the membranes takes place. The PAS domain senses oxidative stress, which might

Table 1. Cold-inducible genes in *Synechocystis* sp. PCC 6803 (data adapted from Inaba et al. (2003) with inclusion of more recent results).

ORF	Gene	Encoded protein	Ratio of transcript levels
slr1291	*ndhD2*	NADH dehydrogenase I chain M	22.0 ± 7.1
ssl2542	*hliA*	High light-inducible protein	20.7 ± 1.0
slr0083	*crhR*	ATP-dependent RNA helicase	16.3 ± 4.8
ssr2595	*hliB*	High light-inducible protein	10.6 ± 3.2
sll1541		Putative lignostilbene-dioxygenase	6.4 ± 0.8
slr1105	*fus*	Elongation factor EF-G	6.2 ± 0.5
sll0086		Arsenical pump-driving ATPase	5.9 ± 1.0
sll1483		Periplasmic protein	5.5 ± 2.9
ssl1633	*hliC*	High light-inducible protein	5.5 ± 1.0
sll0517	*rbpA*	RNA-binding protein	5.4 ± 1.3
sll0384	*cbiQ*	ABC-type cobalt transport system permease protein	4.9 ± 1.1
slr0400		Putative inorganic polyphosphate/ATP-NAD kinase 1	4.7 ± 0.0
sll2012	*sigD*	RNA polymerase sigma factor	4.4 ± 0.9
slr0955		Putative tRNA/rRNA methyltransferase	4.4 ± 0.8
slr0401		Spermidine/putrescine transport system protein	4.4 ± 0.1
slr1974		Putative GTP-binding protein	4.4 ± 0.5
slr1254	*crtP*	Phytoene desaturase	4.3 ± 0.6
slr1392	*feoB*	Ferrous iron transport protein B	4.3 ± 0.8
slr0399	*ycf39*	Quinone-binding protein in PS II	4.3 ± 1.2
slr0423	*rlpA*	Rare lipoprotein A	4.1 ± 1.1
sll1441	*desB*	Fatty acid desaturase	4.0 ± 0.0
sll0385	*cbiO*	ABC-type cobalt transport system protein	4.0 ± 0.7
slr1992	*gpx2*	Glutathione peroxidase	3.7 ± 0.3
slr0426	*folE*	GTP cyclohydrolase I	3.7 ± 0.1
sll1770		ABC1-like	3.6 ± 0.5
sll1147		Glutathione S-transferase	3.6 ± 1.3
sll1772	*mutS*	DNA mismatch repair protein	3.6 ± 0.7
slr1881	*natE*	ABC-type neutral amino acid transport system	3.6 ± 0.4
slr1544		Hypothetical protein	20.6 ± 5.7
slr0082		Hypothetical protein	15.4 ± 4.0
slr1927		Hypothetical protein	6.6 ± 0.9
ssr2016		Hypothetical protein	6.2 ± 0.6
sll1611		Hypothetical protein	5.2 ± 0.8
sll1911		Hypothetical protein	5.1 ± 0.5
slr1747		Hypothetical protein	5.0 ± 0.0
sll0494		Hypothetical protein	4.5 ± 0.1
sll0815		Hypothetical protein	4.4 ± 0.5
slr0236		Hypothetical protein	4.4 ± 0.5
slr1677		Hypothetical protein	4.1 ± 1.3
slr1436		Hypothetical protein	4.0 ± 0.6
sll0355		Hypothetical protein	3.9 ± 0.7
slr0616		Hypothetical protein	3.8 ± 1.7
sll0185		Hypothetical protein	3.7 ± 0.6

Cells that had been grown at 34°C were incubated at 22°C for 30 min. Genes with ratios of transcript levels greater than 3.5 are listed.

accompany cold stress shortly after a drop in temperature (Los and Murata, 2004). Several of the genes that are induced by oxidative stress are also induced by cold stress in *Synechocystis* (Table 1). This observation suggests that cells might experience oxidative stress at the early stages of cold stress. However, our results with DNA microarrays indicate that *Synechocystis* perceives oxidative stress and cold stress as two distinctly different signals (Kanesaki et al., 2007).

The results of analysis by FTIR spectroscopy strongly suggest that Hik33 might recognize a

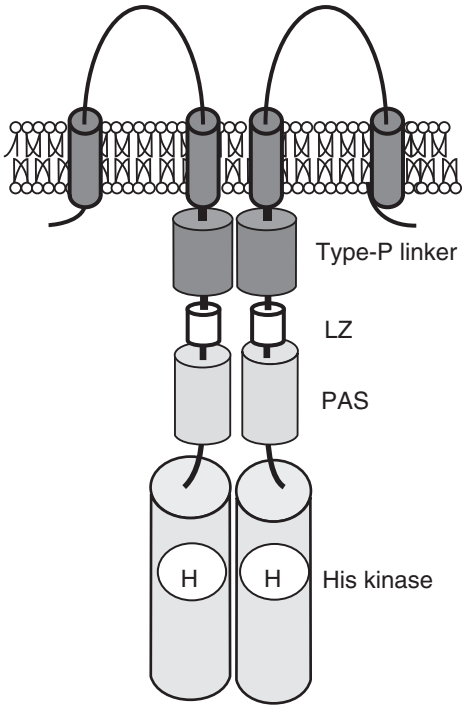

Fig. 2. A hypothetical scheme for the structure and activation of sensory histidine kinase Hik33 of *Synechocystis*. It consists of two transmembrane domains, type-P linker, leucine zipper (LZ), and PAS domain. A decrease in temperature or an increase in outer osmolarity rigidifies the membrane, leading to compression of the lipid bilayer. This compression forces the membrane-spanning domains to move closer together, changes the linker conformation, and leads finally to dimerization and auto-phosphorylation of the histidine kinase domains.

change in membrane fluidity at low temperatures (Murata and Los, 2006). This possibility was tested by mutation of the *hik33* gene in *desA*$^-$/*desD*$^-$ cells. In the resultant *desA*$^-$/*desD*$^-$/*hik33*$^-$ mutant cells, the expression of Hik33-regulated genes (*hliA*, *hliB*, and *sigD*) was no longer inducible by cold (Inaba et al., 2003). Thus, it appears that Hik33 perceives cold-induced membrane rigidification as the primary signal of cold stress.

In *B. subtilis*, the homolog of Hik33, histidine kinase DesK, was identified as a cold sensor that regulates the cold-inducible expression of the *des* gene for the Δ5 desaturase (Aguilar et al., 2001). DesK is also a membrane-bound sensor. Each monomer of DesK has four transmembrane domains and a histidine kinase domain. However, in contrast to Hik33, DesK lacks PAS and leucine zipper domains.

The *desK* gene forms an operon with the *desR* gene, which encodes a response regulator that binds specifically to the promoter region of the gene for the DesK desaturase. Induction of expression of the desaturase in *B. subtilis* by the DesK-DesR two-component system is inhibited by the addition of exogenous unsaturated fatty acids or isoleucine (Aguilar et al., 2001; Cybulski et al., 2002). This observation suggests the presence of a feedback loop between the function of the sensor and the extent of fatty acid unsaturation. It should be noted that the DesK-DesR two-component system regulates the cold-inducible expression of the *des* gene for the Δ5 desaturase while the cyanobacterial Hik33 sensor regulates the expression of many genes induced by different stress factors (see below).

C Cold-Inducible Gene Expression in Plants

Large numbers of cold-inducible genes have been identified in plants (Xiong et al., 1999; 2002; Shinozaki and Yamaguchi-Shinozaki, 2000; Seki et al., 2001; Fowler and Thomashow, 2003). These genes include genes for fatty acid desaturases (Wilhelm and Thomashow, 1993; Ishizaki-Nishizawa et al., 1996; Hayward et al., 2007), confirming the key role of the desaturation of fatty acids in membrane lipids in the regulation of membrane fluidity and the acclimation of plants to low temperatures, as postulated above (Nishida and Murata, 1996; Los and Murata, 2004; Novikova et al., 2007; Upchurch, 2008).

In *Arabidopsis thaliana*, DNA microarray analysis of the expression of 8,000 genes revealed that the expression of more than 200 genes is induced by cold (Fowler and Thomashow, 2003). These genes could be classified into several groups, such as genes for transcription factors, signal transducers, transporters, enzymes involved in the synthesis of cell walls and enzymes involved in the response to oxidative stress.

D Cold Sensing in Plants

Although the induction of the expression of genes for desaturases and the experiments with fluidizers and rigidifiers of membrane lipids (Sangwan et al., 2002) suggest that membrane rigidification might participate in cold signaling, nothing is known

about protein cold sensors in plants. Nevertheless, some components that act below sensors in the cold transduction pathway have been identified (Benedict et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). An analysis of the transcriptional control of two cold-inducible genes (*rd29A* and *cor15a*) in *Arabidopsis* led to the identification of a cold-responsive element, the CRT/DRE [(C-repeat)/(dehydration responsive element)], in their promoters (Shinwari et al., 1998). Members of a family of AP2-domain transcription factors, namely, DREB1 (DRE-binding protein) and CBF (CRT-binding factor), bind to the CRT/DRE element and activate transcription of possessing those domains (Liu et al., 1998; Chinnusamy et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 2006). Expression of the genes for these transcription factors was induced rapidly upon cold treatment of plants. At normal temperatures, the overexpression of CBF1, CBF2, and CBF3 in transgenic *Arabidopsis* enhanced the expression of 41 genes, 30 of which had been identified as cold-inducible genes in wild-type plants (Fowler and Thomashow, 2003; Benedict et al., 2006; Chinnusamy et al., 2006). Thus, CBF transcription factors might regulate the expression not only of cold-inducible genes but also of genes whose expression is induced by other signals. Recently it has been shown that low red to far-red ratio (R/FR) light signal increases CBF gene expression in *Arabidopsis* (Franklin and Whitelam, 2007). By contrast, some of the cold-inducible genes did not appear to be under the control of the CBF pathway, suggesting that some other regulatory system might exist for the cold-inducible regulation of approximately 60 genes.

In the mammalian nervous system, Ca^{2+}-permeable channels have been identified as cold sensors (McKemy et al., 2002; Peier et al., 2002a; Jordt et al., 2003; Patapoutian et al., 2003; Story et al., 2003; McKemy, 2007). Calcium ions play important regulatory roles as second messengers in many signal-transduction pathways (Knight et al., 1996; Trewavas and Malho, 1998). The rapid influx of Ca^{2+} ions into plant cells under cold stress suggests that Ca^{2+} channels or some non-specific ion channels might function as cold sensors in plants (Plieth et al., 1999; Plieth, 2005). However, direct experimental evidence is required to confirm this hypothesis.

E Sensing Hyperosmotic Stress

An increase in extracellular osmolarity causes the outward movement of water from cells, decreasing the cytoplasmic volume and cell turgor and, ultimately, inducing plasmolysis (Wood, 1999). Studies of the expression of genes in response to hypertonic stress have focused mainly on "defense genes" that ensure the synthesis of compatible solutes, such as proline, glycine betaine, trehalose, and sucrose (Wood, 1999; Hohmann, 2003).

Recent analysis of genome-wide patterns of transcription indicated that the expression of more than 200 genes is induced upon exposure of *Synechocystis* cells to hyperosmotic stress (Kanesaki et al., 2002). The products of these genes are responsible for the synthesis of components of cell walls and membranes and the phosphate-transport system. The gene products also include regulators of photosynthesis, signal transduction, gene expression and protein turnover, and they catalyze the synthesis of glucosylglycerol, the main compatible solute in *Synechocystis* (Hagemann et al., 1997; Engelbrecht et al., 1999). Similar phenomena have been observed in studies of genome-wide transcriptional responses to hyperosmotic stress in yeast (Gasch et al., 2000; Causton et al., 2001; Yale and Bohnert, 2001). Moreover, in both *Synechocystis* (Shoumskaya et al., 2005) and yeast (Hohmann, 2003), a relatively small number of genes are induced by hyperosmotic stress specifically.

Most of the genes induced by hyperosmotic stress are also induced by other kinds of stress, such as cold, heat, salt, and strong light (Suzuki et al., 2001; 2005; Hihara et al., 2001; 2003; Mikami et al., 2002; Paithoonrangsarid et al., 2004; Shoumskaya et al., 2005). Among the entire complement of hyperosmotic stress-inducible genes, quite a substantial number of genes encode proteins of as yet unknown function (Murata and Los, 2006). At present, it is difficult to conclude anything about the roles of these genes in acclimation to hypertonic stress. Further investigations with genetically engineered mutants are necessary, if we are to elucidate the roles of these genes in the responses of cells to hyperosmotic stress.

In *E. coli*, a membrane-integrated histidine kinase, EnvZ, is a putative osmosensor that perceives hyperosmotic stress and transmits the

signal to induce the expression of hyperosmotic stress-inducible genes (Nakashima et al., 1993a; Wood, 1999). EnvZ regulates the expression of the *omp* genes that encode outer-membrane porins (Qin et al., 2000).

Application of membrane fluidizers, such as procaine and chlorpromazine, stimulates the phosphorylation of EnvZ and the transfer of a phosphate group to the response regulator OmpR (Nakashima et al., 1991; 1993b). These findings suggest that the activity of the osmosensor might be regulated by changes in the physical state of membrane lipids.

In *Lactococcus lactis*, OpuA, an osmoregulated transporter of quaternary ammonium compounds, has the properties of an osmosensor and an osmoregulator, and it plays a key role in the protection of *L. lactis* from hyperosmotic stress (van der Heide and Poolman, 2000; Los and Murata, 2000; van der Heide et al., 2001). Reconstitution of an active OpuA system in artificial membrane vesicles composed of phospholipids demonstrated that OpuA is both essential and sufficient for the hyperosmotic activation of the uptake of glycine betaine by bacterial cells. The application of cationic (tetracaine and chloropromazine) and anionic (dipyrimidole) amphipathic molecules, which interact with the charged head groups of phospholipid molecules and alter the physical properties of phospholipid membranes, activated OpuA under iso-osmotic conditions to the same extent as the activation observed under hyperosmotic conditions, suggesting strongly that changes in the fluidity of membrane lipids lead to the activation of OpuA (van der Heide and Poolman, 2000).

The osmosensing systems and transduction of osmotic signals in yeast were reviewed by Hohmann (2003). Cells of *S. cerevisiae* have two membrane-integrated proteins, Sho1p and Sln1p, that are putative hyperosmosensors. Sho1p has four transmembrane domains and an SH3 domain for protein–protein interactions at its carboxyl terminus but it lacks kinase and phosphatase regulatory domains (Raitt et al., 2000). Thus, it is unlikely that Sho1p itself is a sensor of hyperosmolarity. However, it might operate in association with some other sensing protein(s), which has not yet been identified. Sln1p is a histidine kinase with a response-regulator domain and two transmembrane domains, and its role as a sensor of hyperosmolarity is well-documented (Ota and Varshavsky, 1993; Maeda et al., 1994). This sensor activates the HOG (high osmolarity glycerol) MAP kinase pathway for the expression of genes for the synthesis of glycerol, which is a major compatible solute in *S. cerevisiae* (Posas et al., 1996). It remains to be determined whether sensing of hyperosmolarity by Sln1p is modulated by membrane fluidity. At present, we can only postulate that this transmembrane sensory protein might perceive membrane rigidification or mechanical stress during the cell shrinkage that occurs as a result of the efflux of water under hyperosmotic stress.

Genome-wide analysis of transcription in *Synechocystis* with DNA microarrays indicated that Hik33, which was identified originally as a cold sensor (see above), controls the inducibility by hyperosmotic stress of nearly 60% of hyperosmotic stress-inducible genes (Kanesaki et al., 2002; Paithoonrangsarid et al., 2004; Shoumskaya et al., 2005). Hik33 controls the expression of genes whose products are involved in the synthesis and maintenance of cell walls and membranes, in formation of a phosphate-transport system, in protection of the photosynthetic apparatus, and in certain other phenomena that are important for acclimation to hyperosmotic stress. In particular, Hik33 controls the osmostress-inducible expression of the *ndpK* gene for nucleoside-diphosphate kinase, which catalyzes the synthesis of nucleoside triphosphates and acts as a regulator of signal transduction via the histidine-specific phosphorylation of the osmosensors EnvZ and CheA. It is very likely that Hik33 recognizes changes in membrane fluidity as the primary signal of both cold stress and hyperosmotic stress.

The histidine kinase AtHK1 of *Arabidopsis* can complement mutations in the osmosensor Sln1p in *Saccharomyces cerevisiae*. Therefore, it is likely that AtHK1 is a plant hyperosmosensor (Urao et al., 1999), and possible salt-stress sensor, which mediates signals with participation of plant hormones, such as abscisic acid and cytokinins (Tran et al., 2007). Such complementation implies that this histidine kinase from *Arabidopsis* can substitute for the osmosensor in yeast and can efficiently transduce a signal to a downstream MAP-kinase cascade. At present, AtHK1 is the only putative osmosensor identified in plants. The

molecular mechanisms of osmosensing in plants remain unknown and further detailed investigations at the submolecular level are required in view of the importance to agriculture of plants that can withstand environmental stress (Novikova et al., 2007).

IV Perception of Membrane Fluidization

A Heat-Induced Gene Expression

Heat-induced gene expression has been studied in *Synechocysis* on a genome-wide level (Inaba et al., 2003; Suzuki et al., 2005). Fifty nine genes were induced more than threefold in *Synechocystis* under heat stress (Suzuki et al., 2005). Mostly the genes were characterized by the transient mode of induction: they were highly induced by heat stress within 10–20 min, but 1 h after the onset of stress their transcription levels significantly declined. These genes encode proteins that belong mostly to the chaperone class (*hspA*, *groESL*, *groEL2*, *dnaJ*, *htpG*, *dnaK2*). Heat stress also induced the expression of *htrA* and *clpB1*genes for proteases, *sigB* for RNA polymerase sigma factor and *hik34* for histidine kinase 34, as well as *sodB* for superoxide dismutase and *hliC* for high light-inducible protein C. The expression of most genes involved in energy and lipid metabolism, pigment biosynthesis and photosynthesis decreased under heat-stress conditions (Suzuki et al., 2005).

Proteomic studies of heat-shock response in *Synechocystis* revealed over 100 spots, corresponding to 65 different proteins alter heat shock (Slabas et al., 2006). Changes occur not only in the classical heat shock proteins but also in the protein biosynthetic machinery, amino acid biosynthetic enzymes, components of the light and dark acts of photosynthesis and energy metabolism. In particular, following heat-shock treatment, there was a twofold increase in the level of the manganese-stabilizing protein, PsbO. Phycobillisome proteins decrease in abundance upon heat shock, and RUBISCO large subunit RbcL decreased over twofold (Slabas et al., 2006).

It should be noted again that the majority of the heat-induced genes and proteins are not entirely specific for heat stress, but they are also induced by osmotic, salt, as well as by oxidative stress, strong visible light and UV-B light (Los et al., 2008).

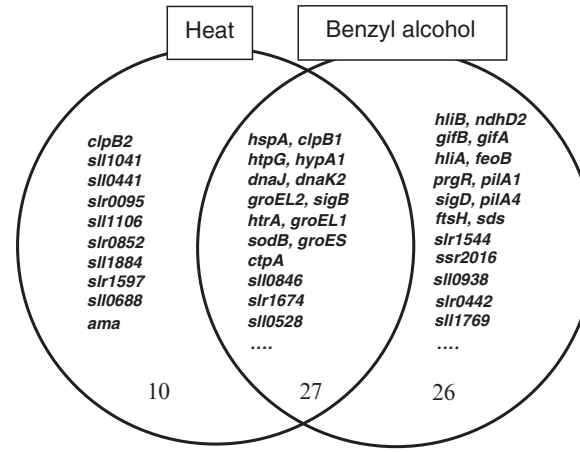

Fig. 3. Some heat stress-inducible genes and benzyl alcohol-inducible genes. The diagram includes genes that are induced during incubation for 10 min after a shift in growth temperature from 34°C to 42°C (heat stress) and after addition of benzyl alcohol at 30 mM for 20 min (fluidization). The diagram represents the tabular data of Inaba et al. (2003) with inclusion of our recent results. Numbers show total number of genes with induction factor higher than 4.0.

The genes that are specifically induced by heat mainly belong to those whose function is as-yet unknown: *slr0095*, *slr1920*, *slr1232*, and some others (Fig. 3).

B Gene Expression Induced by Membrane Fluidization

Heat stress causes membrane fluidization (Horváth et al., 2008). If a cold sensor is activated by rigidification of the plasma membrane (Suzuki et al., 2000; Los and Murata, 2004), it would be reasonable to suggest that a putative heat sensor in the membrane might be activated by membrane fluidization (Vigh et al., 1998). However, no membrane-located candidate for a heat sensor has been identified to date. Nevertheless, it has been demonstrated that, in *S. cerevisiae*, genetic enhancement of the extent of saturation of fatty acids in plasma-membrane lipids enhances the expression of the gene for heat-shock protein 90 (Hsp90) (Carratu et al., 1996). It appears that, in the yeast cells, the expression of the heat-shock gene depends on the fluidity of the membrane. Evidence to support this possibility was obtained in studies of the responses of *Synechocystis* cells to heat shock (Horváth et al., 1998; Török et al., 2001). At normal growth temperatures, the membrane

fluidizer benzyl alcohol activated transcription of the *hspA* gene as efficiently as heat stress. However, other studies do not support the putative involvement of membrane fluidity in heat-induced gene expression. Replacement of polyunsaturated fatty acids by mono-unsaturated fatty acids in the membrane lipids of *Synechocystis* by site-directed mutagenesis (Tasaka et al., 1996) did not affect the heat-induced expression of genes. Moreover, benzyl alcohol as a membrane fluidizer and heat treatment each induced specifically an essentially different set of genes (Fig. 3). Genes whose expression was induced by both the fluidizer and heat treatment comprised a group that included genes whose expression is induced by various kinds of stress, such as heat, salt, and hyperosmotic stress. These genes are so-called heat-shock genes, namely, *hspA*, *cplB*, *htpG*, *dnaJ*, *dnaK2*, *groESL*, *sodB*, *sigB*, and some other genes that are affiliated with different classes of metabolic and regulatory genes (Fig. 3). These findings suggest that treatment with benzyl alcohol is not a substitute for heat shock. Thus, sensing of heat via the detection of increases in membrane fluidity remains a controversial issue. The actual sensor of membrane fluidization caused by chemicals and/or high temperatures remains unknown.

The application of DNA microarray technique allowed identification of the histidine kinase Hik34 as an important contributor to thermotolerance (Suzuki et al., 2005). Overexpressed and purified recombinant Hik34 was autophosphorylated in vitro at physiological temperatures, but not at elevated temperatures, such as 44°C. The Hik34-mutant cells survived incubation at 48°C for 3 h, while wild-type cells and mutant cells with the other Hiks failed to do so. Knock-out mutant in Hik34 displayed the enhanced levels of transcripts of a number of heat-shock genes, including *htpG* and *groESL1*. Alternatively, overexpression of the *hik34* gene repressed the expression of these heat-shock genes. Thus, it seems likely that Hik34 negatively regulates the expression of certain heat-shock genes that might be related to thermotolerance in *Synechocystis*.

In *E. coli*, the heat signal is transduced, in part, via the CpxA-CpxR two-component system (Danese and Silhavy, 1997). CpxA is a transmembrane histidine kinase and CpxR is a response regulator of heat-inducible genes (Danese et al., 1995; Raivio and Silhavy, 2001; DiGiuseppe and Silhavy, 2003). The activity of CpxA is influenced by the composition of membrane lipids (Mileykovskaya and Dowhan, 1997), suggesting that this sensor might perceive changes in membrane fluidity. This regulatory system has, however, been found only in *E. coli*, *Salmonella typhi* and *Yersinia pestis* and it has not been found in any other bacteria examined to date (De Wulf et al., 2000). Thus, these organisms may have heat sensors that differ from CpxA in terms of primary structure and, possibly, in terms of the nature of signal perception.

A histidine kinase, HsfA, and a response regulator, HsfB, were identified in *Myxococcus xanthus* as components of a heat-transducing system (Ueki and Inouye, 2002). HsfA appears to be a soluble protein with a phosphate-receiver domain and, thus, it is likely to be a transducer rather than a heat sensor.

A heat-sensitive transient receptor potential (TRP) channels has been identified as a heat sensors in mammalian cells (Peier et al., 2002b; Patapoutian et al., 2003). Four heat TRP-channels have been characterized as heat sensors so far (Brauchi et al., 2006; McKemy, 2007) but no heat sensors have yet been identified in any photosynthetic organisms.

C Sensors of Hypoosmotic Stress

The molecular mechanisms by which cells perceive hypoosmotic stress are poorly understood. Research in this field has focused mainly on volume-regulated or stretch-activated channels, which were identified many years ago in biophysical and physiological studies of mammalian and bacterial cells. Such channels are thought to play a role in the regulation of cell volume by controlling the export of solutes and ions from the cell. The best studied system is the bacterial MscL (*m*echano*s*ensitive *c*hannel of *l*arge conductance) (Sukharev et al., 1994; Wood, 1999). This protein mediates the nonselective export of solutes from bacterial cells upon their exposure to severe hypoosmotic shock. Modeling based on the crystal structure of MscL (Sukharev et al., 2001), as well as results of cross-linking and mutagenesis experiments, support a two-stage model of channel opening, which leads to an increase in pore diameter of 1–13 Å. In this model, membrane tension leads to a conformational change from

a closed conformation to a closed-expanded and eventually open conformation, in which certain transmembrane helices are twisted within the membrane (Sukharev et al., 2001).

Our studies of the MscL in the freshwater *Synechocystis* revealed that this protein might sense not only membrane hypertension but also the membrane depolarization that occurs under cold stress (Nazarenko et al., 2003). In addition to the role of MscL in the regulation of cell volume, as postulated in *E. coli* (Blount and Moe, 1999; Moe et al., 2000; Poolman et al., 2002), the cyanobacterial MscL functions as an outward Ca^{2+} channel in response to cold stress (Nazarenko et al., 2003). Computer analysis reveals at least eight genes in the genome of *Synechocystis*, which may encode mechanosensitive ion channels, and their functional analysis is the matter of the future studies.

At present, there is no direct evidence of changes in membrane fluidity upon exposure of cells to hypotonic stress. Therefore, the involvement of membrane fluidity in the regulation of a cell's response to hypotonic stress still remains in the field of assumptions and speculations.

V Multifunctional Sensors

There appears to be substantial crosstalk among regulatory pathways that control the responses to various kinds of stress and hormonally regulated cascades (Los and Murata, 2000; Mikami et al., 2002; Xiong et al., 2002; Novikova et al., 2007; Los et al., 2008). It is now clear that different environmental stimuli can be perceived by common sensory proteins. For example, the histidine kinase Hik33 of *Synechocystis* was first identified as a component of a system that endows resistance to drugs that inhibit photosynthesis (Bartsevich and Shestakov, 1995). We subsequently characterized Hik33 as a cold sensor that appears to perceive membrane rigidification under cold stress (Suzuki et al., 2000; Los and Murata, 1999; 2004; Murata and Los, 2006). Then Hik33 was found to be involved in the sensing of hyperosmotic stress (Mikami et al., 2002; Paithoonrangsarid et al., 2004; Murata and Los, 2006), salt stress (Marin et al., 2003; Shoumskaya et al., 2005), and oxidative stress (Kanesaki et al., 2007). Studies with DNA microarrays indicate that Hik33 controls essentially different sets of genes under different kinds of stress. This implies diversification of signals, possibly, through interaction of Hik33 with different response regulators (Fig. 4).

The physical mechanisms by which Hik33 recognizes cold stress, salt stress, osmotic stress, and oxidative stress are still in question. It is very likely that Hik33 perceives changes in membrane fluidity that occur under cold stress, initial stage of salt stress and hyperosmotic conditions (Los and Murata, 2004; Murata and Los, 2006). It remains unclear whether changes in the physical motion of fatty acids in membrane lipids or changes in the surface charges of membrane lipids alter the conformation of Hik33, with its resultant activation. Our recent observation that cold stress causes depolarization of the plasma membrane (Nazarenko et al., 2003) does not exclude the possibility of the involvement of surface charge. Sln1p in yeast, which was characterized initially as an osmosensor, is also involved in perception of oxidative stress and heat stress (Godon et al., 1998; van Wuytswinkel et al., 2000).

DesK of *B. subtilis* is homolog of Hik33, which has four transmembrane domains, but lacks PAS and leucine zipper domains. DesK is a bifunctional enzyme with kinase and phosphatase activities, which is involved in two signaling reactions: phosphorylation in response to membrane rigidification and dephosphorylation in response to membrane fluidization (Albanesi et al., 2004; Mansilla et al., 2004). In fact, the carboxy-terminal portion of DesK (DesKC) acts as an autokinase, as well as a phosphatase; the phosphoryl group of phosphorylated DesKC is transferred to response regulator DesR. The resultant phosphorylated DesR can be dephosphorylated in the presence of DesKC in vitro. These findings suggest that DesK has the ability to modify DesR through both its kinase and its phosphatase activities, depending on the physical state of the membrane. It is likely but, as yet, unproved that transmembrane segments of DesK and Hik33 sense changes in membrane fluidity due to changes in temperature (Mansilla and de Mendoza 2005; Murata and Los, 2006).

The existence of multifunctional sensors has been discussed in plants (Zhu, 2001; Xiong et al., 2002). Cold, drought, and salinity induce a transient influx of Ca^{2+} ions into the cytoplasm of plant cells (Kiegle et al., 2000; Knight and Knight, 2001). Therefore, it has been suggested

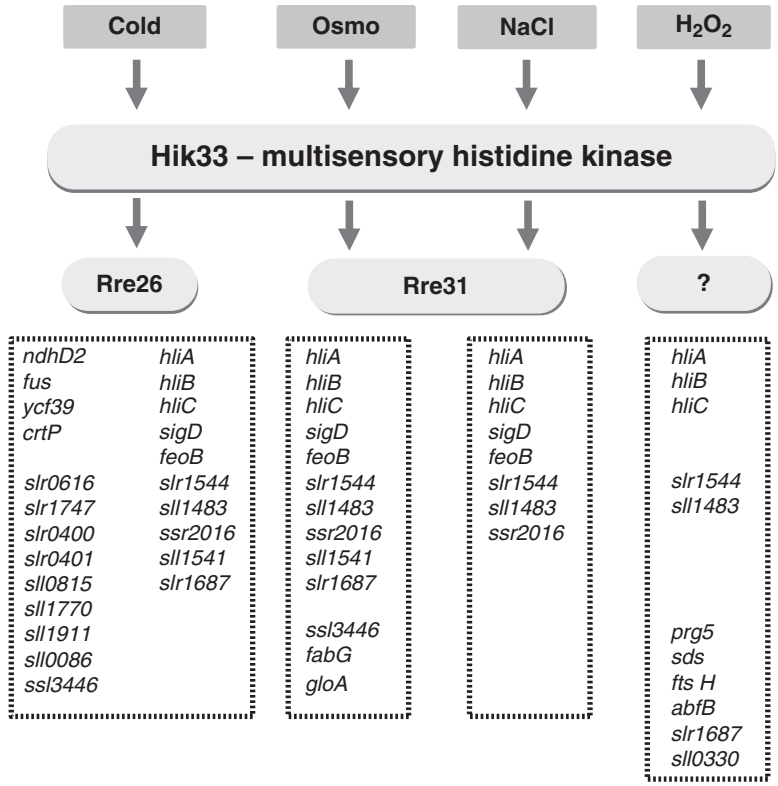

Fig. 4. Multisensory histidine kinase Hik33 controls the expression of genes under cold, salt (NaCl), hyperosmotic (osmo) and oxidative (H_2O_2) stress. Cold stress is transduced via the response regulator Rre26. Salt and osmotic stresses are transduced via Rre31. The transducer for oxidative stress is unknown.

that Ca^{2+} channels might be multifunctional sensors that sense stress-induced perturbations in plasma membranes, which might include changes in the fluidity of membrane lipids (Orvar et al., 2000; Sangwan et al., 2002).

The existence of sensors that perceive a variety of signals related to environmental conditions is an intriguing discovery. Considering membrane fluidity as the key parameter that allows cells to perceive environmental signals appropriately, we can conclude that there might be sensors that perceive changes in the physical state of the membrane, no matter what the nature of the stress that is responsible for the perturbation within the membrane. Such sensors should be bound to membranes or, at least, associated with membranes as are, for example, Hik33 and transmembrane channels.

VI Conclusions and Perspectives

It has been postulated that a change in membrane fluidity might be the primary signal in the perception of cold stress and, possibly, of osmotic stress. However, the molecular mechanisms that control perception and transduction of these signals via membranes have not been fully characterized. The availability of the complete genomic sequences of many organisms and DNA microarrays provide powerful tool for identification of stress sensors, transducers, individual genes and groups of genes that are induced specifically and non-specifically under certain stress conditions. It is very likely that the rigidification of membrane lipids at low temperatures and under hyperosmotic stress is the primary trigger for the corresponding acclimatory responses in cells.

The involvement of membrane fluidity in the perception of heat stress remains controversial (Vigh et al., 1998; Inaba et al., 2003). More precise investigations are necessary if we are to understand how membrane fluidity contributes to the perception of temperature and other environmental signals.

It is possible that some common sensor(s), such as Hik33, recognizes the rigidification of membrane lipids irrespective of the nature of the stimulus (e.g., cold stress, hyperosmotic stress, or salt stress). We do not yet know how sensory transmembrane proteins recognize a change in the membrane's physical state, nor do we know what distinct domains and exact amino acids are involved in signal perception. It is essential that efforts now be made to identify specific lipids or lipid domains that interact with each sensor and that participate in the modulation of its conformation and/or activity.

Acknowledgements

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Chapter 16

Lipid Trafficking in Plant Photosynthetic Cells

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Summary

Each of the various membranes in plant cells has a specific glycerolipid composition, which is kept relatively stable in different cells and different plants. Lipid homeostasis effectors, particularly lipid transporters, remain largely uncharacterized. Recent progresses in the field rely on the analysis of chloroplast lipid homeostasis as a model of choice. Galactolipids are the main lipids of chloroplast membranes. Galactolipid synthesis occurs in the chloroplast envelope membranes and depends on the fine exchange of lipid intermediates between the envelope membranes. This synthesis is also highly dependent on supply of lipid precursors synthesized in the endoplasmic reticulum membranes. Phosphatidic acid is an important lipid intermediate that is generated in the envelope but also in various extraplastidic membranes belonging to the endosomal network. It was recently shown that extraplastidic phosphatidic acid is one of the galactolipid precursors. As it is also a signalling molecule in plant cells, it could be a regulator of the lipid synthesis pathway. Trafficking of phosphatidic acid in the envelope is, therefore, a key step

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for synthesis of chloroplast lipids. After their completion, galactolipids are transferred to the thylakoids possibly through vesicles. The stability of the membrane lipid composition indicates a tight regulation at the subcellular level. This control is, however, modified when the plant is deprived of phosphate resulting in enrichment of digalactosyldiacylglycerol in mitochondrial membranes, the tonoplast and the plasma membrane. The transfers of specific lipids into and out of the chloroplast envelope, particularly the transfer of digalactosyldiacylglycerol to the mitochondria, are enhanced. The molecular mechanisms potentially involved in transport of these glycerolipids are surveyed.

I Introduction

In plant cells, membranes that enclose subcellular compartments have specific lipid compositions. Photosynthesis occurs in chloroplasts, which contain two distinct sets of membranes: a very organized network of internal membranes, the thylakoids, where the light phase of photosynthesis occurs and a pair of delimiting membranes, the chloroplast envelope, essential for chloroplast biogenesis and integration of the photosynthesis function in plant cell metabolism. Galactolipids are the main lipids of chloroplast membranes (see Chapter 3). Monogalactosyldiacylglycerol (MGDG) accounts for about 50% of thylakoid membrane lipids and digalactosyldiacylglycerol (DGDG) for about 30%. Sulfoquinovosyldiacylglycerol (SQDG), another glycolipid, is unique to chloroplast membranes and is generally present at low levels. Phospholipids are present in limited amounts in chloroplasts, mainly represented by phosphatidylglycerol (PG) (see also Chapter 11) and in a minor proportion by phosphatidylinositol (PI). Phosphatidylcholine (PC) is present but constrained to the chloroplast surface. The lipid composition of non-plastid membranes is very different from that of plastid membranes. Non-plastid membranes contain negligible levels of galactolipids but high amount of phosphoglycerolipids mainly PC, phosphatidylethanolamine (PE), PG, PI and phosphatidylserine (PS). Cardiolipin also known as diphosphatidylglycerol (DPG) is restricted to the mitochondria inner membrane (see Chapter 4).

The lipid composition of each type of membrane is relatively constant across different cells or different plants, indicating tight regulation of the lipid homeostasis at the subcellular level. However, under Pi deprivation, the overall phospholipid content of the cell decreases, being consistent with a mobilization of the Pi reserve (see Chapter 3). Conversely, an increase of non-phosphorous lipids, such as DGDG and SQDG, occurs. One form of DGDG with specific fatty acids, i.e., 16:0 at glycerol *sn*-1 position and 18:2 at *sn*-2 position in particular is increased. In correlation, there is a strong modification of the lipid distribution between membranes. DGDG is thus found not only in chloroplast but also in the plasma membrane, mitochondria and tonoplast.

The lipid distribution in membrane compartments cannot be explained solely by metabolic processes confined in the corresponding membranes, because most lipids are not only present at the site where they are synthesized. In the plant cell, there are three main sites of glycerolipid synthesis (Fig. 1). (1) The syntheses of galactolipids, SQDG and chloroplast PG occur in the chloroplast envelope. (2) PC, PI, PS, PG and a part of PE are formed in the ER. (3) Mitochondrial PG, DPG and PE are formed in the mitochondria inner membrane. However, chloroplast glycolipid synthesis requires a transfer of PC-related lipid precursors from ER to chloroplasts whereas mitochondrial PE synthesis relies on PS formation in the ER. DGDG located outside of plastids under Pi deprivation is apparently formed in plastids before its transfer to extra-plastidial membranes. Lipid trafficking is, therefore, essential for the organization of each membrane and for the development of the cell.

In this review, we describe the glycerolipid trafficking events involved in biogenesis and function of plant photosynthetic cells. Firstly, we describe the lipid trafficking involved in formation of chloroplast lipids. The lipid exchanges centralized on the chloroplast envelope are especially important

Abbreviations: DAG – Diacylglycerol; DGDG – Digalactosyldiacylglycerol; DPG – Diphosphatidylglycerol; ER – Endoplasmic reticulum; LTP – Lipid transfer protein; Lyso-PC – Lysophosphatidylcholine; MCSs – Membrane contact sites; MAM – Mitochondria associated membranes; MGDG – Monogalactosyldiacylglycerol; PA – Phosphatidic acid; PC – Phosphatidylcholine; PE – Phosphatidylethanolamine; PG – Phosphatidylglycerol; PI – Phosphatidylinositol; PS – Phosphatidylserine; PAM – Plasma membrane associated membranes; PLAM – Plastid associated membranes; SQDG – Sulfoquinovosyldiacylglycerol

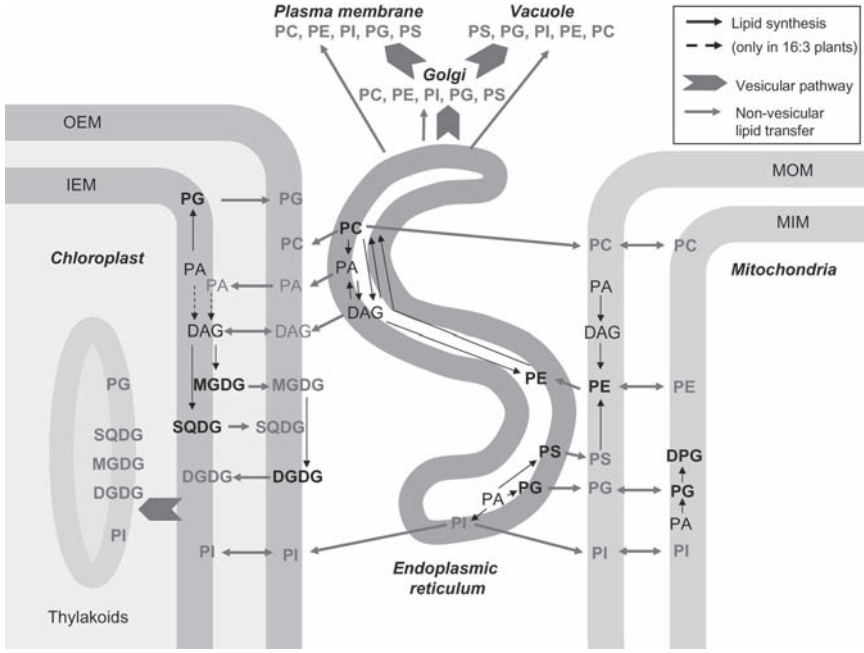

Fig. 1. Glycerolipid synthesis and inter-membrane glycerolipid exchanges in plant cell. Three main sites of lipid synthesis are represented: ER, chloroplast envelope and mitochondria. Vesicular and non vesicular lipid transfers are symbolized by different *arrows* as indicated.

for galactolipid synthesis. Secondly, we describe the effect of Pi limitation on lipid trafficking. Pi deprivation is a physiological situation that the plant partly circumvents by membrane reorganization supported by the mobilisation of certain lipids. Finally, the mechanisms potentially involved in the previously described lipid transfers are analyzed using information issued from plant genetics and proteomics and from several lipid trafficking mechanisms recently discovered in other systems, such as yeast and mammalian cells.

II Lipid Trafficking Involved in Formation of Chloroplast Lipids

A Galactolipid Synthesis in the Chloroplast Envelope

Each glycerolipid class is represented by a range of molecular species differing in their acyl composition at *sn*-1 and *sn*-2 positions of the glycerol backbone. Based on this acyl molecular diversity, two main classes of glycerolipids have been identified (Heinz and Roughan, 1983) markedly represented in chloroplasts at the level of MGDG molecular species (Mongrand et al., 1998). Using the model of bacterial or cyanobacterial lipids, the prokaryotic-type of glycerolipids contains a 16-carbon fatty acid at the *sn*-2 position of glycerol. The eukaryotic type contains an 18-carbon fatty acid at the *sn*-2 position. Some plants, such as *Arabidopsis* and spinach, have both prokaryotic- and eukaryotic-type MGDG, whereas other plants, such as pea and cucumber, have only eukaryotic-type MGDG. DGDG is mostly of eukaryotic type in all plants. Chloroplast PG contains exclusively prokaryotic-type DAG and is unique since it contains a *trans* Δ3−16:1 fatty acid at the *sn*-2 position. In contrast, PC is a typical eukaryotic-type lipid.

MGDG synthase activity was first localized in the chloroplast envelope (Douce, 1974). The enzyme transfers galactose from UDP-gal to DAG. Molecular studies led to the identification of two families of MGDG synthases associated with plastid envelope membranes (Awai et al., 2001). In *Arabidopsis*, AtMGD1 is the unique member of the first MGDG synthase family and is necessary for development of photosynthetic membranes (Jarvis et al., 2000; Kobayashi et al., 2007). MGD1 is highly active and can produce prokaryotic- and eukaryotic-type galactolipids with the same efficiency (Maréchal et al., 1994). Suppression of MGD1 in *Arabidopsis* leads to

the absence of MGDG and DGDG and impairment of photosynthetic growth with an arrest at embryo development (Kobayashi et al., 2007). Invagination of the inner envelope was visible in the *mgd1* mutant, suggesting a blockage in membrane trafficking from the inner envelope to nascent thylakoids (Kobayashi et al., 2007). MGD1 is located in the inner envelope membrane and faces the intermembrane space of the envelope (Block et al., 1983; Miège et al., 1999; Awai et al., 2001; Vojta et al., 2007). Its association with the membrane involves lipid interaction and data suggest that the protein is anchored to the external monolayer of the envelope inner membrane as a monotopic protein (Miège et al., 1999; Awai et al., 2001). Inactivation kinetics after γ-ray irradiation indicated that native and recombinant MGD1 are active as homodimers (Miège et al., 1999). Interestingly, the structure of MGDG synthase as a monotopic homodimer, with separate sites for its substrates, might be analogous to that described for its homolog, i.e., MURG, a bacterial peptidoglycan synthesizing enzyme for which the crystal structure has been resolved (Hu et al., 2003). MURG was used as a template for structure prediction of SoMGD1. On this basis, each MGD1 monomer is likely to be organized in two Rossmann folds (N- and C-domains). Point mutagenesis experiments indicated that the catalytic site is located in the cleft between the N- and C-domains (Botté et al., 2005). Due to difference between MURG and MGD1, position of the DAG binding site in the N-domain could not be resolved. The enzyme activity is enhanced by anionic lipids, phosphatidylglycerol (PG) or phosphatidic acid (PA) (Coves et al., 1988; Ohta et al., 1995; Kelly et al., 2007). Anionic lipids are likely important for the anchoring of the enzyme to the membrane. Visualization of surface hydrophobic regions suggested that MGD1 interacts with the membrane surface through the N-domain whereas the C-domain protrudes above the membrane and possibly facilitates dimerization (Botté et al., 2005). Understanding MGD1 interaction with different kinds of membrane lipids and the physiological significance of these interactions remains to be a challenging question.

Characterization of the *Arabidopsis dgd1* mutant severely impaired in DGDG synthesis led to the first identification of a DGDG synthase gene (Dörmann et al., 1995). The *dgd1* mutant is strongly affected in photosynthesis (Dörmann et al., 1995; Härtel et al., 1997). Based on sequence similarity, a second gene, dgd2, was identified in the *Arabidopsis* genome (Dörmann et al., 1999). In contrast with dgd1, the dgd2 gene is not essential for DGDG synthesis under optimal growth conditions (Kelly et al., 2003). DGD1 is composed of two distinct domains: an N-terminal domain of unknown function and a C-terminal glycosyltransferase domain. DGD2 protein shows only a glycosyltransferase domain. Both DGD1 and DGD2 catalyze DGDG synthesis by transfer of galactose from UDP-galactose to MGDG (Kelly and Dörmann, 2002; Kelly et al., 2003). Difference between MGDG and DGDG fatty acid composition suggests that some MGDG molecular species are selectively used for DGDG synthesis. Selective affinity of DGD1 and DGD2 for some MGDG molecules is possible. Analyses of the *dgd1* and *dgd2* null mutants indicated that the preferential in vivo substrate of DGD1 is MGDG molecular species with 18-carbon fatty acids in both *sn*-positions whereas preferential in vivo substrate of DGD2 is MGDG with 16-carbon fatty acids in the *sn*-1 position and 18-carbon fatty acids in the *sn*-2 position (Kelly et al., 2003). DGD1 and DGD2 are presumably associated with the outer membrane of the chloroplast envelope (Froehlich et al., 2001; Kelly et al., 2003), but additional mitochondrial localization is possible for DGD1 since the protein was detected in a proteome survey of mitochondrial membranes (Heazlewood et al., 2004). It has been previously observed that activity of DGD1 does not depend on direct interaction with MGDG synthase for DGDG biosynthesis (Froehlich et al., 2001). This suggests that the transport of MGDG from the inner envelope membrane, where MGD1 is located, to the outer envelope membrane, where DGD1 is, is not facilitated by a simple MGD1 and DGD1 channelling. The mechanism involved in this necessary lipid transport is, however, not yet known.

B Transfer of Diacylglycerol Backbone from Phosphatidylcholine to Galactolipids

In most plants, a major proportion of MGDG results from galactosylation of eukaryotic DAG. Whereas prokaryotic DAG is formed inside plastids by acylation of glycerol-3-P and dephosphorylation of

phosphatidic acid (PA) (Joyard and Douce, 1977), eukaryotic DAG originates from PC hydrolysis. Studies with labelled lipid precursors indicated that PC provides its DAG-backbone to galactolipids (Heinz and Harwood, 1977; Slack et al., 1977). Desaturation of PC outside of the chloroplast is important for this biosynthesis since the *fad2 Arabidopsis* mutant, deficient in the ER-located desaturation of C18:1, contains a smaller eukaryotic vs prokaryotic MGDG ratio than wild type plants (Okuley et al., 1994). Formation of eukaryotic-type galactolipids consequently requires transfer of PC or of a PC derivative between ER and chloroplast.

PC is present in the outer leaflet of outer envelope membrane (Dorne et al., 1985) but the only reported site of PC de novo biosynthesis is in the endomembrane system, e.g., in the ER. This strongly suggests that PC is transported from ER to the envelope surface. Partial hydrolysis of PC to LysoPC in endomembranes could favour transfer of PC between endomembranes and chloroplast since amphiphilic lysoPC can easily move through the cytosol (Bessoule et al., 1995). In support to this hypothesis, phospholipases A2 are abundant in endomembranes and a lysoPC acyltransferase activity was detected in the chloroplast envelope (Bessoule et al., 1995; Kjellberg et al., 2000).

Conversion of PC into galactolipids requires PC hydrolyzing enzymes. However, the necessary hydrolytic equipments to convert PC into DAG are not present within chloroplasts and, therefore, extra-plastidial phospholipases are necessary for galactolipid formation (Oursel et al., 1987; Andersson et al., 2004). Several studies suggested a possible role of extraplastidic DAG as an intermediate between PC and galactolipids (Williams et al., 2000; Jouhet et al., 2003). Formation of DAG from PC can be mediated by a direct hydrolysis by a phospholipase C (PLC) or by an indirect two-step production. The first step is mediated by a phospholipase D (PLD) that yields phosphatidic acid (PA) and the second step of PA dephosphorylation by a PA phosphatase. The only phospholipase C that was shown to be related to galactolipid formation was NPC4 although this plasma membrane protein has a low expression level in photosynthetic organs and is known to be involved in catabolism of plasma membrane PC during Pi deprivation (see below; Nakamura et al., 2005). Similarly, two phospholipases D, PLDζ1 and PLDζ2 were shown to play a role in galactolipid formation in roots under Pi deprivation (see below; Cruz-Ramirez et al., 2006; Li et al., 2006a, b). However, the PLDζ2 localization in leaf tonoplast suggests an additional role of the enzyme in galactolipid formation possibly through PA signalling (Yamaryo et al., 2008). Finally, although data support a PC-derived hydrophobic moiety is likely to be the molecular species transported from the ER to the chloroplast for galactolipid synthesis, the enzymes necessary for conversion of PC into MGDG and for associated lipid transport remain unidentified.

In some cellular regions, ER and chloroplast envelope are in close proximity. Although vesicles have never been observed between ER and chloroplast, it has been proposed that lipid potentially transit through the secretory pathway. Data indicate that targeting of some chloroplast proteins can involve a brefeldin A-sensitive vesicular compartment (Villarejo et al., 2005). However, brefeldin A treatment of plant cells does not affect galactolipid formation (Merigout et al., 2002) and to date there is no indication for transport of galactolipid precursors through vesicular trafficking. A recent work analyzed the relationship between ER and chloroplast envelope (Andersson et al., 2007). The investigators used confocal microscopy in combination with a laser scalpel and optical tweezers to visualize and optically manipulate ER-chloroplast interface. The ER appeared as an extensive network surrounding chloroplasts, with ER branch end points localized at the chloroplast surfaces establishing membrane contact sites. The chloroplast-ER associations withstood a force exceeding 400 pN and ranged above strong protein–protein interaction. The membrane contact sites are possibly involved in lipid transfer necessary for MGDG formation but no demonstrative evidence has yet been gathered.

C Trafficking of Phosphatidic Acid in the Chloroplast Envelope

Several envelope proteins recently characterized, TGD1, TGD2 and TGD3, contribute to the transport of PA through the envelope membranes (Xu et al., 2003; Awai et al., 2006; Lu et al., 2007). TGD1 is part of an ABC-type transporter localized in the envelope inter-membrane space. TGD2

is a PA-binding protein spanning the inner envelope and TGD3 is a small ATPase localized in the chloroplast stroma. In Gram(−) bacteria, the corresponding proteins are encoded by an operon, suggesting a common biological role. Using the model established for MsbA, an ABC-type lipid transporter biochemically characterized in *E. coli*, TGD1, TGD2 and TGD3 could form a functional complex associated with the inner envelope and be involved in PA import through the envelope membranes.

Lipid metabolism is strongly modified in the *tgd* mutants. Galactolipid metabolism is disturbed with increased amount of trigalactosyldiacylglycerol (TGDG) and reduced amount of MGDG and DGDG. Since content in eukaryotic-type galactolipids is particularly affected, it was proposed that TGD proteins could control the flux of ER-derived PA through the envelope establishing a link between lipid metabolism in ER and thylakoid development. Interestingly, an ER-located TGD4 protein was recently reported to be part of the machinery mediating lipid transfer between the ER and the outer plastid envelope membrane (Xu et al., 2008b).

In the envelope, PA would subsequently be converted into DAG for galactolipid synthesis. A PA phosphatase activity is consistently measured in the inner envelope membrane (Joyard and Douce, 1979; Block et al., 1983) and three chloroplast-located PA phosphatases have been recently characterized (Nakamura et al., 2007). One PA phosphatase, named LPPε2, was detected in a proteomic analysis of *Arabidopsis* chloroplast envelope (Rolland et al., 2009).

Considering the location of enzymes interacting with PA, we can build, at the molecular level, a very constrained three-step pathway for PA metabolism in the envelope. PA is (1) likely delivered to the internal monolayer of the inner envelope (Lu et al., 2007), (2) converted to DAG by transmembrane PA phosphatases (Nakamura et al., 2007) and (3) finally galactosylated by MGD1 in the external monolayer of the inner envelope (Botté et al., 2005; Vojta et al., 2007). In this pathway, one has to consider that PA phosphatase activity is negatively regulated by DAG (Malherbe et al., 1992) and that MGDG synthase is stimulated by PA (Ohta et al., 1995).

The role and fate of eukaryotic PA in the envelope remain unclear. In some plants, such as pea, that contain only eukaryotic galactolipids, the activity of the inner envelope-associated PA phosphatase is very low and apparently inefficient to generate galactolipids (Andrews et al., 1985). In tobacco, a plant that contains both prokaryotic and eukaryotic galactolipids, eukaryotic PA is not detected in the envelope under standard conditions, and only a pool of prokaryotic PA can be detected but at a very low level (Fritz et al., 2007). Moreover, it does not seem that eukaryotic PA can transiently accumulate in the envelope under standard conditions since it was observed that artificially increased formation of eukaryotic PA in the envelope through heterologous expression of a bacterial DAG kinase induced conversion of eukaryotic PA into the eukaryotic PG, which is usually absent in chloroplasts (Fritz et al., 2007). As a secondary effect, this situation induced a severe reduction of plant growth. The very low amount of eukaryotic PA present in the envelope under standard conditions is an argument that PA could function in the envelope as a signalling molecule rather than a metabolic intermediate.

D Lipid Transfer to the Thylakoids

Thylakoids do not contain the lipid-synthesis machinery. Once synthesized in the envelope, chloroplast glycerolipids, i.e., MGDG, DGDG, PG, and also SQDG, are transported to the thylakoids (Siebertz et al., 1980). Vesicle budding from the envelope inner membrane suggests a bulk transfer of lipids through vesicular trafficking to the thylakoids. Vesicles were observed by electron microscopy in young chloroplasts, particularly at low temperature (Carde et al., 1982, Morré et al., 1991). Recent observations on the *mgd1* null mutant support the hypotheses that invagination of the inner envelope initiates the formation of thylakoid membranes from undifferentiated proplastids and that MGDG synthesis is an essential step in this formation (Kobayashi et al., 2007).

A first attempt to analyze the biochemical characteristics of chloroplast vesicular trafficking was done by Räntfors et al. (2000) and bioinformatic studies suggested that a system similar to the COPII vesicular pathway is present in plastids (Andersson and Sandelius, 2004). Several proteins involved in this trafficking have been identified, such as an NSF homolog protein (Hugueney

et al., 1995), a dynamin-like protein, ADL1 (Park et al., 1998), a putative regulator of vesicle coalescence, called Thylakoid Formation 1 or Thf 1 (Wang et al., 2004), and a vesicle-inducing protein, called Vesicle Inducing Protein in Plastid 1 (VIPP1) (Kroll et al., 2001). VIPP1 is a hydrophilic protein found in both the inner envelope and the thylakoid membranes (Li et al., 1994). In a mutant with reduced expression of VIPP1, a disturbed network of thylakoids with widely and irregularly spaced lamellae was observed and vesicle budding from the inner envelope at low temperature was abolished (Kroll et al., 2001). Recent data demonstrated that VIPP1 forms a high molecular mass complex closely associated with the inner envelope membrane and suggest that the C-ter of the protein protrudes from the complex into the chloroplast stroma possibly for interaction with some other proteins (Aseeva et al., 2007). Accordingly, soluble VIPP1 interacts with the HSP70B/CDJ2 chaperone pair (Liu et al., 2005). By analogy with action of the auxilin/Hsc70 chaperone pair with clathrin on clathrin-coated vesicles, HSP70B/CDJ2 might disassemble and/or assemble VIPP1 oligomers to reuse the system for another cycle of vesicle formation/transport (Liu et al., 2005). Although there is no doubt that a vesicular trafficking exists inside the chloroplast and is important for the formation of thylakoid membranes, its relationship with lipid trafficking is not certain. Even though the mutants have distorted internal membrane network they have normal lipid composition, probably because inner envelope membrane and thylakoids have the same lipid composition and, therefore, accumulation of an intermediary pool of membranes cannot change the overall lipid composition.

III Lipid Trafficking Induced by Phosphate Deprivation

A Phosphate Deprivation-Induced Modification of Plant Cell Membranes

Phosphorous (P) is essential for plant growth, development and reproduction. Phospholipids represent up to 30% of organically bound P and it has been established that Pi deprivation decreases phospholipid content. Conversely an increase in levels of non-phosphorous lipids, such as DGDG and SQDG, is observed. It was first demonstrated in leaves of the *Arabidopsis pho1* mutant that was impaired in Pi transport from roots to leaves (Härtel et al., 1998). Moreover, Pi deprivation induces a particular accumulation of a eukaryotic DGDG with specific fatty acid signature (16:0 at glycerol *sn*-1 position and 18:2 at *sn*-2 position) (Härtel et al., 1998, 2000; Klaus et al., 2002).

Since PC and DGDG contents are both reversely modified under Pi starvation and both are bilayer-forming lipids, it has been proposed that DGDG could replace missing PC in cell membranes (Härtel and Benning, 2000; Härtel et al., 2000). This was indeed demonstrated for plasma membrane (Andersson et al., 2003), tonoplast (Andersson et al., 2005) and mitochondrial membranes (Jouhet et al., 2004). Upon advanced Pi starvation, DGDG can form up to 20–25% of the lipid content of these membranes whereas no significant amount of DGDG could be found in other membranes, such as ER or Golgi (Jouhet et al., 2004; Andersson et al., 2005). Plasma membrane DGDG has a clearly different fatty acid composition from chloroplast DGDG and, therefore, possibly a distinct origin. In contrast, mitochondrial DGDG was demonstrated to originate from chloroplast DGDG (Jouhet et al., 2004).

B Digalactosyldiacylglycerol Transfer from Chloroplasts to Mitochondria

The origin of mitochondrial DGDG induced by Pi deprivation was investigated by several methods. Anomeric configuration of galactose was analyzed by proton-NMR (Jouhet et al., 2004). Data indicated a Gal(α1–6) configuration of the second galactose as in DGDG that is synthesized by MGD and DGD-type enzymes. Since several studies reported overexpression of MGD2 and MGD3 (Awai et al., 2001; Kobayashi et al., 2004, 2006), DGD1 and DGD2 (Klaus et al., 2002; Kelly et al., 2003; Benning and Ohta, 2005) under Pi deprivation, the Pi deprivation is likely to induce the DGDG synthesis by these enzymes. Mutant analysis confirmed that MGD2, MGD3, DGD1 and DGD2 are involved in this synthesis (Härtel et al., 2000; Kelly et al., 2003; Kobayashi et al., 2006). Interestingly, a putative mitochondrial protein, DGS1, was proposed to be part of a signal transduction pathway involved in galactolipid homeostasis. Knocking-out of the

dgs1 gene led to overexpression of mgd2 and mgd3 (Xu et al., 2008a). Although MGD1 is not overexpressed under Pi deprivation, galactosylation of DAG by MGD1 is, however, still possible at least in leaves, since: (1) the ratio of prokaryotic/eukaryotic DGDG remains constant upon Pi deprivation (Klaus et al., 2002) and (2) MGD1 is the only enzyme that has a good affinity for prokaryotic DAG (Awai et al., 2001).

MGDG synthase being considered as a marker of plastid envelope, the occurrence of DGDG in mitochondria raised a question of its possible formation in plastid and subsequent transport to mitochondria. Whereas MGD1 is present in the inner envelope, MGD2 and MGD3 associate with the plastid outer envelope (Awai et al., 2001). By confocal imaging of MGD2:GFP expressed in *Arabidopsis* plants, the fusion protein was observed on the surface of chloroplasts even under Pi deprivation (J. Jouhet and M.A. Block, unpublished observations). Furthermore, only background levels of galactosyltransferase activity was detected in highly purified mitochondria from Pi-deprived plant cells, and the low activity present in some mitochondrial fractions was consistent with cross contamination with envelope membranes (Jouhet et al., 2004). Finally, by following the ratio of DGDG vs MGDG during purification of mitochondria from Pi-deprived cells, a selective transfer of DGDG from chloroplast envelope to mitochondria was demonstrated (Jouhet et al., 2004).

Electron microscopy suggested that lipid transfer between the two organelles was not conducted through vesicles but rather through membrane apposition. In support to this, an increased number of contact sites between chloroplast and mitochondria were observed transiently after exposure of cells to Pi deprivation (Jouhet et al., 2004).

C Relationship between Phospholipid Breakdown and Digalactosyldiacylglycerol Synthesis under Phosphate Deprivation

Jouhet and coworkers analyzed the time-course evolution of lipid composition of a cell suspension after exposure of plants to Pi starvation (Jouhet et al., 2003). As an early response, a transient increase of PC was observed. There was a significant increase of DAG with the same fatty acid composition as that of PC. This indicated an activation of both PC synthesis and PC hydrolysis. On one hand, PC was probably generated from degradation of other phospholipids since, for instance, PE content significantly decreased rapidly after transfer of plants to Pi-deprived conditions. On the other hand, this pool of transiently accumulated PC was probably the source of DAG for DGDG production. During Pi deprivation, the evolution of DGDG fatty acid composition is compatible with the galactosylation of PC-derived DAG and the selection of some acyl molecular species by MGDG synthase and DGDG synthase. The processes involved in DAG formation are, therefore, the key to understanding the relationship between degradation of phospholipids and formation of Pi deprivation-induced DGDG.

Phospholipases, PLC and PLD, are involved in early events of Pi deficiency (Misson et al., 2005; Russo et al., 2007). In *A. thaliana* roots, a novel non-specific PLC, NPC4, was induced 6 days after transfer of plants to a Pi-free medium (Nakamura et al., 2005). Expression of a similar PLC, NPC5, is also enhanced when *Arabidopsis* plants are transferred into Pi-deficient conditions but at a relatively lower level than NPC4 (Nakamura et al., 2005). NPC4 is present in the plasma membrane and analysis of *npc4* null mutants indicated a role in PC hydrolysis related to Pi deprivation but little effect on formation of galactolipids. In contrast, the cytosolic NPC5 is required for normal accumulation of DGDG during phosphate limitation in leaves (Gaude et al., 2008). PLD and PA phosphatase activities were the dominant lipase activities induced during prolonged Pi deprivation (Andersson et al., 2005). Detection of a rapidly enhanced high level of expression of PLDζ2 under Pi deprivation suggested a role of this protein in lipid changes associated with Pi deprivation (Misson et al., 2005). Also, under Pi deprivation, absence of PLDζ2 affected the capacity for DGDG formation in roots (Cruz-Ramirez et al., 2006; Li et al., 2006a, b). The PLD gene family in *Arabidopsis* comprises 12 members including the PLDζ subfamily (PLDζ1 and PLDζ2) that is characterized by a long regulatory sequence containing phox homology (PX) and pleckstrin homology (PH) domains usually involved in protein interaction and phosphoinoside binding (Qin and Wang, 2002). PLDζ1 is suspected to play also a role in the adaptation to Pi deprivation although pldζ1 expression is much

less sensitive to Pi deprivation than pldζ2 (Li et al., 2006b). The role of PLDζ2 is possibly to feed PA to the galactolipid synthesis that occurs in the plastid envelope. However, PA is also considered as an intracellular signal for activation of many plant cell processes (Wang, 2004; Testerink and Munnik, 2005) and there is evidence that PLDζ2 is involved in root system architecture, auxin-dependent hypocotyl elongation and vesicle cycling (Li et al., 2006a; Li and Xue, 2007). Furthermore membrane lipid alteration during Pi starvation was shown to be dependent on both Pi signaling and auxin/cytokinin crosstalk (Xu et al., 2003; Awai et al., 2006; Kobayashi et al., 2006). Therefore, the role of PLDζ2 in membrane lipid modification during Pi deprivation remains unresolved and is a challenging question.

PLDζ2 localizes in tonoplast (Yamaryo et al., 2008) and it was observed that vacuolar membrane development was stimulated in the absence of PLDζ2. PLDζ2 was targeted to tonoplast regardless of available Pi. Pi deprivation, however, enhanced transient formation of PLDζ2-enriched domains in the tonoplast and these domains were preferentially positioned in close proximity to mitochondria and chloroplasts. The localization of PLDζ2 in tonoplast is likely to indicate that the protein is active on tonoplast phospholipids. However, the close proximity of PLDζ2-enriched domains with mitochondria can also indicate that PLDζ2 interacts with lipid modification that occurs in mitochondria upon Pi deprivation. As a support to this hypothesis, the increased number of contact sites between mitochondria and chloroplasts observed on cell culture after Pi deprivation (Jouhet et al., 2004) could possibly be related to the transient increase of PLDζ2-enriched tonoplast domains observed at an early stage of plant growth under Pi deprivation (Yamaryo et al., 2008). The position of PLDζ2-enriched domains beside chloroplasts, where mitochondria often position, further supports this hypothesis. High concentration of PLDζ2 could facilitate transfer of lipids between mitochondria and chloroplasts through PA produced by PLDζ2 and/or through a structure induced by the PLDζ2 protein itself.

Pi deprivation is a way to disturb cell membrane homeostasis. This illustrates a connection between membrane biogenesis and primary metabolism. This also shows that regulation of membrane lipid composition in a plant cell goes through a complex mechanism that involves all membrane compartments and a wide network of interaction between membranes. Both lipid metabolism and lipid trafficking are affected. We observed that several types of lipid trafficking between membranes are triggered such as a transfer of PC-derivatives from ER to chloroplast and a transfer of DGDG from chloroplast to mitochondria. They are most likely transported via membrane contacts, although the precise molecular mechanism is not known.

IV Molecular Mechanisms Involved in Lipid Trafficking

A Motion of Glycerolipids

Lipids can spontaneously diffuse in the plane of the membrane bilayer with rapid velocity (in the range of $0.1-1\,\mu m^2\ s^{-1}$) (Vaz et al., 1984). They can also move from one monolayer of the membrane to the other (flip-flop) and outside the source membrane to another membrane through aqueous phase (inter-membrane transfer) although the diffusion rate for these latter types of movement are much lower. Spontaneous flip-flop movement occurs in less than 1 s for DAG, whilst it takes hours for glycerolipids with larger polar heads (Seigneuret and Devaux, 1984). Lipid diffusion between disconnected membranes is rapid for lipids with a single fatty acid chain, like lysoPC that easily partition into the aqueous phase, or for lipids containing two short fatty acyl chains with four to six carbons (Bai and Pagano, 1997). By contrast, there are almost no spontaneous inter-membrane transfers of regular diacyl-lipids although this trafficking is essential for organelle functioning and cell survival.

The vesicular trafficking pathway supports a complex membrane flow, which couples lipid and protein transport. In plant cells, two main membrane flows are documented: the secretory pathway and the vesicular trafficking between the inner envelope and the thylakoids. The former is essentially related with production of lipids in the ER and the latter with production of lipids in the envelope. Through vesicles, lipids are moved in bulk form, as a region of membrane. In this process, in addition to the molecular mechanism necessary for vesicle trafficking, some lipid-manipulating

enzymes are necessary to control membrane curvature (Graham, 2004) or local lipid composition (Devaux and Morris, 2004). Lipid selection can also be achieved in some cases. Alternatively, lipids can be moved as single molecules between membranes. Moving membranes in order to favour membrane apposition is expected to facilitate inter-membrane monomeric lipid transfer.

A number of enzymes are required for the regulation of the various lipid motions. We will describe here some of the enzymatic systems likely involved in plants (Table 1).

B Flip-Flop Movements

Lipid translocases can catalyze flip-flop movements of lipids by several different methods according to their energy requirement. Energy-independent flippases catalyse motion of lipids most likely along the polar surface created by the protein (Kol et al., 2002; Pomorski et al., 2004) and cannot assist the movement of lipids against a physicochemical gradient. They are involved in maintaining bilayer symmetry, for instance, where lipids are generated on a side of the membrane. By contrast, ATP-dependent translocases can accumulate specific lipid classes against equilibrated gradients and are necessary for membrane asymmetry (Seigneuret and Devaux, 1984).

In eukaryotic cells, biosynthesis of phospholipids is localised in the cytosolic leaflet of ER. Therefore, half of the newly synthesized lipids have to be transferred to the other leaflet. In yeast and mammalian cells, the flip-flop movement in the ER membrane is ATP-independent and ten times faster than in membranes that do not contain proteins. This movement is sensitive to proteases (Pomorski et al., 2004) but it is still unclear whether it involves a dedicated class of enzymes or the mere presence of proteins. Interestingly, peptides that mimic the α-helices of transmembrane proteins can stimulate phospholipid flip-flop in liposomes (Kol et al., 2002).

The first ATP-independent flippase ever characterized in eukaryotic cells is the scramblase, a calcium-dependent flippase in the plasma membrane of red blood cells (Zhou et al., 1997). In membranes of red blood cells, sphingomyelin and most of the PC are located in the outer leaflet of plasma membrane whereas PS and a part of the PE are in the cytosolic leaflet. An increase of calcium influx activates the scramblase, which equilibrates the phospholipid composition between the two plasma membrane leaflets by catalysing bidirectional transfer of each lipid species. Movement of PS to the outer leaflet can subsequently induce blood coagulation (Zhou et al., 1997). In plants, a change in distribution of PS was also observed in the plasma membrane after treatment with camptothecin, an apoptosis inducer in animal cells (O'Brien et al., 1997). Although apoptosis is not strictly analogous in plant cells, it is possible that a scramblase-like protein plays some roles in cell development. The At2g04940-encoded protein has a weak but significant sequence similarity with the animal scramblase (Jouhet et al., 2007) but it is not known whether it is important for plant development. A second well characterized ATP-independent flippase is the RFT1 protein, a yeast reticulum pyrophosphoryloligosaccharide-dolichol flippase (Helenius et al., 2002). RFT1 transfers dolichol from the reticulum cytosolic leaflet, where it is synthesized, to the lumenal leaflet where it is eventually located. In *Arabidopsis*, one RFT1 homolog (At5g07630) can be found, but is not yet functionally characterized.

Several asymmetric distributions of lipids are well described in plant membranes, indicating the importance of flippase/translocase activities. In the tonoplast membrane, a slight asymmetrical distribution of the major phospholipids was observed. PE is approximately 20% more abundant in the outer monolayer than in the inner monolayer while PC is equally distributed between both leaflets of tonoplast membrane (Tavernier and Pugin, 1995). The thylakoid membrane shows a marked asymmetry with an enrichment of MGDG and PG in the outer leaflet while DGDG and SQDG are essentially confined to the inner leaflet (Rawyler et al., 1987). Since these lipids are linked to specific structures of the photosystems, lipid asymmetry in the thylakoids is likely to reflect the structural organisation of the photosynthetic machinery (Tremolieres et al., 1994). It may result from topology of lipid-synthesising enzymes in the inner envelope. Initial asymmetry in the envelope inner membrane would be transferred as a whole to the thylakoids through membrane trafficking (Rawyler et al., 1995). A high asymmetry of lipids is also expected in the outer membrane of the envelope since it was observed in spinach chloroplasts that PC is present only in

Table 1. Arabidopsis thaliana genes potentially involved in lipid trafficking (revised from Jouhet et al. 2007).

LTP	Bound lipid	*Arabidopsis* locus	Protein	Evidence	TM	Localization
Flippases						
RFT1	?	At5g07630		Sequence similarity	9	–
Scramblase	?	At2g04940		Sequence similarity	0	M
P-type ATPases						
Aminophospholipid translocase	PS, PE	At5g04930	ALA1	Functional complementation	10	Plasma Mb
Aminophospholipid translocase	?	At5g44240	ALA2	Sequence similarity	9	–
Aminophospholipid translocase	PE, PS, PC	At1g59820	ALA3	Functional compl/ALIS1	8	Golgi
Aminophospholipid translocase	?	At1g17500	ALA4	Sequence similarity	10	M
Aminophospholipid translocase	?	At1g72700	ALA5	Sequence similarity	10	M
Aminophospholipid translocase	?	At1g54280	ALA6	Sequence similarity	10	M
Aminophospholipid translocase	?	At3g13900	ALA7	Sequence similarity	10	M
Aminophospholipid translocase	?	At3g27870	ALA8	Sequence similarity	10	M
Aminophospholipid translocase	?	At1g68710	ALA9	Sequence similarity	10	M
Aminophospholipid translocase	?	At3g25610	ALA10	Sequence similarity	10	M
Aminophospholipid translocase	?	At1g13210	ALA11	Sequence similarity	10	M
Aminophospholipid translocase	?	At1g26130	ALA12	Sequence similarity	10	M
ABC transporters likely involved in lipid translocation						
ABC acyl transporter	?	At1g54350		Sequence similarity	5	P
ABC transporter	?	At1g19800	TGD1	EMS mutant characterization	6	P
Lipid-binding proteins of the OSBP family						
OSBP	?	At2g31020		Sequence similarity	0	P
OSBP	?	At4g12460	Osh1	Sequence similarity	0	–
OSBP	?	At4g22540	Osh2	Sequence similarity	0	–
OSBP	?	At1g13170	Osh3	Sequence similarity	0	P
OSBP	?	At1g77730		Sequence similarity	0	P
OSBP	?	At4g08180		Sequence similarity	0	–
OSBP	?	At3g09300		Sequence similarity	0	P
OSBP	?	At5g02100		Sequence similarity	0	P
OSBP	?	At5g59420		Sequence similarity	0	–
OSBP	?	At2g31030		Sequence similarity	0	–
OSBP	?	At2g31035		Sequence similarity	0	–
OSBP	?	At4g25850		Sequence similarity	0	–
OSBP	?	At4g25860		Sequence similarity	0	–
OSBP	?	At5g57240	KES1	Sequence similarity	0	–
Lipid-binding proteins with a START domain						

(continued)

Table 1. (continued)

LTP	Bound lipid	*Arabidopsis* locus	Protein	Evidence	TM	Localization
StART lipid binding protein	PC	At1g64720	CP5	Sequence similarity	1	S
StART lipid binding protein	PC	At1g55960		Sequence similarity	1	–
StART lipid binding protein	PC	At3g13062		Sequence similarity	1	–
StART lipid binding protein	PC	At3g23080		Sequence similarity	1	–
StART lipid binding protein	PC	At4g14500		Sequence similarity	2	S
StART lipid binding protein	PC	At5g54170		Sequence similarity	1	–
StART lipid binding protein	?	At2g28320	EDR2	Sequence similarity	0	–
StART lipid binding protein	?	At3g54800		Sequence similarity	0	M
StART lipid binding protein	?	At5g45560		Sequence similarity	1	M
Lipid-binding proteins of the GLTP family						
GLTP	Sphingosine	At2g34690	ACD11	T-DNA mutant characterization, functional complementation and in vitro expression	0	–
GLTP	?	At2g33470	GLTP1	Sequence similarity	0	–
GLTP	?	At1g21360	GLTP2	Sequence similarity	0	–
GLTP	?	At3g21260	GLTP3	Sequence similarity	0	–
GLTP	?	At4g39670		Sequence similarity	0	–
PI-transfer proteins						
Type SEC14	PI/PC	At1g55840	SEC14	Functional complementation	0	–
Type SEC14	PI	At4g34580	AtSfh1/COW1	T-DNA mutant characterization and functional complementation	1	– (Golgi)
Sterol carrier protein						
SCP	PL, stérol	At5g42890	SCP2	In vitro expression	0	– (Perox)
Non specific lipid transfer proteins						
Lipid transfer protein type 1	–	At2g38540	LTP1	In vitro expression	0	S
Lipid transfer protein type 1	–	At2g38530	LTP2	In vitro expression	0	S
Lipid transfer protein type 1	–	At5g59320	LTP3	Sequence similarity	0	S
Lipid transfer protein type 1	–	At5g59310	LTP4	Sequence similarity	1	S
Lipid transfer protein type 1	–	At3g51600	LTP5	Sequence similarity	0	S
Lipid transfer protein type 1	–	At3g08770	LTP6	Sequence similarity	0	S
Lipid transfer protein type 1	–	At2g18370	LTP7	Sequence similarity	0	S
Lipid transfer protein type 1	–	At2g15050	LTP8	Sequence similarity	0	S
Lipid transfer protein type 1	–	At2g15325	LTP9	Sequence similarity	1	S
Lipid transfer protein type 1	–	At5g01870	LTP10	Sequence similarity	0	S
Lipid transfer protein type 1	–	At4g33555	LTP11	Sequence similarity	0	S

Lipid transfer protein type 1	–	At3g51590	LTP12	Sequence similarity	0	S
Lipid transfer protein type 1	–	At4g08530	LTP15	Sequence similarity	0	–
Lipid transfer protein type 2	–	At1g48750		Sequence similarity	1	S
Lipid transfer protein type 2	–	At1g66850		Sequence similarity	0	S
Lipid transfer protein type 2	–	At1g73780		Sequence similarity	0	S
Lipid transfer protein type 2	–	At3g18280		Sequence similarity	1	S
Lipid transfer protein type 2	–	At3g57310		Sequence similarity	0	S
Lipid transfer protein type 2	–	At5g38160		Sequence similarity	0	S
Lipid transfer protein type 2	–	At5g38170		Sequence similarity	1	S
Lipid transfer protein type 2	–	At5g38180		Sequence similarity	1	S
Lipid transfer protein type 3	–	At1g32280		Sequence similarity	0	S
Lipid transfer protein type 3	–	At3g07450		Sequence similarity	0	S
Lipid transfer protein type 3	–	At3g52130		Sequence similarity	1	S
Lipid transfer protein type 3	–	At4g30880		Sequence similarity	0	S
Lipid transfer protein type 3	–	At4g33550		Sequence similarity	0	S
Lipid transfer protein type 3	–	At5g07230	A9	Sequence similarity	0	S
Lipid transfer protein type 3	–	At5g48485		Sequence similarity	1	S
Lipid transfer protein type 3	–	At5g48490		Sequence similarity	0	S
Lipid transfer protein type 3	–	At5g52160		Sequence similarity	1	S
Lipid transfer protein type 3	–	At5g55410		Sequence similarity	0	S
Lipid transfer protein type 3	–	At5g55450		Sequence similarity	0	S
Lipid transfer protein type 3	–	At5g55460		Sequence similarity	0	S
Lipid transfer protein type 3	–	At5g56480		Sequence similarity	0	S
Lipid transfer protein type 3	–	At5g62080		Sequence similarity	0	S
Lipid transfer protein type 4	–	At3g53980		Sequence similarity	0	S
Lipid transfer protein type 4	–	At5g05960		Sequence similarity	0	S
Lipid transfer protein type 5	–	At1g18280		Sequence similarity	0	S
Lipid transfer protein type 5	–	At1g27950		Sequence similarity	2	S
Lipid transfer protein type 5	–	At1g36150		Sequence similarity	1	S
Lipid transfer protein type 5	–	At1g55260		Sequence similarity	0	S
Lipid transfer protein type 5	–	At1g62790		Sequence similarity	2	S
Lipid transfer protein type 5	–	At1g70240		Sequence similarity	0	
Lipid transfer protein type 5	–	At1g73550		Sequence similarity	1	S
Lipid transfer protein type 5	–	At1g73560		Sequence similarity	0	S
Lipid transfer protein type 5	–	At1g73890		Sequence similarity	1	S
Lipid transfer protein type 5	–	At2g13820		Sequence similarity	0	S
Lipid transfer protein type 5	–	At2g27130		Sequence similarity	1	S
Lipid transfer protein type 5	–	At2g37870		Sequence similarity	0	S
Lipid transfer protein type 5	–	At2g44290		Sequence similarity	1	S
Lipid transfer protein type 5	–	At2g44300		Sequence similarity	2	S
Lipid transfer protein type 5	–	At2g48130		Sequence similarity	0	M

(continued)

Table 1. (continued)

LTP	Bound lipid	*Arabidopsis* locus	Protein	Evidence	TM	Localization
Lipid transfer protein type 5	–	At2g48140		Sequence similarity	0	–
Lipid transfer protein type 5	–	At3g22570		Sequence similarity	0	S
Lipid transfer protein type 5	–	At3g22580		Sequence similarity	1	S
Lipid transfer protein type 5	–	At3g22600		Sequence similarity	0	S
Lipid transfer protein type 5	–	At3g22620		Sequence similarity	0	S
Lipid transfer protein type 5	–	At3g43720		Sequence similarity	2	S
Lipid transfer protein type 5	–	At4g08670		Sequence similarity	1	S
Lipid transfer protein type 5	–	At4g12360		Sequence similarity	2	S
Lipid transfer protein type 5	–	At4g14815		Sequence similarity	1	S
Lipid transfer protein type 5	–	At4g22630		Sequence similarity	2	S
Lipid transfer protein type 5	–	At4g22640		Sequence similarity	1	S
Lipid transfer protein type 5	–	At5g09370		Sequence similarity	0	S
Lipid transfer protein type 5	–	At5g13900		Sequence similarity	0	S
Lipid transfer protein type 5	–	At5g64080		Sequence similarity	2	S
Lipid transfer protein type 6	–	At4g22490		Sequence similarity	0	S
Lipid transfer protein type 6	–	At4g22520		Sequence similarity	0	S
Lipid transfer protein type 7	–	At3g58550		Sequence similarity	0	S
Lipid transfer protein type 8	–	At4g28395	ATA7	Sequence similarity	0	–

Protein localization determined by Target P (Emanuelsson et al., 2000) is indicated by P letter for plastid, M for mitochondria and S for secretory peptide presence. TM: transmembrane domain number.

the cytosolic leaflet of the membrane (Dorne et al., 1985). Furthermore, PC is restricted to the chloroplast surface and is absent from the chloroplast inner membranes, i.e., inner envelope membrane and thylakoids. It is not known how PC asymmetry of the outer envelope is maintained. It may be linked to the role of PC in formation of the eukaryotic-type galactolipids. PA may play a role in maintaining of the asymmetry since overexpression of a recombinant DAG kinase in the chloroplast envelope led to the presence of PC in thylakoids of transformed tobacco whereas PC was absent from thylakoids of control plants (Fritz et al., 2007). Whether enhanced production of PA in the envelope may destabilize a system maintaining PC asymmetry or a mechanism of PC transfer is unknown.

Based on sequence similarity, several plant proteins exhibit homology with flippases/translocases characterized in other organisms, mainly in yeast and animals. Some of these ATP-dependent translocases have furthermore been characterized in plants. There are two families of ATP-dependent translocases to be considered. One family that includes aminophospholipid translocases belongs to the P4-type ATPase superfamily. The first identified enzyme of this family was the yeast DRS2 translocase (Tang et al., 1996). The yeast *drs2* mutant is cold-sensitive and does not show asymmetrical accumulation of PS in the inner leaflet of the plasma membrane. Although the initial study was controversial, new data supports that DRS2 might play a role in the translocation of lipids in the trans-Golgi and endosomal compartments during budding of membrane vesicles (for review, see Pomorski et al., 2004). In *Arabidopsis*, 12 genes belong to this family (ALA1 to ALA12; Table 1). ALA1 exhibits the strongest similarity to DRS2 and its expression in *drs2* yeast mutant restores cold resistance and internalisation of the plasma membrane PS (Gomes et al., 2000). ALA3 localizes to the Golgi and requires a β-subunit, ALIS1, to function in translocation of lipids, mainly PE but also PS and PC, and in formation of secretory vesicles (Poulsen et al., 2008).

The other family of ATP-dependent translocases belongs to the ABC (ATP-binding cassette) protein superfamily that comprises transporters for a whole variety of organic and inorganic compounds. Typically, ABC proteins possess two nucleotide-binding domains and two transmembrane domains. Multi-drug resistance studies in cancerous cells and in yeast first suggested that some ABC transporters participated to externalisation of plasma-membrane lipids and numerous studies now indicate that various lipid-transport processes in cellular membranes relies on ABC proteins (for reviews, see Raggers et al., 2000; Pohl et al., 2005). *Arabidopsis* contains 129 genes belonging to this family (Sanchez-Fernandez et al., 2001). Data on plant ABC transporters indicate that they play a key role in a number of processes necessary for plant development: chlorophyll biosynthesis, iron–sulphur cluster formation, stomata movement and possibly ionic fluxes (for review, see Martinoia et al., 2002). Recent reports have pointed out their role in lipid transport in plants.

Among the best characterized mechanisms of lipid trafficking in plants that involve ABC-like proteins, the analysis of suppressor mutants for the *Arabidopsis dgd1* mutation led to the identification of several components of a potential PA-transport complex associated with the chloroplast envelope (see above). Among the identified proteins, TGD1 is similar to the permease component of bacterial ABC transporters and TGD2 is similar to membrane-tethered substrate-binding proteins associated with these bacterial ABC transporters (Xu et al., 2003; Awai et al., 2006). A third component, TGD3, was recently identified as a small ATPase that most likely associates with TGD1 and TGD2 (Lu et al., 2007).

C Vesicular Lipid Transfer

Lipids are bulk transported by vesicular transfer as a portion of a membrane bilayer from a donor to a target membrane. This allows long distance transfer inside the cell by distribution of vesicles along cytoskeleton filaments. In plants, this mode of transfer concerns the secretory/endocytic pathway and most likely the chloroplast vesicular pathway.

The general mechanism of vesicular transfer is carried out in several steps (for reviews, see Nebenfuhr, 2002; Hawes and Satiat-Jeunemaitre, 2005) and involves different kinds of protein (Fig. 2). In brief, small-type Ras GTPases, coupled to GTP exchange factors, support the recruiting of protein complexes and their anchoring to the donor membrane. Coat proteins, like adaptatin

or clathrin, can be a part of these complexes. Vesicles extrude them from the donor membrane (following mechanisms that remain to be fully characterized), and migrate away using the actin cytoskeleton and myosin. Dynamin, a large GTPase is involved in the scission of nascent vesicles from the parent membrane. Anchoring and fusion to the target membrane are mediated by SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptors) proteins, located both on the vesicles (v-SNARE) and on the target membrane (t-SNARE), and small GTPases. Most of the known plant protein actors are presented in Fig. 2.

Vesicular transfer also requires some specific lipids that are essential for vesicle targeting and formation. Phosphorylated species of PI are involved in vesicle sorting to different compartments. Use of PI kinase inhibitors and plant mutants helped to elucidate the role of different PI forms. Transport of vesicles to the storage vacuole is dependent on PI-4P availability (Matsuoka et al., 1995). PI-3P contributes to vesicle targeting to the lytic vacuole via the prevacuolar compartment (Kim et al., 2001), the endosome (Emans et al., 2002) or multivesicular bodies (Tse et al., 2004; Vermeer et al., 2006). PI-3P modulates the reorganisation of actin filaments

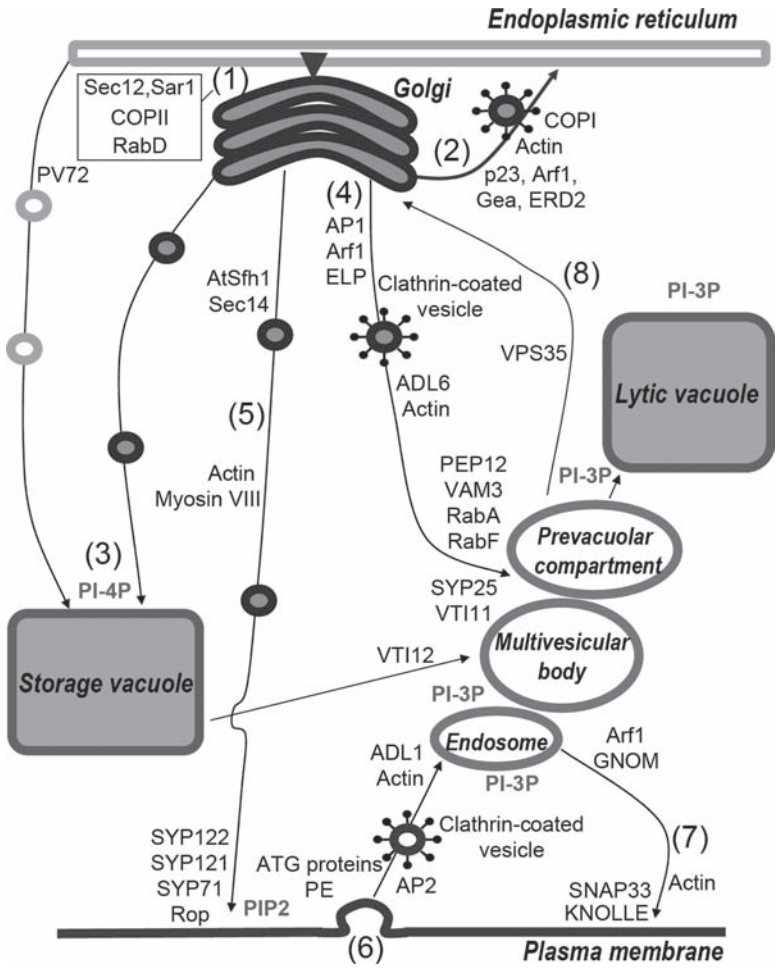

Fig. 2. Scheme of the plant secretory pathway. This scheme represents different routes of vesicle trafficking starting from the ER and indicates names of key proteins involved in these different routes. Phosphorylated forms of PI address vesicles to specific compartments: PI-4P to the storage vacuole, PI-3P to the lytic vacuole and PIP2 to the plasma membrane. (1) Transfer from ER to Golgi; (2) transfer from Golgi to ER; (3) transfer from ER and Golgi to storage vacuole; (4) transfer from Golgi to lytic vacuole; (5) transfer from Golgi to plasma membrane; (6) endocytosis; (7) exocytosis; (8) vesicle recycling to Golgi.

(Choi et al., 2008) and recruitment of proteins involved in vesicle transfer, such as EpsinR2, which interact with adaptatin and clathrin (Lee et al., 2007), or the dynamin ADL6 which binds to adaptatin and actin (Lee et al., 2002). PIP_2 is present at the plasma membrane (van Leeuwen et al., 2007) where it promotes vesicle targeting and stimulates plasma-membrane expansion during root hair elongation (Vincent et al., 2005) or pollen-tube growth (Kost et al., 1999). The PI-PLC activity maintains the polarised PIP_2 distribution and thereby cell expansion (for review, see Kost et al., 1999). PIP_2 might favour vesicle transfer by its interaction with villin that regulates actin remodeling (Xiang et al., 2007) and by activating Rop GTPase (Kost, 2008).

PE is also involved in the endocytic pathway during autophagic process (for review, see Thompson and Vierstra, 2005). Autophagy is important in plants for nutrient recycling, response to abiotic and biotic stresses, programmed cell death and vacuole formation (Bassham, 2007). Autophagic vesicle formation requires autophagy proteins (called ATG) and conjugation of a ubiquitin-fold polypeptide ATG8 to PE (Phillips et al., 2008). Under nitrogen or carbon deficiency, *atg* mutants exhibit increased chlorosis and increased degradation of organelles (Doelling et al., 2002). Interestingly, autophagy is apparently not enhanced under Pi deprivation probably indicating that another membrane reprocessing is acting more efficiently focusing on phospholipid recycling.

Lipid selection and extent of lipid traffic via the vesicular pathway is poorly known. It is generally considered that (a) the ER-Golgi trafficking has no glycerolipid specificity whereas (b) there is a selective trafficking from the Golgi apparatus to the plasma membrane. By using monensin, which blocks the secretory pathway, it is possible to determine which lipid classes are transferred to plant plasma membrane via vesicles. In the presence of monensin, PS was absent from plasma membrane, PE and PC amounts were reduced by half, and PI content was not affected (Moreau et al., 1998). These results suggested that PS was exclusively transferred to the plasma membrane by the vesicular pathway, PE and PC were partly transferred by the vesicular pathway, and PI transported by a distinct process. Although it was not demonstrated, it is believed that there is no glycerolipid selection during vesicular transfer from chloroplast envelope to thylakoids since the inner envelope membrane and thylakoids have a similar lipid composition.

D Transfer of Lipids through Membrane Contact Sites

1 General Features

Besides bulk transfer of lipids by vesicular trafficking, lipids are alternatively moved between membranes as monomers preferentially at membrane contact sites (MCSs). In plant cells, different types of MCSs have been reported possibly involved in lipid transfer. As discussed above, contacts between chloroplasts and mitochondria are likely involved in DGDG transfer that is induced by Pi deprivation (Jouhet et al., 2004). Optical manipulation was used to show physical association between ER and chloroplast envelope (Andersson et al., 2007). MCSs have also been observed between ER and a number of organelle membranes (Levine and Loewen, 2006). The network of ER membranes associated with mitochondria was called MAM (Mitochondria Associated Membranes). MAM were abundantly studied in mammalian cells and yeast. They act as a conduit for the transfer of PS from ER to mitochondria before decarboxylation of PS into PE in mitochondria (For a review, see Vance, 2008). In plants, such a MAM network is most likely to play a similar role in PE synthesis in mitochondria (Nerlich et al., 2007). Several other ER-related MCSs have been observed, in particular, with plasma membrane PAM (Plasma membrane Associated Membranes) and with vacuoles (Staehelin, 1997). It is possible that PAM are involved in non-vesicular transfer of PE, PC or PI to the plasma membrane (Moreau et al., 1998). The isolation of a fraction containing ER associated with chloroplasts PLAM (Plastid Associated Membranes) and a preliminary biochemical characterization of this fraction was recently described (Andersson et al., 2007).

Several works contributed to understanding how some proteins can enable transfer of lipid monomers between two membranes at MCSs. The emerging view is that macromolecular complexes form between donor and acceptor membranes and serve to dock the compartments and facilitate lipid transport (Levine and Loewen, 2006). None of these systems have been characterized in plants.

In the following sections, we will present a few examples mostly in animal cells or in yeast, which unravelled this novel type of lipid transfer. In particular, we will describe the type of proteins involved, their contribution to building of a bridge between two membranes, and their relationship with lipid synthesis.

2 The Transfer of Phosphatidylserine at Membrane Contact Sites

In yeast, PS is synthesized in the ER and transported to the PS decarboxylases: Pds1p in the mitochondria, or Psd2p in the Golgi. This transfer has been investigated in depth by genetic and biochemical analyses by Voelker and collaborators (Voelker, 2005; Choi et al., 2006). The transport of PS from MAM to mitochondria is regulated by at least three genes: encoding a ubiquitin ligase (MET30), a transcription factor (MET4), and one or more genes of unknown function whose transcription is regulated by MET4. MET30-dependent ubiquitination is required for the MAM to function as a donor membrane and for the mitochondria as an acceptor membrane. Non-vesicular transport of PS to the Golgi-located PS decarboxylase is under the control of at least three genes, encoding a PI 4-kinase (STT4), a lipid-binding protein (PSTB2), and the PS decarboxylase. Production of PI-4P is necessary for this lipid transport. The lipid-binding protein and the PS decarboxylase must be present on the Golgi acceptor membrane for PS transport to occur. PS decarboxylase contains a C2 (a Ca^{2+} and phospholipid binding sequence) domain that is required for lipid transport. Reconstitution of lipid transport with chemically defined donor membranes demonstrated that membrane domains enriched in the anionic lipids, PS, PI-4P and PA, function as the most efficient PS donors to the PS decarboxylase present in acceptor membranes. Macromolecular complexes dependent on protein–protein and protein–lipid interactions form between donor and acceptor membranes and serve to dock the compartments and facilitate lipid transport.

3 The Inter-Membrane Transfer of Cardiolipin through Oligomeric Kinase Bridges

In mammalian cells, oligomeric structures of kinases can build bridges between the outer and inner membranes of mitochondria and facilitate transfer of cardiolipin along these bridges. MCSs between the two mitochondrial membranes can fulfil various functions, such as import of mitochondrial precursor proteins, channelling of high-energy phosphates, and formation of mitochondrial permeability transition pores that are involved in apoptotic signalling. During apoptosis, transfer of cardiolipin occurs from the inner to the outer membrane. Bcl2 protein binding to the mitochondrial surface is enhanced by exposed cardiolipin and accelerates transbilayer diffusion of cardiolipin to the outer membrane. Two oligomeric kinases, i.e., a creatine kinase and a nucleotide diphosphate kinase, localized at specific contact sites contribute through oligomerization to formation of mitochondrial inter-membrane bridges (Speer et al., 2005). Membrane contact and binding through these structures are stabilized by electrostatic interaction of the oligomeric kinases with anionic lipids, such as cardiolipin (Epand et al., 2007a). By in vitro assays on liposomes, it was demonstrated that these kinase oligomeric structures facilitate the cardiolipin transfer between the two membranes (Epand et al., 2007b). In chloroplasts, contact sites are also present between the two envelope membranes and could contribute to lipid transfer although junctions through oligomeric protein structures have not been observed.

4 CERT, a Module Protein Involved in Inter-Membrane Lipid Transfer

A novel mechanism of inter-membrane transfer of monomeric lipids was recently described in mammalian cells. This concerns transfer of ceramide and involves a multi-module protein called CERT (CERamide Transfer Protein). CERT contains three main regions: an amino-terminal region with a PH (pleckstrin homology) domain, a carboxy-terminal region with a StART (Steroidogenic Acute Response Transfer) domain, and in between a region with a lipid-binding motif FFAT (two phenylalanines in an Acidic Tract). CERT extracts newly synthesized ceramide from the ER through lipid binding properties of the StART region, then targets to the Golgi apparatus dependent on its PI-4P recognizing PH domain. Interaction with G-proteins, PI-4P, and a PI4 kinase are determinant for targeting of CERT

to the trans-Golgi. After release of ceramide in the Golgi, CERT returns to the ER through interaction of the FFAT motif with an ER protein called VAP (Vesicle-Associated Protein). Generally, VAP proteins interact with SNARE and serve as adaptators for association of ER membranes with other membranes. CERT-mediated transfer of ceramide from ER to Golgi is believed to occur at MCSs. It is possible that MCSs are specifically positioned at domains of the *trans* Golgi membrane enriched in sphingomyelin synthase. The distance between the two membranes, approximately 10 nm, matches the distance between the FFAT motif and the START domain in the CERT protein, indicating that CERT could contribute to the binding of ER to the Golgi membrane.

CERT homologs are apparently absent in plants. There are, however, various proteins, which have a partial sequence similarity with CERT or which could act as a part of a lipid transfer system between membranes. Some proteins of the OSBP (OxySterol Binding Protein) family have two characteristic domains, a PH domain and a lipid-binding domain that is specific for sterol or PA (Li et al., 2008). They are apparently involved in cellular lipid metabolism, vesicle transport and signal transduction. Fourteen homologs of the OSBP family are known in *Arabidopsis*. Nine *Arabidopsis* proteins contain a StART domain and either a PC-binding motif or a PH domain. The GLTP (GLycolipid Transfer Protein) family includes soluble proteins involved in transfer of ceramide, glucosylceramide or sphingolipids. The GLTP family comprises five members in *Arabidopsis* (Table 1). One of these proteins, the *Arabidopsis* ACD11 protein, is involved in the cell death mechanism and in transfer of sphingosine. No evidence was found of the GLTP involvement in galactolipid transfer in plants but because there are similarities between galactocerebrosides and galactosylglycerides, this hypothesis remains possible.

V Conclusions

The stability of subcellular membrane composition relies on combined lipid biosynthesis and trafficking, inside and between vesicularly connected or disconnected membranes. Although the process of lipid biosynthesis has been well characterized in the last two decades and has given important roles to ER and plastid envelope membranes as dual sources of glycerolipids, the necessary transfer of lipid intermediates combining ER-generated and plastid-generated structures in all cell membranes is not well understood. Firstly, protein components routing lipid intermediates outside their origin membranes, transporting and integrating them in the target membranes are largely unknown for both vesicular and non-vesicular transfer. Inventory of these proteins is currently sought by different groups, mainly by genetic approaches that proved successful for the identification of lipid-synthesizing enzymes. Complementary strategies rely on the comparison with non-plant systems and the mining of transcriptomic information. Secondly, the regulatory processes underlying the lipid homeostasis of each membrane, and controlling massive lipid remodelling under some conditions, such as phosphate deprivation, are also important mechanisms to be understood. Likewise, combination of genetic approaches and transcriptomic/proteomic data mining, as well as biochemical approaches, will be crucial to understanding these processes. Thirdly, collected information has allowed a better comprehension of lipid metabolism of the cell. Transfer of this knowledge to understand the relationship of plants with their environment should also require integration with the "soluble" metabolism since lipids are also key metabolites in carbon, phosphorus, nitrogen and sulphur fluxes.

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Chapter 17

Regulatory Roles in Photosynthesis of Unsaturated Fatty Acids in Membrane Lipids

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Summary

The diversity of lipids in thylakoid membranes and their unique characteristics, in addition to their specific orientation in these membranes, strongly suggest that they play specific and important roles in the thylakoid membrane. In the chloroplasts of plants and algae, as well as in cyanobacterial cells, most of the photosynthetic machinery is embedded in thylakoid membranes, which are composed of proteins, lipids and pigments. Alterations in the extent of unsaturation of fatty acids in membrane lipids are expected to affect the physical characteristics of the membranes and, consequently, the activities of the photosynthetic machinery.

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The availability of entire genome sequences and an understanding of the functions of the individual genes for fatty acid desaturases in cyanobacteria led to the successful site-directed mutagenesis of such genes that reduced the extent of unsaturation of fatty acids in membrane lipids in a step-wise manner and, also, to the genetic transformation of cyanobacterial cells and whole plants that increased the extent of unsaturation of fatty acids in lipids of thylakoid membranes. Characterization of the photosynthetic properties of the transformed cyanobacteria and higher plants revealed that polyunsaturated fatty acids are essential for protection of the photosynthetic machinery against environmental stresses, such as strong light, salt stress, and high and low temperatures. Moreover, the available evidence suggests that the unsaturation of fatty acids enhances the repair of the photosystem II complex that has been damaged by strong light under stress conditions.

I Introduction

Does the unsaturation of fatty acids in membrane lipids affect photosynthesis and, if so, how does it do so? This deceptively simple question has a very long history. It was asked first in the 1960s but clear answers only became available in the 1990s as an understanding of the genetics of lipid metabolism developed and techniques became established for site-directed mutagenesis and genetic engineering of photosynthesizing cells and plants.

It has been known for several decades that certain mutations that affect the extent of unsaturation of fatty acids influence photosynthetic activity in vivo and in vitro (Chapman et al., 1983), in higher plants (McCourt et al., 1987; Hugly et al., 1989) and in cyanobacteria (Murata, 1989; Wada and Murata, 1990). Two groups, those of Somerville in the USA and Murata in Japan, used genetic approaches to demonstrate that the unsaturation of fatty acids in thylakoid lipids plays important roles in the acclimation of the photosynthetic machinery to changes in various forms of environmental stress.

Thylakoid membranes of chloroplasts and of cyanobacterial cells contain four major glycerolipids: monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) (Nishida and Murata, 1996; see also Chapters 3, 8 and 12). The molecular motion of these glycerolipids is determined mainly by the extents of unsaturation of the fatty acids that have been esterified to their glycerol backbones (Szalontai et al., 2000; Chapter 15). The extent of unsaturation is, in turn, determined by the activity of fatty acid desaturases, which are enzymes that introduce double bonds into specific positions of fatty-acyl chains of lipids (Los and Murata, 1998). It was anticipated that changes in the unsaturation of fatty acids would affect various functions of membrane-bound proteins, such as the photochemical and electron-transport reactions that occur in thylakoid and other membranes (e.g., the plasma membranes of cyanobacterial cells).

The unsaturation of the fatty acids in the glycerolipids of biological membranes can be modified by various environmental factors (Los and Murata, 1998, 2004), and ambient temperature is a major factor that influences the unsaturation of fatty acids. In higher plants and cyanobacteria, a decrease in growth temperature induces the enzymatic desaturation of fatty acids in the membrane lipids. Such temperature-induced changes in unsaturation can be explained in terms of the regulation of membrane fluidity (see Chapter 15), which decreases at low temperatures and is restored by the enzymatic desaturation of fatty acids. It has been proposed that the desaturation of fatty acids is the key mechanism for the maintenance of membrane fluidity and the optimal performance of biological membranes (Macartney et al., 1994; Los and Murata, 2004).

Temperature is the environmental factor with the greatest effect on the unsaturation of fatty acids that can be easily manipulated in the laboratory (Nishida and Murata, 1996). However, a decrease in ambient temperature not only induces the desaturation of fatty acids but also affects other cellular components, such as enzymes and

Abbreviations: ACP – Acyl-carrier protein; DGDG – Digalactosyldiacylglycerol; FTIR – Fourier transform infrared; GPAT – Glycerol-3-phosphate acyltransferase; MGDG – Monogalactosyldiacyglycerol; PG – Phosphatidylglycerol; PS I – Photosystem I; PS II – Photosystem II; SODG – Sulfoquinovosyldiacylglycerol; X:Y(Z) – Fatty acid in which X and Y indicate numbers of carbon atoms and double bonds, respectively, and Z in parenthesis indicates the position of double bond as counted from the carboxyl terminus of the fatty-acyl chain.

metabolites. Thus, in the 1990s, efforts to understand relationships between the unsaturation of fatty acids and photosynthesis focused on the establishment of the suitable model systems in which the extent of unsaturation of fatty acids could be precisely and reproducibly controlled by genetic manipulations (for publications in this field prior to 1997, see the review by Gombos and Murata, 1998, in an earlier volume in this series).

The most effective system for decreasing the unsaturation of fatty acids in a step-wise manner was created by targeted mutagenesis of fatty acid desaturases in the cyanobacterium *Synechocystis* sp. PCC 6803 (hereinafter *Synechocystis*; Tasaka et al., 1996). In this system, genes for the Δ12 (Wada et al., 1990), Δ6 (Reddy et al., 1993), and ω3 or Δ15 (Sakamoto et al., 1994) desaturases were sequentially inactivated. The specific site of desaturation by these enzymes is defined by reference to the carboxyl terminus (Δ-position) or the methyl terminus (ω-position) of the fatty acid (Los and Murata, 1998). The most suitable mutant for studies of photosynthetic reactions proved to be the *desA*$^-$/*desD*$^-$ mutant, in which the genes for the Δ12 and Δ6 desaturases are defective. The mutant cells produce only the Δ9 monounsaturated and saturated fatty acids, whereas the wild-type parental cells synthesize polyunsaturated fatty acids with two, three, and even four double bonds (the latter only at low temperatures), in addition to saturated and monounsaturated fatty acids.

In other studies, identification and cloning of the individual genes for specific fatty acid desaturases allowed genetic transformations that altered the extent of unsaturation of fatty acids in a variety of photosynthetic organisms. The first successful transformation was achieved using the cyanobacterium *Anacystis nidulans* (now designated *Synechococcus* sp. PCC 7942, hereinafter *Synechococcus*), which was transformed with the *desA* gene for Δ12 desaturase of *Synechocystis* (Wada et al., 1990). Wild-type *Synechococcus* cells contain only Δ9 desaturases and synthesize monounsaturated fatty acids (mainly 16:1 and 18:1) and, thus, they resemble the *desA*$^-$/*desD*$^-$ double-mutant cells of *Synechocystis*. The *desA*$^+$-transformed cells of *Synechococcus* produced diunsaturated fatty acids with desaturation at the Δ9 and Δ12 positions (Sakamoto et al., 1994).

The experimental systems that allow changes in the extent of unsaturation of membrane lipids without any concomitant and nonspecific changes in environmental conditions, such as temperature, have proved to be very useful tools for studies of the roles of membrane lipids in photosynthesis.

II Engineered Decreases in Unsaturation of Fatty Acids in Membrane Lipids in *Synechocystis*

A Targeted Mutagenesis of Fatty Acid Desaturases

As mentioned above, a reduction in the extent of unsaturation of fatty acids was achieved by targeted mutagenesis of the fatty acid desaturases in the cyanobacterium *Synechocystis* (Tasaka et al., 1996). The effects of the unsaturation of fatty acids on the thermotropic behavior of thylakoid membranes from wild-type and mutant cells was examined first by differential scanning calorimetry. The temperatures for the onset of the phase transition were 14°C and 21°C for thylakoid membranes isolated, respectively, from wild-type and *desA*$^-$/*desD*$^-$ mutant cells that had been grown at 25°C (Tasaka et al., 1996). Thus, replacement of polyunsaturated fatty acids in membrane lipids by a monounsaturated fatty acid raised the temperature for the phase transition of the lipids in the thylakoid membranes.

Another approach that has been used to characterize the physical properties of such membranes is Fourier transform infrared (FTIR) spectrometry (Szalontai et al., 2000; Inaba et al., 2003). Lipid disorganization and changes in membrane dynamics are characterized by shifts in the frequency of the $\nu_{sym}CH_2$ stretching mode at approximately 2,852 cm^{-1}. Moreover, the contributions of the *trans* and *gauche* segments of fatty-acyl chains to the $\nu_{sym}CH_2$ band can be separated. Thus, the actual frequency of the $\nu_{sym}CH_2$ stretching mode can be interpreted in terms of thermally induced changes in the dynamics of membrane lipids and of protein–lipid interactions.

Direct measurements of the rigidity of plasma membranes from wild-type and *desA*$^-$/*desD*$^-$ cells were made by FTIR spectrometry, which demonstrated that the plasma membranes from *desA*$^-$/*desD*$^-$ cells were more rigid than those from wild-type cells,

in particular, at 22°C, the temperature at which we examined the expression of cold-regulated genes. The rigidification of plasma membranes, as a result of the mutation of fatty acid desaturases, reflected the absence of polyunsaturated fatty-acyl chains in *desA*$^-$/*desD*$^-$ cells. The difference between wild-type and mutant cells was greater at lower temperatures and was smaller at higher temperatures (Szalontai et al., 2000). Moreover, FTIR analysis indicated that lipids in plasma membranes were rigidified with decreases in temperature and that the rigidification was enhanced by the saturation of fatty acids that occurred after the inactivation of the fatty acid desaturases. Analysis of the frequencies of $\nu_{sym}CH_2$ bands in FTIR spectra, which are indicators of the fluidity (microviscosity) of the fatty-acyl chains in membrane lipids, demonstrated that the genetically engineered decrease in the extent of unsaturation of fatty acids reduced the fluidity of membrane lipids in the same way as a decrease in ambient temperature. The difference between temperatures at which membrane fluidity was the same in wild-type and mutant cells was 7–10°C within a range of ambient growth temperatures from 5°C to 25°C (Szalontai et al., 2000; Inaba et al., 2003).

The effects of the unsaturation of fatty acids in membrane lipids on the growth of cells were examined at various temperatures (Tasaka et al., 1996; Los and Murata, 2004). Maximum growth of wild-type cells was observed at approximately 30–35°C and the cells were able to grow even at 20°C. By contrast, *desA*$^-$/*desD*$^-$ mutant cells, which contained 18:1(9) but no polyunsaturated fatty acids, yielded a growth profile that was very different from that of wild-type cells. Although growth at 30–35°C was hardly affected, growth at lower temperatures was markedly depressed by the mutation: at 25°C, the *desA*$^-$/*desD*$^-$ cells grew very slowly after a long lag period; at 20°C, they failed to grow at all. These results indicated that replacement of polyunsaturated fatty acids by a monounsaturated species had a significant effect on the proliferation of cells at low temperature.

Since membrane-bound protein complexes, such as Photosystem I (PS I), Photosystem II (PS II), the cytochrome b_6/f complex and ATP synthase, are associated directly with lipids in thylakoid membranes, it is reasonable to predict that their activities are affected by the properties of membrane lipids. In this context, Dilley et al. (2001) examined the effects of decreases in the unsaturation of fatty acids in *desA*$^-$/*desD*$^-$ mutant cells on the transport of electrons (from water to ferricyanide), as well as on the uptake of protons and on the synthesis of ATP (both of which are coupled to the cyclic transport of electrons that is mediated by phenazine methosulfate). They observed that these activities in wild-type cells were reduced with a decrease in temperature but were retained at certain low levels at temperatures close to freezing. By contrast, *desA*$^-$/*desD*$^-$ mutant cells lost the ability to synthesize ATP but not the ability to transport electrons and take up protons at approximately 5°C. These findings suggest that a decrease in the unsaturation of fatty acids might block the ability of thylakoid membranes to couple a bioenergetically competent proton-motive force to the synthesis of ATP at low temperatures. Nevertheless, it remains to be determined how the unsaturation of fatty acids in membrane lipids or a decrease in the fluidity of thylakoid membranes might affect such coupling.

Using thylakoid membranes isolated from *Synechocystis* mutant cells similar to *desA*$^-$/*desD*$^-$ cells, Mamedov et al. (1993) examined the effects of decreases in the unsaturation of fatty acids on the stability to heat of electron-transport reactions mediated by PS I and PS II, singly or in combination, as well as the synthesis of ATP. They observed that changes in unsaturation had almost no effect at all on the heat stability of these reactions.

B Decreased Unsaturation of Fatty Acids Stimulates Photoinhibition at Low Temperature

The inactivation of PS II by strong light is a phenomenon known as photoinhibition, which is due to an imbalance between the rate of photodamage to PS II and the rate of repair of the damaged PS II. Photodamage is a result, initially, of the direct effect of light on the oxygen-evolving complex and the photochemical reaction center is inactivated subsequently (Ohnishi et al., 2005; Hakala et al., 2005). In addition, strong light induces the production of reactive oxygen species, which inhibit the repair of photodamaged PS II by suppressing, primarily, the synthesis of proteins de novo (Nishiyama et al., 2005, 2006; Murata et al., 2007; Takahashi and Murata, 2008). The effects

of environmental stress on damage to and repair of PS II can be examined separately and it appears that environmental stresses, such as low temperature, act primarily by inhibiting the repair of PS II (Gombos et al. 1992, 1994; Wada et al., 1994; Murata et al., 2007).

A comparison of the turnover of the Dl protein, which is an important component of the photochemical reaction center of PS II, in wild-type and *desA*$^-$/*desD*$^-$ cells of *Synechocystis* revealed that posttranslational carboxy-terminal processing of the precursor to the D1 protein was extremely sensitive to low temperature and was also dependent on the extent of unsaturation of fatty acids in the lipids of thylakoid membranes (Kanervo et al., 1995, 1997). When the temperature was decreased from 33°C (growth temperature) to 18°C, the inability of *desA*$^-$/*desD*$^-$ mutant cells to recover from photodamage was accompanied by their failure to process the newly synthesized precursor to the D1 protein, which accumulated in the mutant cells. The precursor to D1 is integrated into the monomeric form of PS II complexes, even at low temperatures, but no evolution of oxygen occurred in such complexes in mutant cells that were defective in the unsaturation of fatty acids (Kanervo et al., 1997). These results indicated that the unsaturation of fatty acids mitigates photoinhibition by counteracting the inhibitory effects of low temperature on the processing of the precursor to D1.

C Decreased Unsaturation of Fatty Acids Stimulates Irreversible Photoinhibition

As mentioned above, photosynthetic organisms are often exposed to strong light and, under such conditions, the PS II is subject to photoinhibition. Under normal conditions, PS II is repaired by replacement of the D1 protein in the photodamaged PS II by newly synthesized D1 protein (Adir et al., 2003; Aro et al., 2005; Nishiyama et al., 2006; Murata et al., 2007; Takahashi and Murata, 2008). However, upon exposure of *Synechocystis* cells to very strong light (e.g., 2,500 µmol photons m^{-2} s^{-1}) for 2 h, almost all PS II complexes are photodamaged, and when such photodamaged PS II is irradiated still further by strong light, reversible photoinhibition may be converted to irreversible photoinhibition (Allakhverdiev et al., 2005; Mohanty et al., 2007). It is likely that the processing of the precursor to the D1 protein is blocked in irreversibly photoinhibited PS II.

As mentioned above, photoinhibition is stimulated at low temperatures as a result of the inhibition of the repair of PS II (Kanervo et al., 1995, 1997; Allakhverdiev and Murata, 2004), and this effect is enhanced by the desaturation of fatty acids. Similarly, a decrease in the unsaturation of fatty acids enhances the rate and extent of the irreversible photoinhibition. It seems likely that a decrease in the unsaturation of fatty acids tends to maintain PS II in a (reversibly) photoinhibited state, which is the target of irreversible photoinhibition.

D Decreased Unsaturation of Fatty Acids Enhances Sensitivity to Salt Stress

The ability of the photosynthetic machinery to tolerate salt stress has been examined in detail in wild-type and *desA*$^-$/*desD*$^-$ mutant cells of *Synechocystis*. The disappearance of the oxygen-evolving activity of PS II under salt stress due to 0.5 M NaCl, both in darkness and in light, was much more rapid in *desA*$^-$/*desD*$^-$ cells than in wild-type cells (Fig. 1; Allakhverdiev et al., 1999). Moreover, the photosynthetic activity of wild-type cells recovered more rapidly than that of the mutant cells after removal of NaCl from the growth medium (Fig. 1). Recovery in the presence of NaCl depended on light and ATP was required for the synthesis de novo of the proteins necessary for reactivation of the oxygen-evolving machinery (Allakhverdiev et al., 1999, 2001).

The recovery of the oxygen-evolving activity depended on the activity of the Na^+/H^+ antiporter system. This latter activity appeared to be strongly dependent on the unsaturation of fatty acids. Incubation of wild-type and *desA*$^-$/*desD*$^-$ cells in the presence of 1.0 M NaCl in darkness resulted in total inactivation of the Na^+/H^+ antiporter system. Upon exposure to low light, the activity of the Na^+/H^+ antiporter system in wild-type cells returned to 30% of the original level while, in *desA*$^-$/*desD*$^-$ cells, it returned to only to 5% of the original level. The NaCl-induced inactivation of the oxygen-evolving complex in thylakoid membranes isolated from *desA*$^-$/*desD*$^-$ cells also occurred much more rapidly than that in membranes from wild-type cells and this effect was similar in darkness and in weak light, such as

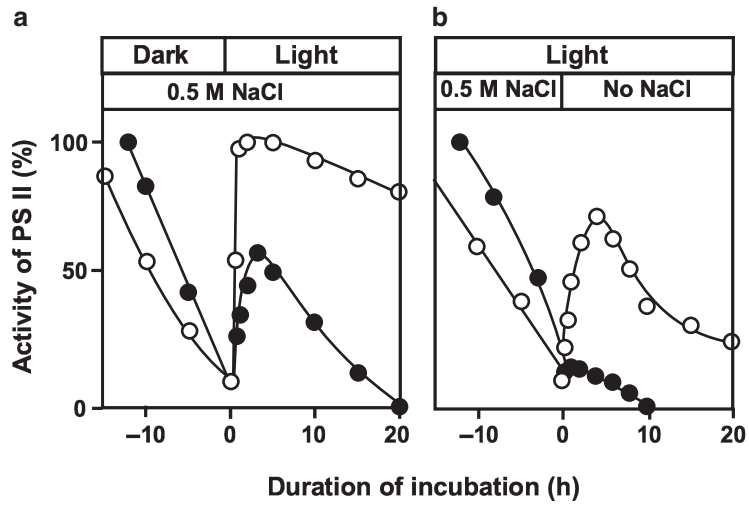

Fig. 1. The defects in polyunsaturated fatty acids in the *desA*$^-$/*desD*$^-$ mutant cells of *Synechocystis* accelerated the NaCl-induced inactivation of PS II and reduced the light-dependent recovery of PS II activity. (**a**) Wild-type and *desA*$^-$/*desD*$^-$ cells were incubated for 25 and 12 h, respectively, in darkness in the presence of 0.5 M NaCl. Then, cells were exposed to light at 50 μmol photons m^{-2} s^{-1}. (**b**) Wild-type and *desA*$^-$/*desD*$^-$ cells were incubated with 0.5 M NaCl in light at 50 μmol photons m^{-2} s^{-1} for 45 and 25 h, respectively. Then, cells were collected by centrifugation, resuspended in fresh medium with no added NaCl, and incubated in light at 50 μmol photons m^{-2} s^{-1}. Activity of PS II was monitored by means of the light-dependent evolution of oxygen in the presence of 1.0 mM 1,4-benzoquinone. ○, Wild-type cells; ● *desA*$^-$/*desD*$^-$ cells (reproduced with permission from Allakhverdiev et al., 1999).

light at 50 μmol photons m^{-2} s^{-1} (Allakhverdiev et al., 1999).

The above-described observations showed that incubation with 0.5 M NaCl resulted primarily in damage to the oxygen-evolving site in the PS II complex and that the oxygen-evolving site in the PS II complex from wild-type cells was more tolerant to 0.5 M NaCl than was the PS II complex from *desA*$^-$/*desD*$^-$ cells. In prior studies in vitro (Miyao and Murata, 1983; Murata and Miyao, 1985), high concentrations of NaCl led specifically to dissociation of the extrinsic proteins from the oxygen-evolving machinery of the PS II complex. The inactivation of the oxygen-evolving complex in *Synechocystis* is probably also caused by dissociation of the extrinsic proteins from the oxygen-evolving machinery.

In general, *desA*$^-$/*desD*$^-$ cells were characterized by enhanced sensitivity to NaCl and a reduced ability to recover from salt stress, as compared to wild-type cells. This observation can be explained in terms of a combination of the following scenarios.

1. The oxygen-evolving machinery in thylakoid membranes isolated from *desA*$^-$/*desD*$^-$ cells was more sensitive to NaCl than that from wild-type cells. This finding suggests that the unsaturation of fatty acids in membrane lipids might act directly to protect the oxygen-evolving machinery against salt-induced inactivation.
2. The activity of the Na^+/H^+ antiporter system was higher in wild-type cells than in *desA*$^-$/*desD*$^-$ cells, suggesting that the unsaturation of fatty acids in membrane lipids might activate the Na^+/H^+ antiporter system via enhanced fluidity of the membrane. This hypothesis is supported by results, obtained in studies of other membrane systems, that indicate that the activities of certain membrane-bound enzymes can change with changes in membrane fluidity (Kamada et al., 1995).
3. The activity of the Na^+/H^+ antiporter system was more sensitive to NaCl in *desA*$^-$/*desD*$^-$ cells than in wild-type cells, and the recovery of Na^+/H^+ antiporter activity in wild-type cells occurred much more efficiently than in *desA*$^-$/*desD*$^-$ cells. These findings suggest that the unsaturation of fatty acids might also stimulate the synthesis of one or more components of the Na^+/H^+ antiporter system.

The extent of unsaturation of fatty acids is clearly important in the protection of the oxygen-evolving

machinery of the PS II complex against salt-induced inactivation. NaCl, but not sorbitol, interfered with the photosynthetic evolution of oxygen and, thus, it seems likely that the salt-induced inactivation was due to the effects of cations and not to the effects of osmotic stress. It also seems likely that the activity and synthesis of the Na^+/H^+ antiporter system might be suppressed under salt-stress conditions and that this effect might be reversed, in part, by the unsaturation of fatty acids in membrane lipids (Allakhverdiev et al., 1999; Allakhverdiev and Murata, 2008).

III Engineered Increases in Unsaturation of Fatty Acids in Membrane Lipids in *Synechococcus*

A Transgenes for Fatty Acid Desaturases

As mentioned in Section 1, the first successful attempt at increasing the unsaturation of fatty acids by genetic engineering was made using *Synechococcus*, which was transformed with the gene for the Δ12 desaturase of *Synechocystis* (Wada et al., 1990). *Synechococcus* contains monounsaturated and saturated fatty acids but no polyunsaturated fatty acids. The genome of this organism contains only one *desC* gene for the Δ9 desaturase; *desC*, and it catalyzes the introduction of double bonds at the *sn*-1 and *sn*-2 positions of the glycerol moiety of glycerolipids (see Chapter 8). No other genes for fatty acid desaturases have been reported in this strain so far (http://www.genome.jp/dbget-bin/www_bget?refseq+NC_007604).

Thus, *Synechococcus* synthesizes only monounsaturated fatty acids, mainly 16:1(9) and 18:1(9). The *desA*$^+$-transformed *Synechococcus* cells were able to introduce a double bond at the Δ12 position of the fatty acid that was esterified to the *sn*-1 position of the glycerol moiety (Sakamoto et al., 1994). The change in the fatty acid composition, due to the transformation, was recognized as an increase in the level of diunsaturated fatty acids at the expense of monounsaturated fatty acid (Murata and Wada, 1995; Nishida and Murata, 1996). As a result, the transformed cells contained almost equal amounts of monounsaturated and diunsaturated fatty acids.

Wada et al. (1994), using *Synechococcus* mutant cells that were similar to *desA*$^+$-transformed cells, examined the heat stability of electron transport from water to 1.4-benzoquinone. The result clearly demonstrated that the heat stability was unaffected by the increase in unsaturation of fatty acids although it was strongly affected by changes in growth temperature. These findings suggest that the growth temperature-dependent change in heat stability of electron-transport reactions is not related to the change in unsaturation of fatty acids, but is related to changes in levels of other components.

B Increased Unsaturation of Fatty Acids Mitigates Photoinhibition at Low Temperature

As mentioned above, photoinhibition in vivo reflects the effects of photodamage to PS II and subsequent repair (Nishiyama et al., 2006; Murata et al., 2007). The damage corresponds to the light-induced inactivation of PS II (probably because of damage to the D1 protein), while repair consists of a series of steps, namely, degradation of the D1 protein in the photodamaged PS II, synthesis of the precursor to the D1 protein de novo, incorporation of the precursor into the PS II complex and, finally, processing of the precursor to yield the mature D1 protein (Tyystjärvi, 2008 and references therein).

The *desA*$^+$-transformed *Synechococcus* cells, whose glycerolipids included diunsaturated fatty acids, were more resistant to photoinhibition at low temperatures than were wild-type cells, which contained monounsaturated fatty acids exclusively (Gombos et al., 1997). Analysis of the light-induced inactivation and subsequent recovery of the activity of the PS II complex revealed that the recovery process was markedly accelerated by the transformation with the *desA* gene. Pulse-labeling experiments also revealed that the synthesis of the D1 protein de novo at low temperature, which was necessary for restoration of the activity of the PS II complex, was much faster in the *desA*$^+$-transformed cells than in the wild-type cells. These findings demonstrated that increased unsaturation of membrane lipids enhanced the ability of photosynthesizing cells to tolerate strong light by accelerating the synthesis of the D1 protein de novo (Gombos et al., 1997).

The D1 protein is encoded by three *psbA* genes in *Synechococcus*. The *psbAI* gene encodes D1

form I (D1:1) and the *psbAII* and *psbAIII* genes encode the transiently expressed D1 form II (D1:2). Further studies of the mechanism of the enhanced tolerance of *desA*$^+$-transformed cells revealed the following information (Sippola et al., 1998). Transfer of cultures of *Synechococcus* cells from 32°C to 25°C under light at 100 μmol photons m^{-2} s^{-1} resulted in replacement of D1:1, the prevailing form at the higher temperature, by D1:2 in both wild-type cells and the *desA*$^+$-transformed cells, with no loss of PS II activity. A further downward shift in temperature to 18°C caused a dramatic decrease in PS II activity in wild-type cells, while the *desA*$^+$-transformed cells were affected to a much smaller extent. It appeared that wild-type cells were incapable of accumulating D1:2 to compensate for the loss of D1:1, with resultant disassembly of PS II at the low temperature of 18°C (Sippola et al., 1998). These results suggested a crucial role for the extent of unsaturation of fatty acids in promotion of the exchange of the prevailing forms of the D1 protein and, thus, in the maintenance of the activity of PS II at low temperature.

C Increased Unsaturation of Fatty Acids Enhances Tolerance to Salt Stress

In *Synechococcus*, the inactivation of PS II under salt stress, as well as under osmotic stress, was more prominent in wild-type cells than *desA*$^+$-transformed cells and the effect of the transformation was especially pronounced under illumination (Allakhverdiev et al., 2001). A similar effect of transformation with the *desA* gene was also observed when the activity of PS I was examined: the reduction of $P700^+$ in the PS I complex was more resistant to the damaging action of a high concentration of NaCl in *desA*$^+$-transformed cells than in wild-type cells.

The NaCl-induced inactivation of PS I and PS II, as well as that of the Na^+/H^+ antiporter system, consists of a rapid phase and a slow phase (Allakhverdiev et al., 2000; Allakhverdiev and Murata, 2008). The rapid phase is reversible and is induced by the osmotic effect, which reduces the amount of water in the cytosol via the efflux of water through water channels. The slow phase is irreversible and is induced by ionic effects that are due to the influx of Na^+ ions through K^+/Na^+ channels.

Unsaturation of fatty acids in membrane lipids protects PS I and PS II against both rapid phase and slow phase of NaCl-induced inactivation (Allakhverdiev et al., 2001). Experiments with isolated thylakoid membranes also demonstrated that the NaCl-induced inactivation occurred more slowly in membranes isolated from *desA*$^+$-transformed cells than in those from wild-type cells.

Recovery of the activities of PS I and PS II, which was assessed directly in illuminated intact cells, was much more pronounced in *desA*$^+$-transformed cells than in wild-type cells. The available evidence suggests that the unsaturation of fatty acids has two effects: it mitigates the NaCl-induced damage to PS I and PS II both in vivo and in vitro and it enhances the repair of damaged PS I and PS II in vivo. These phenomena might be due, in turn, to the activities of the Na^+/H^+ antiporter system and H^+-ATPase(s), as well as to water channels and $K^+(Na^+)$ channels, which might be influenced by the fluidity of membranes that is dependent on the extent of unsaturation of component fatty acids.

The data summarized above provide direct evidence that the unsaturation of fatty acids in membrane lipids is important for the maintenance of the photosynthetic machinery under salt stress. The data mirror results obtained with wild-type *Synechocystis* and its *desA*$^-$/*desD*$^-$ mutant (see above).

IV Modulation of Unsaturation of Fatty Acids in Membrane Lipids in Higher Plants

A Mutation of Fatty Acid Desaturases and Changes in Lipid Composition

In parallel with the development of techniques for the genetic engineering of various aspects of plant metabolism, including the unsaturation of fatty acids, extensive efforts have been made to isolate and characterize mutant plants with defects in the unsaturation of fatty acids by a classical genetic approach (reviewed by Wallis and Browse, 2002). In an *Arabidopsis* mutant, in which the activities of the Δ12 desaturases were reduced and which accumulated high levels of 16:1 and 18:1 in lipids, chloroplast ultrastructure, the protein and chlorophyll contents of thylakoid membranes, electron-transport activity,

and the thermal stability of photosynthetic membranes were all abnormal (Hugly et al., 1989). These observations suggested a central role for diunsaturated fatty acids in lipids in determining chloroplast structure and maintaining normal photosynthetic functions and they demonstrated that the unsaturation of fatty acids has a direct effect on the thermal stability of photosynthetic membranes.

The *fab1* mutant of *Arabidopsis* is defective in ketoacyl synthase, which catalizes the first step of elongation of 16:0 to 18:0. This mutant had elevated levels of saturated 16:0 fatty acids, although mutant plants were indistinguishable from wild-type plants when grown at 22°C or 12°C (Wu and Browse, 1995). However, during prolonged growth of plants at 2°C, the photosynthetic capacity of *fab1* mutants was impaired. The loss of photosynthetic activity in mutant plants was accompanied by extensive disruption of thylakoid and chloroplast structure, and reductions in the content of chlorophylls and glycerolipids in chloroplasts (Wu et al., 1997). Five lines of the *fab1* supressors have been obtained that could survive after 16 weeks at 2°C and produce viable seed. Three of the suppressors displayed rather expected changes in leaf fatty acid composition towards elevated unsaturation when compared to *fab1*. In one line, however, further increase in lipid saturation was detected. Those plants were deficient in one allele of the chloroplast 16:0 Δ9 desaturase (namely, Fad5-2, which desaturates 16:0 esterified to the *sn*-2 position of MGDG and DGDG), and they contained 31% of 16:0 compared with 23% in *fab1* and 17% in wild type (Barkan et al., 2006). The raised amounts of 16:0 would be expected to accelerate rather than prevent low-temperature damage. Though, the reality appeared a bit far from expectations. Authors proposed instead that a change in shape of the MGDG mediated by the *fad5-2* mutation, may compensate for changes in lipid structure resulting from the original *fab1* mutation (Barkan et al., 2006). This suggestion requires further experimental support, but anyhow, it provides evidence on mechanisms other than increased unsaturation, which might be important in acquisition of cold resistance and which might be considered in the studies of the stress-tolerance of the photosynthetic machinery.

The importance of lipid shape and structure was also indicated in systematic studies of fish living at different latitudes (Logue et al., 2006). Determination of fatty acid composition in individual molecular species of lipids revealed cold-adaptive increase in unsaturation. Particularly, in phosphatidylcholine, the cold-adaptive increase in unsaturation was mostly associated with increased proportions of polyunsaturated fatty acids in the *sn*-2 position. In phosphatidylethanolamine compositional adaptation involved exchanges between saturates and monounsaturates exclusively at the *sn*-1 position. Thus, temperature-dependent adjustment of membrane physical properties relies on the balance between bilayer-stabilizing and destabilizing tendencies. This balance may depend on molecular shapes of the two phosphoglycerides that balance *sn*-1 and *sn*-2 fatty acids to compensate for difference in habitat temperature (Logue et al., 2006).

Although the latter story is far from photosynthesis, it is worth the reader's attention. Recently, manipulations of lipid biosynthesis of a warm *Caenorhabditis elegans* have been conducted independent of temperature (Murray et al., 2007). Worms transferred from 25°C to 10°C develop over several days a much-increased tolerance of lethal cold (0°C) and also an increased phospholipid unsaturation, as in other model organisms. The only cold inducible Δ9-desaturase, *fat7*, among three Δ9-desaturases of *C. elegans*, was suppressed with the corresponding RNAi to prevent the formation of cold-induced monounsaturates. This, however, caused the compensatory induction of *fat5* desaturase. Finally, combined RNAi of *fat7* with a *fat5* knockout was constructed, which displayed the expected negative linear relationship between lipid saturation and cold tolerance at 0°C. However, it was pointed that "the detected change in lipid saturation explained only about 16% of the observed difference between cold tolerance of animals held at 25°C and 10°C". The conclusion from that report was that genetic manipulations with lipid composition in *C. elegans* have negligible or non effect on its thermal acclimation. It was postulated that other nonlipid mechanisms of acquired cold protection clearly dominate in warms (Murray et al., 2007).

In *Synechosytis*, however, the Δ12-desaturase, which introduces the second double bond in fatty acids esterified at *sn*-1 position, was shown to

be crusial for cold acclimation and survival at low temperatures (Tasaka et al., 1996; Los and Murata, 2004). Unicellular prokaryotic cyanobacteria, however, cannot compete with animals and plants in the complexity of their inherent adaptive pathways and mechanisms. Nevertheless, in some eukaryotes (e.g., protozoan *Acanthamoeba castellanii*) the key role of the Δ12-desaturase in cold acclimation had been demonstrated (for review see Harwood, 2007). It is also interesting to note that one of the identified genes for Δ12-desaturase in *A. castellanii* appeared to be bifunctional, and it also catalizes Δ15-desaturation (Sayanova et al., 2006). In *Synechocystis*, Δ12 and Δ15 (ω3) desaturases, although encoded by different genes, are highly homologous to each other, and there was quite intricate method applied in order to clone the gene for the latter enzyme (Sakamoto et al., 1994).

It was also suggested chilling tolerance might be correlated with desaturation of sphingolipids in various plant species (Imai et al., 1997). This is still a questionable matter, and it is unlikely that desaturation of sphingolipids directly affects photosynthetic machinery (for review on the roles of shingolipids in plants see Dunn et al., 2004; Chapter 5).

The complete absence of trienoic fatty acids from the lipids of thylakoid membranes of *Arabidopsis*, due to triple mutation (*fad3-2*/*fad7-2*/*fad8*) of genes for ω3 desaturases (Vijayan and Browse, 2002) revealed that plants that lacked trienoic fatty acids were much more susceptible to photoinhibition than wild-type plants. The rate of photodamage to PS II was the same in wild-type and triple-mutant plants. However, the recovery of photodamaged PS II was slower in triple-mutant than in wild-type plants at temperatures below 27°C. These results support the proposed importance of polyunsaturated fatty acids in the recovery of PS II activity at low temperatures (Gombos et al., 1994, 1997; Moon et al., 1995). Similar conclusions have been drawn from the lessons learned from the studies of cyanobacteria (Tasaka et al., 1996; Allakhverdiev et al., 1999; 2000).

It should be mentioned that cold-induced desaturation in cyanobacteria occurs mainly due to transcriptional induction and due to the increase in desaturase mRNA stability (Los et al., 1997; Panpoom et al., 1998; Los and Murata, 1999). In plants, as well as in fish (Tiku et al., 1996) desaturases, in addition to transcriptional level, are regulated at the post-transcriptional and post-translational levels including phosphorylation and modulation of protein stability (Matsuda et al., 2005; Tang et al., 2005; Kachroo et al., 2007).

The effect of changes in membrane lipids on the oxygen-evolving capability of isolated thylakoids was recently studied (Popova et al., 2007). Lipid composition was altered by incorporation of the plant sterol stigmasterol, which caused rigidification of the membranes, thus mimicking cold and/or hyperosmotic stress. As expected, rigidification of the membranes was accompanied by a reduction of the activity of PSII and by even more pronounced inhibition of PSI-mediated electron transport.

The transport of proteins across membranes occurs via one of three distinct pathways (Gutensohn et al., 2006). A recent study of three mutant lines of *Arabidopsis*, namely, *fad6*, *fad5*, and *fad3*/*fad7*/*fad8*, which had depressed unsaturation of fatty acids in their thylakoid membranes, has provided useful information relevant to the characterization of these three pathways (Ma and Browse, 2006). The mutations in desaturases reduced, by up to 50%, the rate of transport of the 18-kDa extrinsic protein of PS II via the Tat pathway, which depends on a pH gradient across membranes. By contrast, the mutations substantially increased the transport of the 33-kDa protein of PS II via the Sec pathway. This increased capacity for protein import might compensate partially for the reduced capacity for thylakoid transport via the Tat pathway. The transport of the CF_0II protein of the ATPase complex via the so-called "spontaneous" pathway was unaffected by the depressed unsaturation of fatty acids. These results suggest that the transport of proteins across chloroplast membranes might depend on the unsaturation of fatty acids in membrane lipids and, thus, probably on membrane fluidity. Moreover, impaired transport of proteins in the mutants might contribute to their low temperature-sensitive phenotype (Ma and Browse, 2006).

B Changes in Unsaturation of Fatty Acids by Transgenes for Acyltransferases

Glycerol-3-phosphate acyltransferase (GPAT), encoded by the *ATS1* gene (Nishida et al., 1993) and localized in chloroplasts (and plastids),

catalyzes the transfer of the acyl group of acyl-ACP to the *sn*-1 position of glycerol 3-phosphate (Frentzen et al., 1983; Nishida and Murata, 1996). In chilling-resistant plants, such as spinach (*Spinacea oleracea*) and *Arabidopsis*, this enzyme uses 18:1-ACP specifically as its substrate (Frentzen et al., 1983). By contrast, in chilling-sensitive plants, such as squash (*Cucurbita moschata*), the enzyme uses both 18:1-ACP and 16:0-ACP (Nishida et al., 1987; Frentzen et al., 1987). Thus, the extent of unsaturation of fatty acids was altered in transgenic tobacco (*Nicotiana tabacum*) that expressed GPAT from plants that differed in terms of chilling sensitivity (Murata et al., 1992). In transgenic lines of tobacco that expressed GPAT derived from squash the rigidity of thylakoid membranes, determined by shifts in the frequency of the $\nu_{sym}CH_2$ stretching mode, was increased in response to the increase in the proportion of saturated plus *trans*-monounsaturated molecular species of PG from 24% to 65% (Szalontai et al., 2003).

Tobacco plants transformed with cDNA for the chilling-sensitive GPAT of squash displayed more pronounced sensitivity to photoinhibition at low temperatures than wild-type plants (Moon et al., 1995). Moreover, the recovery of PS II from photoinhibition in leaves of wild-type plants was faster than that in leaves of the transgenic tobacco plants. These results suggested that the unsaturation of fatty acids of PG in thylakoid membranes might stabilize the photosynthetic machinery against photoinhibition at low temperature by accelerating the repair of PS II (Moon et al., 1995).

Chilling stress also damaged inflorescences: the abscission of flower buds and inflorescence meristems occurred more frequently in transgenic plants than wild-type plants. Thus, it is likely that decreases in the proportion of *cis*-unsaturated PG enhance the sensitivity to chilling of reproductive organs (Sakamoto et al., 2003). The decrease in the *cis*-unsaturation of fatty acids in PG enhanced growth at 20°C and greening of cotyledons at 10°C (Fig. 2).

Recently, Sui et al. (2007a) overexpressed cDNA for GPAT of tomato in tomato plants under the control of the 35S promoter of cauliflower mosaic virus (CaMV). They observed that overexpression of GPAT increased the relative level of *cis*-unsaturated PG, enhanced the tolerance of plants to cold stress, and accelerated the recovery of PS II after photodamage during photoinhibition at low temperature. The same group also suppressed the expression of the *ATS1* gene for GPAT in tomato by an antisense method (Sui et al., 2007b) and observed an increase in the relative level of saturated fatty acids in PG from leaves. They did not, however, examine changes in the composition of lipid classes or changes in GPAT activity. Nevertheless, it is of interest that the antisense-mediated decreases in unsaturation of fatty acids reduced male fertility, increased the size of tapetal cells, and mitigated photoinhibition of PS II under mild-heat conditions (Sui et al., 2007b).

A similar approach has been used in rice (*Oryza sativa* L.), and levels of *cis*-unsaturated fatty acids in PG in leaves have been genetically raised by introduction of cDNA for GPAT from spinach (Yokoi et al., 1998; Ariizumi et al., 2002). The photosynthetic activity at chilling temperature, such as 14° and 17°C, of transgenic plants was higher than that of wild-type plants. However, at 25°C both types of plant revealed similar activities of photosynthesis. Moreover, growth at chilling temperature of transgenic plants was greater than that of wild-type plants (Ariizumi et al., 2002). These results demonstrate the strict dependence of photosynthetic reactions on the lipid environment.

C Increases in Unsaturation of Fatty Acids due to Transgenes for Fatty Acid Desaturases

Another method that has been developed to modulate the unsaturation of fatty acids in chloroplastic PG involves transformation of tobacco plants with the *desC* gene for Δ9 desaturase from *Synechococcus* (Ishizaki-Nishizawa et al., 1996). The expression of this acyl-lipid desaturase increased the relative level of monounsaturated fatty acids in the PG of chloroplasts in tobacco leaves. The excess of monounsaturated fatty acids served as substrates for further desaturation. As a result, such transformation of tobacco plants dramatically increased levels of polyunsaturated fatty acids and decreased levels of saturated fatty acids from 44% to 24%. These changes dramatically enhanced the ability of the transgenic tobacco plants to tolerate low temperatures.

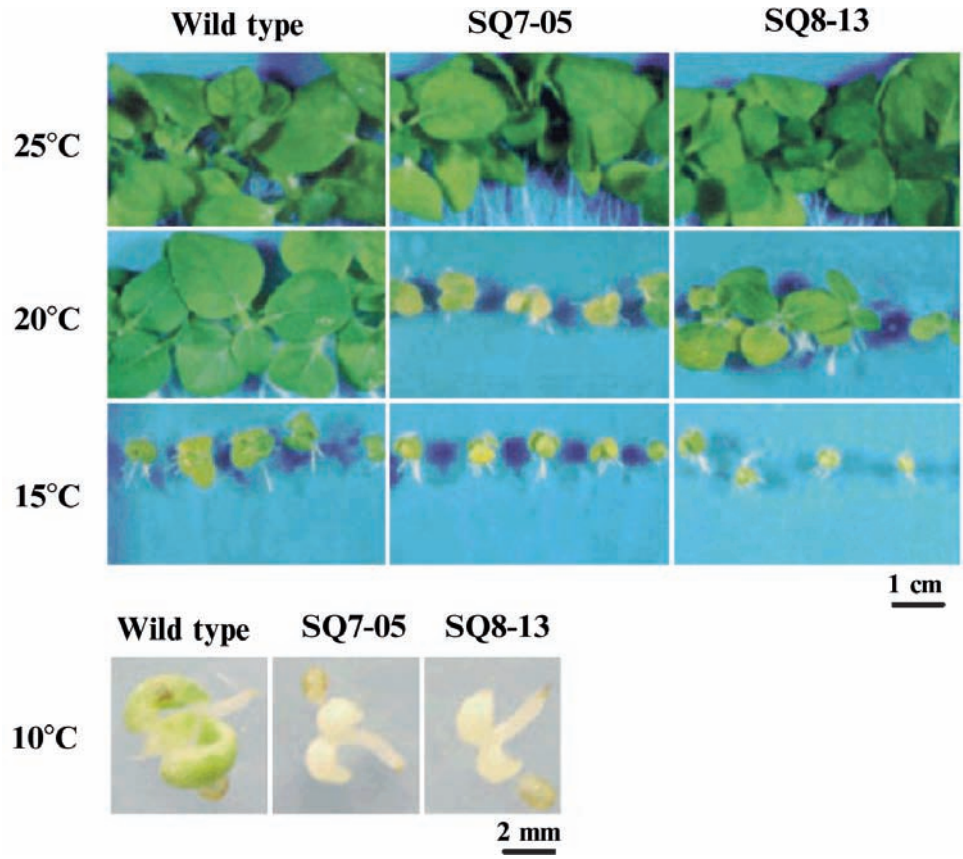

Fig. 2. A decrease in the unsaturation of fatty acids in chloroplastic PG in transgenic tobacco plants suppressed growth at 20°C and inhibited the greening of cotyledons at 10°C. Seeds were allowed to germinate and seedlings were allowed to grow for 1 month at designated temperatures under a 16-h light/8-h dark cycle. SQ7–05 and SQ8–13 were two independent lines of transgenic tobacco (reproduced with permission from Sakamoto et al., 2003) (See Color Fig. 17 on Color Plate 15).

The tobacco plants have also been transformed with the *desC* gene from the thermophilic cyanobacterium *Synechococcus vulcanus* (Orlova et al., 2003). The lipid content and extent of unsaturation of fatty acids were significantly greater in leaves of transgenic plants than in those of parental plants. The chilling tolerance of plants was also increased by the introduction of the *desC* gene, as was estimated from the leakage of electrolytes from tissues upon exposure to low temperatures. Seeds of wild-type plants and of plants that expressed the *desC* gene were allowed to germinate at low temperatures. The mutant seeds exhibited greater chilling tolerance than the control seeds.

In a recent study, overexpression in tomato plants of the *fad7* gene for ω3 fatty acid desaturase from tomato increased the level of α-linolenic acid (18:3) in the glycerolipids of thylakoid membranes (Liu et al., 2008). Upon exposure to chilling stress at 4°C under light at 100 μmol photons m^{-2} s^{-1}, the *fad7*$^{+}$-transgenic plants maintained greater oxygen-evolving activity and greater photochemical efficiency than wild-type plants.

Together with other mutants with defects at certain steps in desaturation pathways, these transgenic plants should serve as useful models for direct assessment of the roles of the unsaturation of fatty acids in photosynthesis and related physiological activities in higher plants.

V Conclusions

Plants and microorganisms respond to changes in ambient temperature by modulating the extent of unsaturation of the fatty acids in their

membrane lipids. Upon exposure to a downward shift in temperature, they increase the extent of unsaturation of these fatty acids, thereby enhancing their tolerance to low temperature. Upon exposure to an upward shift in temperature, they decrease the extent of unsaturation and increase the extent of saturation of the fatty acids, enhancing their ability to tolerate the elevated temperature. Classical physiologists, whose conclusions were based on correlations between observed phenomena, postulated that the extent of unsaturation or saturation of fatty acids would make a predominant contribution to the acclimation of plants and microorganisms to a change in environmental temperature. However, in the late 1900s and early 2000s, this hypothesis was reexamined with the application of newly developed techniques, namely, targeted mutagenesis and the introduction of transgenes into plants and microorganisms, in combination with newly determined genomic sequences. The results of these recent studies have provided good evidence for direct relationships between the lipid environment and the temperature-dependent characteristics of the photosynthetic machinery. It has become clear that the extent of unsaturation of fatty acids in membrane lipids is related to the acclimation of photosynthetic activities to low temperatures but not to high temperatures.

The unsaturation of fatty acids affects the synthesis of ATP but not the photosynthetic transport of electrons at low temperatures. Increases in the unsaturation of fatty acids also enhance the tolerance of PS I and PS II to salt stress. The most striking effect of the unsaturation of fatty acids is the enhanced tolerance of PS II to environmental stress. In particular, increases in the unsaturation of fatty acids in membrane lipids accelerate the recovery of the PS II complex from damage that has been induced by strong light in combination with other kinds of environmental stress, such as low-temperature and salt stress. This phenomenon is related to the accelerated translation, in particular, of genes that encode the precursor to the D1 protein, as well as to the accelerated posttranslational processing of the precursor into the mature Dl protein in the PS II complex.

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Chapter 18

Oxidation of Membrane Lipids and Functions of Oxylipins

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Summary

The glycerolipids that make up the thylakoid bilayer contain exceptionally high levels of polyunsaturated fatty acids. These fatty acids are very susceptible to oxidation, and the activated oxygen species generated as biproducts of photosynthesis will accelerate the initiation of peroxidation. Fortunately, the chloroplast is well protected from damage caused by fatty acid oxidation (as well as other oxidation reactions) by several antioxidant systems. Despite these systems, chemical peroxidation does occur – particularly after tissue damage from wounding or pathogen infection. The oxylipin compounds produced, including reactive electrophile species (RES), contribute to the induction of defense-gene expression and also act directly in defense against insects and microbes. Plants have evolved enzymatic pathways to facilitate the synthesis of particular oxylipin products, including several that are not synthesized by the chemical peroxidation reactions. The best known of these is the defense hormone, jasmonate, which acts through a specific signaling pathway to regulate plant responses. Jasmonate has additional roles in plant development and metabolic regulation. The recent discovery of a family

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of repressor proteins, the JAZ proteins that are targets of jasmonate signaling provide new tools to understand the mechanism of jasmonate action.

I Introduction

The chloroplast thylakoid is a uniquely constructed bilayer membrane. In all photosynthetic eukaryotes, the complement of atypical glycerolipid molecules that form the foundation of this membrane are characterized by sugar headgroups and a very high level of unsaturation in the fatty acid chains, which compose the central portion of the thylakoid lamella bilayer (McConn and Browse, 1996; Mène-Saffrané et al., 2007). Indeed, the thylakoid is one of the most highly unsaturated biological membranes and contains 75–80% polyunsaturated fatty acids. Monogalactosyldiacylglycerol, the major thylakoid lipid, typically contains >90% of α-linolenic acid (18:3), or a combination of 18:3 and hexadecatrienoic (16:3) acids, depending on the plant species (Jamieson and Reid, 1971). For details of chloroplast glycerolipid synthesis and desaturation, see Chapters 3 and 8.

These very high levels of trienoic fatty acids are noteworthy because free radicals that are byproducts of the photosynthetic light reactions (Niyogi, 1999; Rao and Davis, 2001) stimulate oxidation of polyunsaturated fatty acids. Because this oxidation might be expected to mediate against a high degree of unsaturation, it has been inferred that there is a strong selective advantage to having such high levels of trienoic fatty acids in the thylakoid. Therefore, it could be reasoned that these lipid structures must have some critical role in maintaining photosynthetic function.

In actuality, characterization of mutants lacking trienoic acids revealed only subtle effects on photosynthesis under normal growth conditions (McConn and Browse, 1996). Low-temperature or high-light treatments were required to expose deficiencies caused by the replacement of 18:3 and 16:3 fatty acids with their 18:2 and 16:2 precursors (Routaboul et al., 2000; Vijayan and Browse, 2002). However, mutants that contain little or no polyunsaturated fatty acids (<12% of total leaf fatty acids) are not able to grow autotrophically (McConn and Browse, 1998). When grown on sucrose-containing media, these mutants are robust plants with strong leaf and root development. Apparently, most membrane functions are adequately supported in the absence of polyunsaturated lipids. Only the chloroplast membranes require high levels of polyunsaturation for assembly, maintenance and function of the photosynthetic machinery. The importance of thylakoid unsaturation to photosynthetic function is discussed in more detail in Chapters 13 and 17.

II Chemical and Enzymatic Pathways of Oxylipin Synthesis

A Polyunsaturated Fatty Acids Are Sensitive to Oxidation

Regardless of the subtle effects of removing trienoic fatty acids, the conservation of the high trienoic content of thylakoid membranes through evolutionary time attests to the selective advantage of this trait. The challenge of avoiding excessive membrane damage in the chloroplast is clear. Under many conditions, fatty acids form radicals that react with molecular O_2 to initiate radical chain reactions in the following reaction sequence:

$$\mathrm{RH} \rightarrow \mathrm{R}^{\cdot} \qquad \text{Initiation}$$

$$\left.\begin{aligned} \mathrm{R}^{\cdot} + \mathrm{O_2} &\rightarrow \mathrm{ROO}^{\cdot} \\ \mathrm{ROO}^{\cdot} + \mathrm{RH} &\rightarrow \mathrm{ROOH} + \mathrm{R}^{\cdot}, \text{ etc.} \end{aligned}\right\} \text{Propagation}$$

The peroxy-fatty acid formed (ROOH) is a substrate for many further reactions including the production of RO and OH radicals, and both breakdown and polymerization of fatty acids occurs. The reactivity of fatty acids increases disproportionately with the number of double bonds so that oils high in 18:3, such as linseed oil, form polymerized coatings, alone or in varnish and paint formulations. The reactive oxygen species (ROS) and free radicals produced during photosynthesis are ideal initiators and reactants in the peroxidation cascade, so they are a very great threat to the integrity of the thylakoid membrane. This threat extends to the protein and genetic components of the chloroplast because the free-radicals products produced (including malondialdehyde – see

Abbreviations: JA – Jasmonate; JA-Ile – Jasmonoyl-isoleucine; OPC-8:0 – 3-Oxo-2(2′-pentenyl)cyclopentane-1-octanoic acid; OPDA – 12-Oxo-phytodienoic acid; RES – Reactive electrophile species

below) also react with and damage proteins and DNA (Marnett, 2002; Alméras et al., 2003).

And yet, the trienoic acids and other lipid components of the thylakoid are not subject to rapid degradation, even under high light, which independently accelerates fatty acid oxidation. The reason for this, of course, is that several very-active antioxidant systems operate in the chloroplast to quench free radical reactions that would otherwise lead to runaway lipid breakdown (as well as destruction of other components of the photosynthetic machinery). These mechanisms include superoxide dismutase (SOD) enzymes, tocopherol and carotenoid redox systems, glutathione, ascorbate/ascorbate peroxidase and peroxiredoxins (Niyogi, 1999). These mechanisms will not be discussed in detail in this review, but it is clear that they are highly redundant with respect to protecting photosynthesis from damage.

One illustration of how well protected the chloroplast lipids are comes from the investigation of mutants deficient in tocopherol synthesis. The *Arabidopsis vte2* mutant is deficient in homogentisate phytyl transferase, the first committed step in tocopherol synthesis. *vte2*-Mutant plants are compromised during seedling establishment (when extensive seed oil reserves are being mobilized); however, established plants are essentially indistinguishable from wild-type in the extent of damage caused by high-light stress. The *vte2* plants were more strongly impacted during high-light treatment at low temperatures (2–8°C), but these effects were attributed to damage to non-photosynthetic membranes in phloem transfer cells (Maeda et al., 2008). Combined high-light, low-temperature stress is more damaging to a *vte1 npg1* double mutant that is deficient in the synthesis of both tocopherols and carotenoids (Havaux et al., 2005), indicating the overlapping protection provided by these two classes of compounds. The phenotypes seen in *vte2* seedlings have been shown by genetic and biochemical approaches to be the result of malondialdehyde production from 18:3 and 16:3 fatty acids (Mène-Saffrané et al., 2007).

B Production and Role of Reactive Electrophile Species

Notwithstanding the protective mechanisms in the chloroplast, non-enzymatic, free-radical oxidation does occur, particularly when leaf tissue is subject to mechanical wounding, attack by insects, or infection by microbial pathogens. A single polyunsaturated fatty acid, such as 18:3, can give rise to scores of different compounds including a complex mixture of six-carbon aldehydes and alcohols known as green-leaf volatiles that constitute the aroma of newly-cut grass (Matsui, 2006). This class of compounds includes n-hexanal, 2,- and 3,-hexenals and the corresponding alcohols. Other products include longer (e.g., 2-nonenal, 4-hydroxy-2-nonenal) and shorter chain molecules (e.g., malondialdehyde, methyl-vinyl ketone), mainly derived from cleavage of the 18:3 (or 18:2) chain at the C=C double bonds. Several of the compounds produced have been shown to inhibit bacterial and fungal growth and may act directly in plant defense against pathogens (Blée, 2002). In addition, there is clear evidence that green-leaf volatiles act as inter-plant (and intra-plant) signals that potentiate defense processes (Matsui, 2006).

An important structural feature of a subset of these compounds, including 2-hexenal, malondialdehyde (in the form of the β-hydroxyacrolein isomer) and methyl vinyl ketone, is the presence of an α,β-unsaturated carbonyl group. This chemical structure is a strong electrophile and readily enters into Michael addition reactions with nucleophiles, such as sulphydryl groups. Compounds containing α,β-unsaturated carbonyl groups are included in a larger class of molecules termed reactive electrophile species, RES. Over the last 5–10 years, studies of low molecular weight RES, including 2-alkenals, methyl vinyl ketone and malondialdehyde, have shown that they activate expression of many defense genes (Bate and Rothstein, 1998; Alméras et al., 2003; Weber et al., 2004; Farmer and Davoine, 2007). Interestingly, methyl vinyl ketone and malondialdehyde induce overlapping but distinct sets of genes suggesting a level of complexity in the transcriptional response to RES (Alméras et al., 2003; Weber et al., 2004). It is not clear whether methyl vinyl ketone and malondialdehyde directly contribute to the induction of gene expression, or instead damage cellular components with this damage then activating stress response pathways (Farmer and Davoine, 2007).

Because they react with many nucleophiles, RES may also influence cellular biochemistry by covalently modifying enzymes and other proteins. For example, 4-hydroxy-2-nonenal appears

to specifically inhibit pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and the alternative oxidase in plant mitochondria (Millar and Leaver, 2000; Winger et al., 2005).

As well as fragmenting into the compounds discussed above, peroxidized fatty acids may undergo ring formation and intramolecular rearrangement to form members of a large class of phytoprostanes (Thoma et al., 2004). The phytoprostanes are characterized by a five-carbon ring with one or more keto or hydroxy groups and two side chains derived from the parental 18- or 16-carbon fatty acid. Several of the isoprostanes contain α,β-unsaturated carbonyl groups and are thus RES. These compounds, as well as other phytoprostanes, have been shown to induce phytoalexin synthesis and thus may act as mediators of plant defense against pathogens (Mueller, 2004; Thoma et al., 2004; Loeffler et al., 2005; Sattler et al., 2006; Mueller et al., 2008).

C Enzymatic Pathways of Oxylipin Synthesis

The chemical reactions that produce the wide range of fatty acid derivatives briefly discussed in the first part of this review are matched by enzyme-catalyzed reactions that give rise to a smaller (but still sizeable) number of compounds. In these pathways, fatty acid peroxides are generated by the action of 9- and 13-lipoxygenase enzymes. From 18:3, the 13-lipoxygenase produces (13S)-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid, 13-HPOT (Fig. 1). This compound can be cleaved by hydroperoxide lyase (HPL) into (3Z)-hexenal, which can isomerize to 2E-hexenal, and

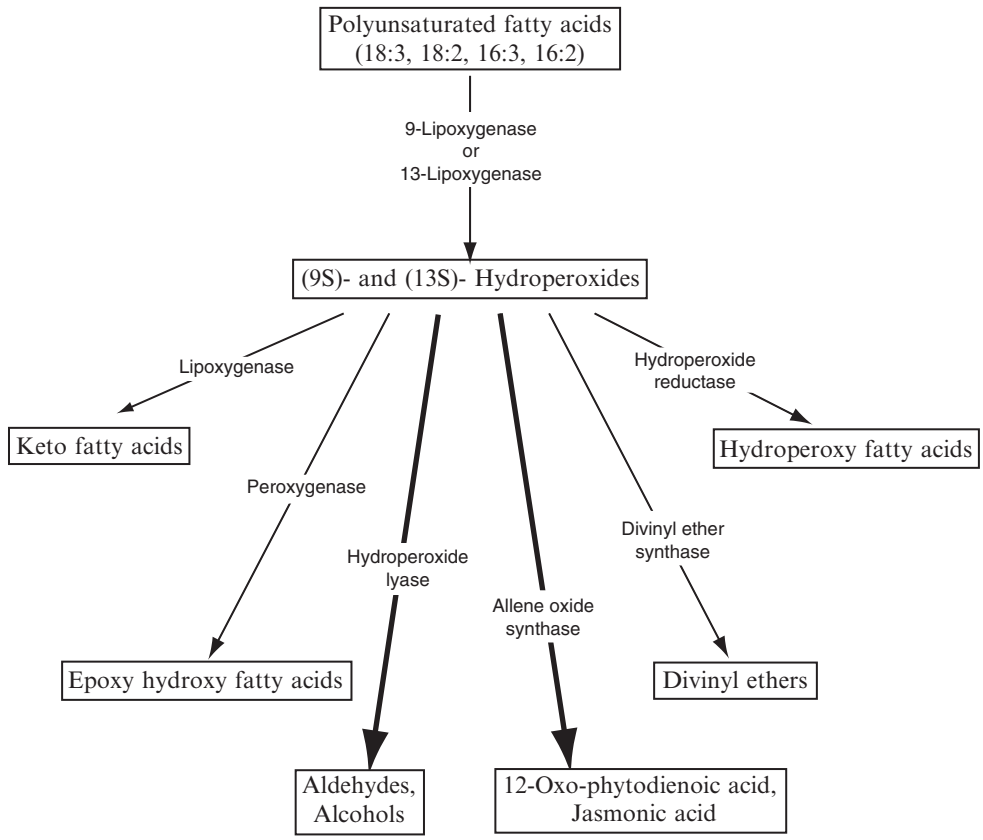

Fig. 1. The major enzymatic routes to oxylipin synthesis in plants. Position-specific 9- or 13-lipoxygenase isozymes act on polyunsaturated fatty acids to produce hydroperoxide derivatives. The major pathways for further metabolism are those initiated by hydroperoxide lyase and allene oxide synthase. Additional classes of oxylipins are produced by pathways involving lipoxygenase, peroxygenase, divinyl ether synthase and hydroperoxide lyase as shown (see text for details).

12-oxo-(9Z)-dodecenoic acid, which can isomerize to 12-oxo-(10E)-dodecenoic acid (traumatin). Enzymatic reduction of the aldehyde groups in these molecules (to form the corresponding alcohol), or oxidation to carboxyl groups gives rise to additional derivatives including several of those produced by the less-specific chemical peroxidation pathway from 18:3 described in Section II.B.

A similar range of compounds are obtained from the conversion of 18:2 to 13-hydroperoxy (9Z,11E)-octadecadienoic acid (13-HPOD) by 13-lipoxygenase followed by the action of HPL and other downstream enzymes. The 9-lipoxygenase isozymes produce 9-HPOT (from 18:3) and 9-HPOD (from 18:2) and these compounds are also cleaved by HPL isozymes in pathways that lead to the synthesis of additional aldehyde and alcohol compounds (Vellosillo et al., 2007). The details of enzymology and products of the hydroperoxide lyase pathway are covered in several detailed reviews and some of these also discuss other conversions of HPOT and HPOD intermediates via peroxygenase, divinyl ether synthase and other enzymes (Noordermeer et al., 2001; Blée, 2002; Grechkin, 2002; Matsui, 2006). A number of compounds produced are RES as discussed above and some non-RES products are also believed to act in plant defense (Blée, 2002; Matsui, 2006).

D An Evolutionary Perspective

Clearly, peroxidation of fatty acids has been occurring since the first development of oxygenic environments and is particularly extensive in wounded tissues rich in highly unsaturated fatty acids. It is reasonable to suggest that the induction of biochemical and genetic stress-response pathways evolved so that the organism could respond to both the damage caused by the peroxidation products and to the primary causative agent in the case of insect or pathogen attack. Subsequently, the evolution of enzyme-catalyzed pathways of fatty acid oxidation would have a selective advantage because specific, efficacious compounds could be produced.

The mechanism of gene activation by RES is not known; however, given that RES react with a range of cellular components, it may involve a general, diffuse cell-stress response. Because defense is critical to plant survival and reproductive fitness, it is perhaps not surprising that enzymatic pathways have evolved to produce unique products of fatty acid peroxidation that act as specific ligands in receptor-mediated defense signaling pathways. The jasmonate pathway is the best established example and it is described in detail below. It is noteworthy that neither jasmonate nor its precursor 12-oxo-phytodienoic acid (OPDA) is a product of chemical peroxidation reactions.

III The Synthesis and Function of Jasmonate in Higher Plants

A An Overview of Defense and Other Functions in Plants

In the last 15 years, jasmonate (JA) has emerged as a key regulator of an astonishingly wide range of plant processes, and this oxylipin is now firmly established as a major plant hormone. The ability of methyl jasmonate to induce proteinase inhibitors in tomato was first reported in 1990 (Farmer and Ryan, 1990) and papers describing the induction of some pathogen-defense genes followed (Xu et al., 1994). Demonstrations that JA was essential for defense against some insects, fungi, and bacteria came later with the production of prosystemin antisense tomato plants (McGurl et al., 1992) and with the isolation of *Arabidopsis* and tomato mutants deficient in JA synthesis or perception (McGurl et al., 1992; Howe et al., 1996; McConn et al., 1997; Staswick et al., 1998; Thomma et al., 1998; Vijayan et al., 1998). Characterization of one of these mutants (*fad3 fad7 fad8*) also led to the discovery that JA is an essential signal for the final stages of pollen maturation and anther dehiscence (McConn and Browse, 1996). More recently, JA has been shown to act in signaling of abiotic stresses, including UV radiation (Conconi et al., 1996) and ozone (Rao and Davis, 2001). In its defense role, JA downregulates the synthesis of many proteins involved in normal plant metabolism including many chloroplast proteins (Reinbothe et al., 1993). In healthy, unwounded plant tissue, JA is involved in carbon partitioning (Mason and Mullet, 1990), mechanotransduction (Weiler et al., 1993), root growth (Staswick et al., 1992), and the maturation and release of pollen (McConn and Browse, 1996; Stintzi and Browse, 2000; Ishiguro et al., 2001;

Park et al., 2002; von Malek et al., 2002). Much of our new understanding of the actions of JA signaling pathways has developed from the isolation and characterization of mutants in *Arabidopsis* and other plants (Turner et al., 2002; Devoto and Turner, 2003; Browse, 2005).

B The Biochemistry and Cell Biology of Jasmonate Synthesis

1 Chloroplast and Peroxisome Enzymes of Jasmonate Synthesis

The pathway for jasmonate synthesis from 18:3 was first proposed by Vick and Zimmerman (1983). This overall chemistry has been substantially confirmed by many recent studies, which have also added important details about the enzymology, regulation and subcellular location of the pathway reactions (Liechti and Farmer, 2002; Howe and Browse, 2007; Wasternack, 2007). In the canonical presentation (Fig. 2), the first step is release of 18:3 (or 16:3) from membrane glycerolipids (Ishiguro et al., 2001; Hyun et al., 2008). 13-Lipoxygenase converts 18:3 to 13-HPOT and then allene oxide synthase (AOS) produces 12,13-epoxyoctadecatrienoic acid, which is acted on by allene oxide cyclase (AOC). The AOC enzyme determines the stereoconfiguration of the product as (9S,13S)-12-oxo-phytodienoic acid (OPDA) (Ziegler et al., 2000), and this stereoisomer is the exclusive (>99%) isomer detected in wounded plants. The same enzymes act on 16:3 to form dinor-OPDA (Weber et al., 1997). Interestingly, OPDA has also been identified as a substituent at *sn*-1 of the chloroplast lipid, monogalactosyldiacylglycerol (Stelmach et al., 2001; Andersson et al., 2006). Because a substantial proportion of total leaf OPDA is esterified to chloroplast glycerolipids, it is possible that release of this intermediate by lipases is the regulated step in production of JA. It is now known that a specific isozyme of OPDA reductase (encoded by the *OPR3* gene) is required to reduce (9S,13S) OPDA to 3-oxo-2(2′[Z]-pentenyl)cyclopentane-1-octanoic acid (OPC-8:0) (Sanders et al., 2000; Schaller et al., 2000; Stintzi and Browse, 2000), which is then converted to (3R,7S)-jasmonic acid by three cycles of β-oxidation (Vick and Zimmerman, 1983).

Considerable evidence indicates that the synthesis of OPDA occurs in the chloroplast (plastid) (Blée, 1998; Schaller et al., 2000), while the final production of JA occurs in the peroxisome – the only known site of β-oxidation in plants (Schaller et al., 2000; Ziegler et al., 2000). Import of OPDA into the peroxisome is partly dependent on the ATP-binding cassette transporter PXA1 (= CTS = PED3) (Footitt et al., 2007) and the OPC-8:0 produced by OPR3 is activated by a carboxyl-CoA ligase encoded by *OPCL1* (Koo et al., 2006). In plants, β-oxidation is catalyzed by acyl-CoA oxidase, the multifunctional protein (MFP) (which exhibits 2-*trans*-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, D-3-hydroxyacyl-CoA epimerase, and $\Delta^3\Delta^2$-enoyl-CoA isomerase activities), and L-3-ketoacyl-CoA thiolase.

In *Arabidopsis*, there appear to be five genes encoding acyl-CoA oxidases (*ACX1-ACX5*, with varying chain-length specificities), two encoding MFP, and three encoding the thiolase (Hayashi et al., 1998, 1999; Richmond and Bleecker, 1999; Eastmond and Graham, 2000; Eastmond et al., 2000). Mutant analysis indicated that ACX1 and ACX5 act redundantly in initiating β-oxidation of OPC-8:0-CoA (Schilmiller et al., 2007). Besides fatty acids, β-oxidation is involved in the metabolism of a number of compounds including OPC-8:0, indole butyric acid (Bartel, 1997), and 2,4-diphenoxybutyric acid (2,4-DB) (Hayashi et al., 1998). Available evidence suggests that each of the MFP and thiolase isozymes will act in β-oxidation of 2,4-DB (Hayashi et al., 1998; Richmond and Bleecker, 1999; Eastmond and Graham, 2000), and the same may well be true for metabolism of OPC-8:0 to JA. Certainly none of the mutations in these genes has provided a JA-reversible, male-sterile phenotype, the diagnostic consequence of a severe JA-synthesis defect in *Arabidopsis* (Hayashi et al., 1998; Richmond and Bleecker, 1999; Eastmond et al., 2000). Finally, there is probably also a thioesterase that produces JA from jasmonoyl-CoA after three cycles of β-oxidation.

2 Plants Synthesize Numerous Jasmonate Derivatives

(9S, 13S) OPDA yields the (3R, 7S) isomer of JA (also called (+)-7-*iso*-JA or cis-(epi)-JA), and this is believed to be the isomer used for synthesis of the active jasmonoyl–isoleucine conjugate (see Section III.D.3). However, (3R, 7S)-JA is

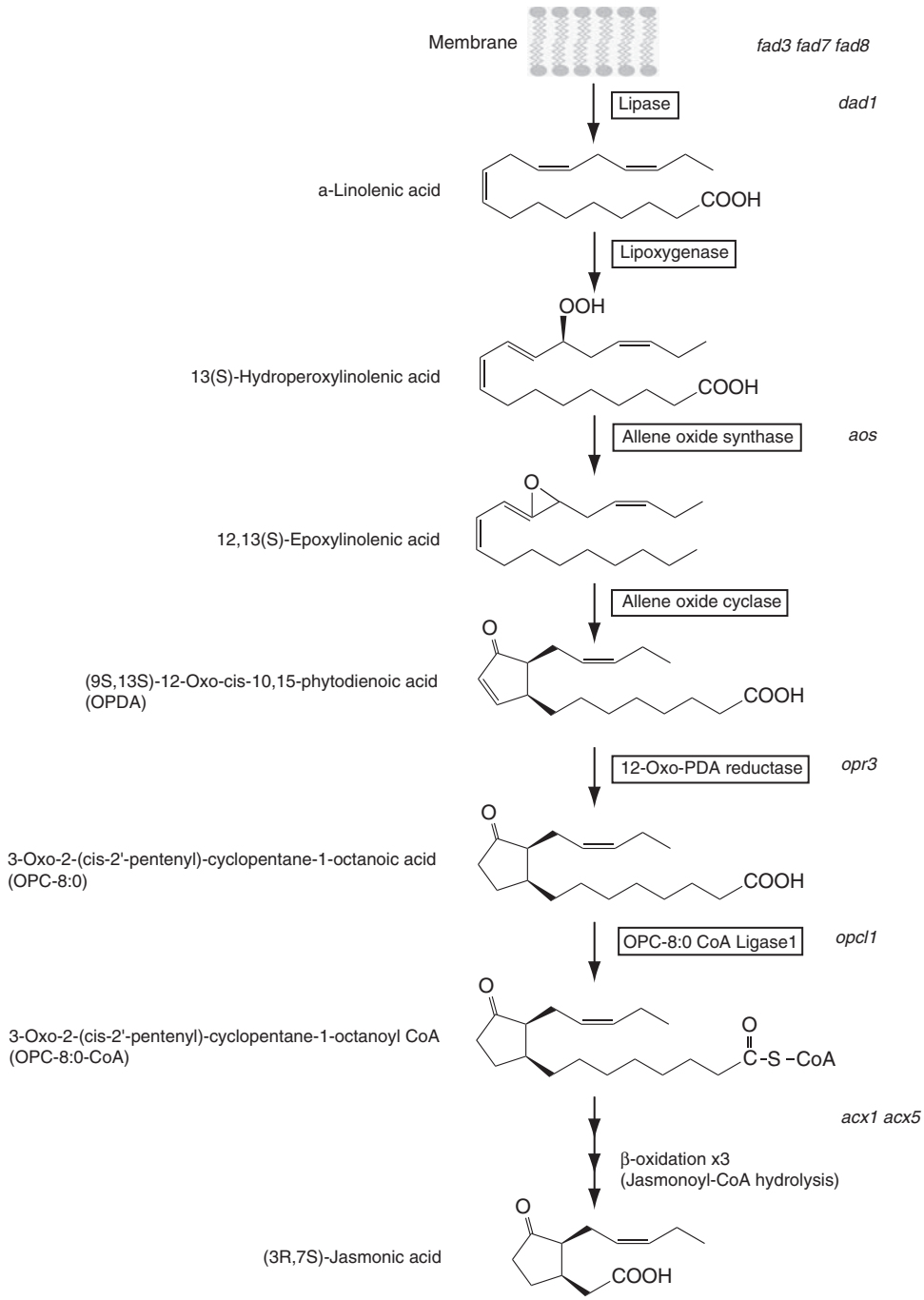

Fig. 2. The pathway for biosynthesis of jasmonic acid from 18:3. The names of *Arabidopsis* mutants that have contributed to our knowledge of jasmonate synthesis and function are shown. The projections shown represent the absolute stereoconfigurations of the side chains.

sterically hindered (both side chains are on the same side of the cyclopentanone ring) and isomerizes the more stable (3R, 7R) isomer ((−)-JA or trans-JA), which is believed to be substantially inactive. Both isomers are observed in plant extracts, with (3R, 7R)-JA the predominant form; however, it is not clear to what extent conversion occurs in planta or during sample work up. JA is

subject to various enzymatic transformations to generate a suite of derivatives that differ in their biological activities. Among the major routes of JA metabolism are: (i) methylation of C1 to yield volatile MeJA, which was originally identified as a fragrant component of jasmine flowers; (ii) decarboxylation of C1 to form another volatile compound, *cis*-jasmone; (iii) hydroxylation at C12 (or C11) yielding tuberonic acid and related derivatives that can be modified further by sulfation or glycosylation; (iv) reduction of C6 to yield cucurbic acid, which may also be esterified to sugar residues; and (v) amide-linked conjugation of the carboxyl group to isoleucine (Ile) and other amino acids, yielding JA–Ile and other jasmonoyl–amino acid conjugates, respectively (Browse and Howe, 2008).

In *Arabidopsis*, JA–Ile is synthesized by an enzyme encoded by the *JAR1* gene. Mutants at the *jar1* locus were found to be partially deficient in JA responses (Staswick et al., 1998; Staswick and Tiryaki, 2004; Kang et al., 2006). Taken together, these findings indicated that JA–Ile is an important primary signal for at least some JA responses. As described in Section III.D.3, JA–Ile and structurally related conjugates are the only jasmonates currently known to be active at the molecular level.

C Jasmonate Is the Defense Hormone

1 An Essential Role for Jasmonate in Insect Defense

Our current understanding of local and systemic signaling processes in plant defense against insects is based on several decades of work in tomato. Local signals, such as oligouronides (Hahn et al., 1981; Bishop et al., 1984) and systemic signals, such as the peptide hormone systemin (Pearce et al., 1991), interact with receptors to trigger a series of cellular responses. These include the opening of plasma membrane ion channels, increases in cytoplasmic [Ca^{2+}], and triggering of a MAP kinase (Felix and Boller, 1995; Usami et al., 1995; Moyen and Johannes, 1996; Stratmann and Ryan, 1997; Schaller and Oecking, 1999), as well as activation of a phospholipase that releases 18:3 (or OPDA) from membrane lipids and initiates the synthesis of JA (Lee et al., 1997; Nárvaez-Vásquez et al., 1999). These initial responses take place within 2–10 min and are too rapid to involve changes in transcription, although JA signaling subsequently activates many of the genes involved in these processes. Although other signaling processes occur, mutant analysis demonstrates that JA synthesis and JA signaling are essential for successful defense (Howe et al., 1996; McConn et al., 1997; Howe and Jander, 2008). Within 30 min of wounding, the production of JA triggers the activation of genes encoding the enzymes of the octadecanoid pathway (Ryan, 2000) and genes encoding other proteins involved in signaling, such as prosystemin from which the 18 aa systemin peptide is derived (Ryan, 2000). Expression of these 'early' genes peaks at 2–4 h after wounding, and then declines. By contrast, the defense genes (including proteinase inhibitors and polyphenol oxidase, for example) are activated later with transcript levels starting to rise at 4 h and peaking at 8–10 h after wounding (Constabel et al., 1995). We now know that both temporal and spatial differences in gene expression are important in understanding JA responses at a mechanistic level (Glauser et al., 2008).

Many studies of JA-related processes have relied on a small number of 'archetypal' genes to document and follow changes in expression using RNA blot analysis. More recently, broad transcriptional profiling has revealed the extraordinary extent of JA regulation (Reymond et al., 2000; Schenk et al., 2000; Devoto et al., 2005; Mandaokar et al., 2006; Goda et al., 2008). At least 2,000 of the 26,000 genes in *Arabidopsis* are activated or repressed by JA signaling. In the JA pathway, as in most signaling pathways, many target genes are induced (or repressed) by the action of transcription factors, which often act on a set of related genes that might, for example, encode the enzymes of a biochemical pathway – for instance, the induction of alkaloid synthesis in periwinkle (*Catharanthus roseus*). Expression of many (but not all) enzymes required for the synthesis of these alkaloids has been shown to be regulated by the JA-responsive transcription factors, ORCA2 and ORCA3 (Menke et al., 1999; van der Fits and MemLink, 2000). These are members of the AP2/EREBP family, which has 144 members in *Arabidopsis* (Riechmann et al., 2000).

These studies established JA as a chemical signal mediating defense responses against insect attack. However, the signaling pathways that allow plants to mount defenses against chewing insects are known to be complex, and clear

demonstrations on the efficacy of JA signaling are required. Definitive evidence for the essential role of JA in insect defense came from studies of tomato and *Arabidopsis* mutants deficient in synthesis or accumulation of JA (Howe et al., 1996; McConn et al., 1997). The *Arabidopsis fad3–2 fad7–2 fad8* triple mutant is deficient in all three desaturase enzymes that can convert 18:2 to 18:3 and 16:2 to 16:3 (McConn and Browse, 1996). The plants therefore lack the fatty acid precursors for JA synthesis and contain negligible levels of JA. Mutant plants showed extremely high mortality (approximately 80%) from attack by larvae of a common saprophagous fungal gnat, *Bradysia impatiens* (Diptera:Sciaridae), even though neighboring wild-type plants were largely unaffected. Application of exogenous JA substantially protected the mutant plants and reduced mortality to approximately 12%. These experiments precisely define the role of JA as being essential for the induction of biologically-effective defense in this plant/insect interaction. Perhaps not surprisingly, this detailed knowledge of JA-mediated defense obtained in the laboratory has recently been shown to have relevance to plants grown in natural environments (Kessler et al., 2004). Experimental evidence indicates that OPDA also activates defense genes (Stintzi et al., 2001; Taki et al., 2005) but it is currently unclear whether this is through its activity as a reactive electrophile species (Stintzi et al., 2001) or through COI1-dependent signaling (Ribot et al., 2008).

2 Jasmonate Is a Translocated Signal

A key aspect of wound-defense signaling in tomato and other plants is the finding that wounding of a single leaf results in the expression of defense genes throughout the plant (Ryan and Pearce, 1998). This means that a systemic signal must be generated in the wounded leaf to activate defenses at distal sites. Several chemical compounds, as well as a proposed electrical signal, have been tested for possible involvement in long-distance signaling. In tomato, the 18-aa peptide systemin has a clearly established role in activation of defense genes throughout the plant. However, it is now clear that JA (or a related oxylipin) is an essential component of the translocated signal. The *spr-2* mutant of tomato is deficient in JA synthesis but responds normally to applied JA (Li et al., 2002, 2003). Conversely, the *jai-1* mutant synthesizes JA but induction of defense genes does not occur in response to wounding or JA (Li et al., 2004). These mutants were used in grafting experiments with wild-type plants (Li et al., 2002) and the key observations are cartooned in Fig. 3. (1) Wounding of a lower leaf on a wild-type plant (a) resulted in generation of a graft-transmissible signal and induction of defense genes measured in an unwounded leaf on the scion (grafted upper part of the plant). (2) Wounding of an *spr-2* rootstock (b) does not lead

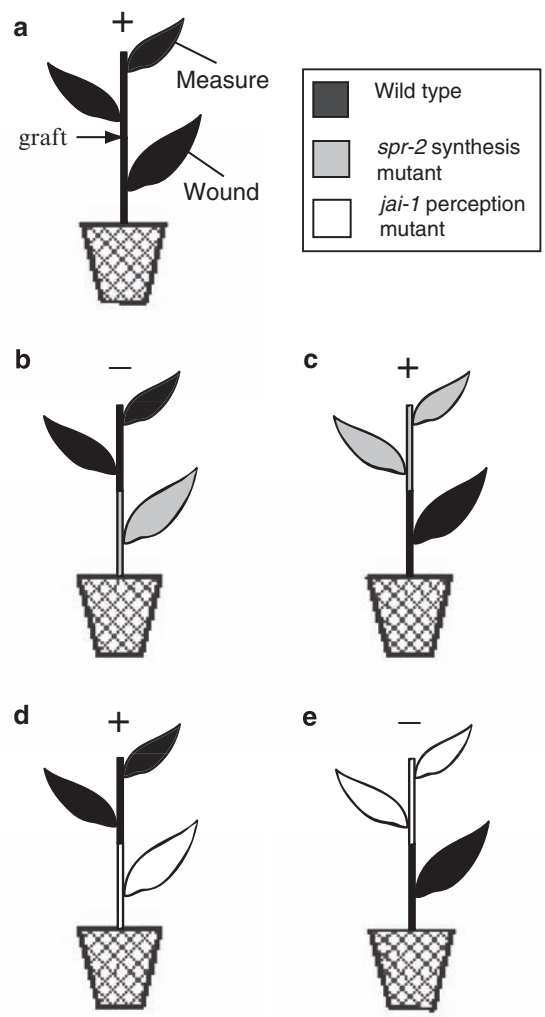

Fig. 3. Grafting experiments with tomato mutants establish a role for jasmonate as the translocated wound signal. A leaf of the rootstock was wounded and transmission of the signal was assayed by measuring induction of defense genes (+ or −) in a leaf of the graft scion see text for details).

to a signal even though an *spr-2* scion (c) can respond to the signal generated by wounding of a wild-type rootstock. (3) A rootstock from the *jai-1* mutant (d) is able to generate a signal that results in defense gene induction in a wild-type scion. However, a *jai-1* scion (e) is insensitive to the signal produced by wounding a wild-type rootstock. These results indicate that JA is synthesized in wounded leaves and moves through the plant to activate defense responses.

3 Jasmonate Also Acts in Defense against Microbial Pathogens

The signals, such as salicylic acid, that have been identified as activating defenses against many fungal pathogens, are distinct from those involved in insect defense and in fact stimulation of salicylic acid synthesis inhibits jasmonate signaling. However, it is now known that JA plays an essential role in defense against some fungal pathogens, particularly necrotrophic pathogens, such as *Pythium* and *Alternaria brassicicola*. These pathogens kill mutants that cannot synthesize JA, while neighboring wild-type plants remain healthy (Vijayan et al., 1998). Application of exogenous JA substantially protects mutant plants, reducing the incidence of disease to a level close to that of wild-type controls, but JA treatment does not protect the JA-insensitive mutant *coi1* from infection. *Pythium* species are ubiquitous in soil and root habitats world-wide, but most are considered to be minor pathogens. Thus, JA is essential for plant defense against *Pythium* and, because of the high exposure of plant roots to *Pythium* inoculum in soil, may well be fundamental to survival of plants in nature. Subsequent work has demonstrated that JA and ethylene are key signaling molecules in non-host defense of plants against many fungi and other microbial pathogens, by regulating the production of defense proteins and inducing biochemical pathways for the synthesis of defense compounds (Kunkel and Brooks, 2002; Glazebrook, 2005; Shah, 2005; Broekaert et al., 2006).

The combinatorial actions of JA and ethylene in defense signaling are coordinated through the induction of genes encoding key transcription factors. ERF1 is one of five Ethylene Response Factors in *Arabidopsis*. The *ERF* genes were first characterized as being induced by ethylene (Lorenzo et al., 2002); however, subsequent analysis demonstrated that both ethylene- and JA-signaling are required for high *ERF1* expression. Thus, ERF1 is a likely candidate for a transcription factor that integrates signals for the expression of genes whose products protect plants from necrotrophic fungal pathogens, such as *Pythium* spp. and *Alternaria brassicicola*. Consistent with this notion, constitutive expression of *ERF1* (controlled by the cauliflower mosaic virus 35S promoter) resulted in expression of many defense genes. Furthermore, mutants that are normally susceptible to pathogens as a result of defective JA or ethylene signaling (*coi1* and *ein2*, respectively) were protected by expression of the *35S:ERF1* transgene (Lorenzo et al., 2002). More recently, cloning of the *JASMONATE-INSENSITIVE1* locus of *Arabidopsis* (*JAI1/JIW1*), and yeast one-hybrid screens in tomato have identified b-HLH transcription factors (MYC2 in *Arabidopsis*; JAMYC2 and JAMYC10 in tomato) that perform a complementary role in JA signaling. *JIN1* expression is induced by JA and the MYC2 protein that it encodes is required for activation of many JA-responsive genes – particularly those that are induced in response to wounding and insect attack (Lorenzo et al., 2004). However, MYC2 represses many genes that are induced in response to pathogens by action of the ERF1 transcription factor. Conversely, ERF1 represses wound-responsive genes. Thus, there are (at least) two sub-pathways of JA-induced defense signaling. In one, wound-responsive genes are up-regulated through the MYC2 transcription factor. In the second, JA and ethylene act synergistically through ERF1 to activate pathogen-responsive genes. The antagonistic cross regulation of MYC2 and ERF1 provides the means to elicit different responses based on appropriate integration of input signals.

D Discovery of the JAZ Repressors and the Mechanism of Jasmonate Signaling

1 Eight JAZ Genes Induced by Jasmonate Treatment

The discovery that *COI1* encodes an F-box protein led to the suggestion that core JA signaling depends on the action of the E_3 ubiquitin ligase, SCF^{COI1} (Xie et al., 1998). Specifically, it was proposed that JA signaling involves ubiquitina-

tion of specific target proteins by the SCF^{COI1} complex and their subsequent degradation by the 26S proteasome. Extensive genetic screens for positive effectors, negative effectors, and components downstream of COI1, as well as searches for COI1-interacting proteins failed to identify verifiable SCF^{COI1} targets (Thines et al., 2007; Balbi and Devoto, 2008). Recently, substrates of SCF^{COI1} were discovered through transcriptional profiling of stamen development in response to JA treatment (Mandaokar et al., 2006; Thines et al., 2007). This approach led to the identification of eight JA-inducible early response genes that encode proteins of unknown function and contain a so-called ZIM motif. These genes were thus named *JASMONATE ZIM-DOMAIN* (*JAZ*) genes (Chini et al., 2007; Thines et al., 2007). The JAZ family of proteins in *Arabidopsis* consists of 12 members including eight whose cognate transcripts are rapidly and strongly induced by jasmonate treatment of plants. Overall, homology among the JAZ proteins is confined to three regions (Fig. 4a): the N-terminal region exhibits the weakest sequence similarity; the central region contains the recognized ZIM motif; and the C-terminal region contains a highly conserved $SLX_2FX_2KRX_2RX_5PY$ stretch of amino acids referred to previously as Domain 3 (Thines et al., 2007) or the CT domain (Chini et al., 2007), but now referred to as the Jas motif (Yan et al., 2007). Several JAZ proteins have been localized to the nucleus but, unlike the *Arabidopsis* ZIM and ZIM-like proteins, which have zinc-finger DNA-binding domains (Shikata et al., 2003), none of the JAZ proteins contains a known DNA-binding domain.

2 A Modified JAZ Protein Blocks Jasmonate Signaling

Null mutations in four *JAZ* genes (*jaz2*, *jaz5*, *jaz7* and *jaz9*) did not cause male sterility or other strong JA-related phenotypes (Thines et al., 2007). These results suggest that the JAZ proteins may have overlapping functions, and this could explain the failure of forward-genetic screens to identify recessive mutations in the *JAZ* genes. However, these results do not preclude the possibility that mutations in these or other *JAZ* genes cause subtle JA-related phenotypes, or that production of multiple-mutant lines will provide informative phenotypes. Although overexpression of genes encoding full-length JAZ proteins also failed to induce any phenotype, expression of a truncated JAZ1 protein (JAZ1Δ3A) lacking

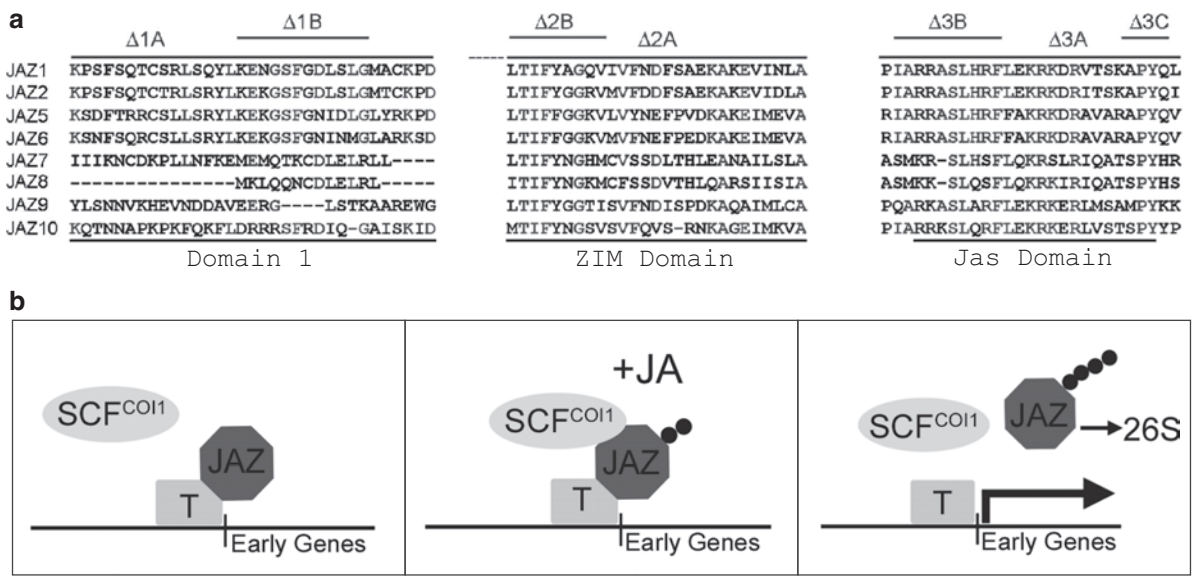

Fig. 4. (**a**) Sequence alignment of three conserved domains from the eight *Arabidopsis* JAZ proteins that are induced by JA, showing the seven deletions made in JAZ1. (**b**) A simplified model of possible SCFCOI1 action. In response to jasmonate (JA), SCF^{COI1} interacts with JAZ repressors blocking transcription complexes (T) on jasmonate-responsive genes. JAZ ubiquitination (*black circles*) results in degradation through the 26S proteasome and derepression of the jasmonate-response pathway.

residues 202–228, which includes the Jas motif, yielded plants that were male sterile. The sterile *35S-JAZ1Δ3A* plants showed other phenotypes typical of jasmonate-response mutants, including resistance to the inhibition of root growth caused by jasmonate, resistance to infection by a coronatine-producing strain of *Pseudomonas syringae*, and weak induction of JA-responsive genes (Thines et al., 2007).

The dominant action of JAZ1Δ3A in blocking JA responses suggested a model in which JAZ proteins are repressors that prevent transcription of JA-responsive genes. In this scenario, JA facilitates interaction between JAZ and COI1, leading to degradation of the JAZ substrates through the ubiquitination-26S proteasome pathway; destruction of JAZ repressors in response to a JA signal would allow rapid expression of early response genes (Fig. 4b). Deletion of the C-terminal region of JAZ proteins prevents their degradation and allows continued suppression of JA-responsive genes. Experiments with plants expressing JAZ1-GUS reporters support this model. For example, it was shown that JA-induced turnover of JAZ1-GUS requires COI1 and the 26S proteasome, as well as the C-terminal region of JAZ1 (Thines et al., 2007). These results imply that the C-terminal region of JAZ contains the sequence determinants for JA-dependent interaction with COI1.

3 Jasmonoyl-Isoleucine Promotes JAZ-COI1 Interaction

Direct evidence for COI1–JAZ1 interaction has come from both yeast two-hybrid experiments and protein pull-down assays. The latter assay took advantage of a transgenic line of tomato expressing a functional c-Myc-tagged tomato COI1 and recombinant tomato JAZ1 containing a 6x-His tag (Thines et al., 2007). Strikingly, these experiments showed that JA–Ile is highly active in promoting COI1–JAZ1 interaction in a dose-dependent manner. JA–Leu exhibited weak activity in the pull-down assay, whereas non-conjugated jasmonates, including JA, MeJA, and OPDA, were inactive. These findings provide direct evidence that JA–Ile is an active form of the hormone. Subsequent experiments have determined that the JA–Ile-dependent interaction with COI1 is not unique to JAZ1 (Melotto et al., 2008) and that jasmonoyl-valine is a second, highly-active conjugate for the JAZ-COI1 interaction (Katsir et al., 2008), although it is present at low levels in *Arabidopsis* tissues. In principle, it is possible that JA and other non-conjugated jasmonates promote COI1 interaction with other members of the JAZ family, but this seems unlikely on current evidence. With the identification of JAZ proteins as substrates for SCF^{COI1}, protein–protein interaction assays can now be used to study the mechanism of jasmonate perception in vitro. A major advantage of this approach is that it minimizes the extent to which exogenous hormone is metabolized by intact plant cells prior to receptor binding. The experiments of Katsir et al. (2008) indicate that COI1 and JAZ proteins are the receptor for JA–Ile, the active form of jasmonate hormone.

Interestingly, a common splice variant of *JAZ10* (also known as *JAS1*; At5g13220.3) encodes a protein that lacks part of the Jas motif and, when overexpressed in *Arabidopsis*, provides partial resistance to the effects of JA on seedling growth (Yan et al., 2007). Also, the dominant JA-insensitive phenotype of the *jai3–1* mutant is caused by expression of a truncated JAZ3 protein (also known as JAI3) that lacks the Jas-motif-containing C-terminal region (Chini et al., 2007). Characterization of the wild-type and truncated derivatives of JAZ3 showed the C-terminal region interacts with the transcription factor MYC2. This is an important finding because MYC2 activity is central to JA responses in plants, particularly those involved in wounding and defense against pathogen attack (Lorenzo et al., 2004). MYC2 also positively regulates the expression of many of the *JAZ* genes (Chini et al., 2007). The interaction of MYC2 with JAZ3 was proposed to inhibit the activity of MYC2 as a transcriptional activator. Removal of the JAZ repressors via the SCF^{COI1}/26S proteasome pathway, in response to a JA signal, would then allow MYC2 to transcribe early response genes.

4 Alternative Models of Jasmonate Signaling

Chini et al. (2007) reported that, even in the absence of JA, COI1 interacts with the ZIM-motif-containing N-terminal region of JAZ3, but not with the C-terminal region that contains the Jas motif. This observation led them to propose a model in which the C-terminally truncated form

of JAZ3 binds to and inhibits the ubiquitin ligase activity of SCF^{COI1}, thus allowing for the persistence of other JAZ proteins and continued repression of MYC2 activity, even in the presence jasmonate. However, their studies did not address whether the COI1–JAZ interaction that they observed was promoted by jasmonate. In light of other results showing that COI1 and JAZ1 interact in a JA–Ile-dependent manner (Thines et al., 2007), additional work is needed to clearly define the sequence determinants that target JAZ proteins to SCF^{COI1}, and how jasmonate influences this interaction. Recent evidence (Melotto et al., 2008) strongly indicates that the Jas domain is responsible for JA–Ile-dependent binding of both JAZ1 and JAZ9 to COI1 and that residues required for COI1 binding (Arg205 and Arg206 in JAZ1) are distinct from those mediating interactions with MYC2. While there is much to be done, it is clear that discovery of the JAZ proteins has provided researchers with new tools to understand the molecular mechanism of jasmonate signaling.

The emerging picture of jasmonate signaling is remarkably similar to that of the plant hormone auxin. Recent studies have shown that auxin regulates gene expression by stimulating SCF^{TIR1}-ubiquitin-ligase-catalyzed degradation of Aux/IAA transcriptional repressors, and that direct binding of auxin to TIR1 (the auxin receptor) mediates TIR1 interaction with Aux/IAA substrates (Dharmasiri et al., 2005). The x-ray crystal structure of the TIR1-Aux/IAA-auxin complex revealed that auxin binding to TIR1 promotes substrate recruitment by creating a surface that facilitates Aux/IAA binding (Tan et al., 2007). Based on the predicted structural similarity between COI1 and TIR1 (Tan et al., 2007), it is conceivable that JA–Ile acts in a similar way to promote COI1–JAZ binding.

IV Conclusions and Perspectives

Polyunsaturated fatty acids are readily oxidized by activated oxygen species generated by photosynthesis. The polyunsaturated membrane lipids of the chloroplast are largely protected by antioxidant systems, but chemical oxidation does occur to some extent. Enzymatic oxidation by lipoxygenase enzymes initiates several pathways of oxylipin metabolism, including the pathway leading to JA synthesis. JA regulates many aspects of growth, development and environmental responses in plants, particularly defense responses against herbivores and necrotrophic pathogens. Mutants of *Arabidopsis* helped define the biochemical pathway for synthesis of jasmonoyl–isoleucine (JA–Ile), the active form of JA hormone, and demonstrated that JA is required for plant survival of insect and pathogen attacks, and for plant fertility. Transcriptional profiling led to the discovery of the Jasmonate Zim-domain (JAZ) proteins that are repressors of JA signaling. JA–Ile relieves repression by promoting binding of the JAZ proteins to the F-box protein, COI1, and their subsequent degradation by the ubiquitination/26S-proteasome pathway. We now have a much better understanding of the molecular mechanism of JA action, but many questions remain. As these questions are answered, we can look forward to improved knowledge of JA signaling that will provide new tools for efforts to improve crop protection and reproductive performance. Experimental evidence indicates that other oxylipins also have important roles in signaling and plant protection. Future work on the different classes of oxylipins is needed to identify the signaling pathways and chemistry of action through which these compounds contribute to plant growth, development and defense.

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Chapter 19

Biosynthesis and Biotechnology of Seed Lipids Including Sterols, Carotenoids and Tocochromanols

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Summary

This chapter will take a broad look at non-acyl and acyl lipids found in seed. Initially it will focus on certain non-acyl lipids, such as sterols, carotenoids and tocochromanols. It will provide a brief overview of the function of these molecules in plants with a focus on seeds, review information on biosynthetic pathways and attempts at altering quality and quantity of these molecules through metabolic engineering. Our subsequent review of acyl lipid biosynthesis will mainly focus on recent findings related to modification of the pathways providing energy, reductant and carbon for fatty acid biosynthesis that allows for high levels of acyl lipid accumulation during seed filling. This section will have a specific focus on the interaction of primary metabolism and fatty acid biosynthesis. We will then briefly review fatty acid biosynthesis in seed plastids and cytosolic assembly of seed storage and membrane lipids in seed. Finally developmental regulation of seed storage lipid accumulation and transgenic approaches to increase the total oil content of seeds will be summarized.

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I Introduction

A Seed Lipids and their Biosynthetic Origin

Seed lipids in general have a diverse biosynthetic origin. Fatty acids, the building blocks of acyl lipids, are synthesized in plastids using intermediates derived from the sucrose-fed hexose phosphate pool that are channeled towards fatty acid biosynthesis through reactions of glycolysis, oxidative pentose phosphate pathway (and seed photosynthesis). Further desaturation of acyl moieties and assembly of seed storage and membrane lipids requires additional interactions with phosphate and nitrogen metabolism for assembly of lipid head groups. Sterols, on the other hand, are derived from a relatively simple cytosolic pathway that is fed by acetyl-CoA. Other prenyl lipids, such as carotenoids and tocochromanols, are generated by a plastidic pathway that generates isoprenoid monomers from intermediates of glycolysis. Synthesis of tocochromanols also requires intermediates of aromatic amino acid biosynthesis for the biosynthesis of the oxidizable head group.

B Lipid Function

Triacylglycerols provide the seed storage reserve with the highest energy density that is well adapted for dehydration during late stages of seed development. Free and glycosylated forms of sterol lipids have important functions in determining membrane fluidity. Acylated sterols represent storage forms that can provide sterols for membrane insertion. Sterol lipids are also important substrates for synthesis of brassinosteroids, a class of hormones that controls essential aspects of embryo development. Tocochromanols and carotenoids are lipid-soluble antioxidants and/or photosynthetic pigments. Carotenoids serve as precursor in the biosynthesis of abscisic acid, an important regulator of seed development, plant growth and drought responses.

C Objectives and Outline

A large number of recent reviews have covered biochemical and biotechnological aspects of fatty acid biosynthesis, specifically the progress that has been made to generate oil seeds that produce significant amounts of non-native acyl lipids for industrial applications or human nutrition (Jaworski and Cahoon, 2003; Cahoon and Kinney, 2005; Cahoon et al., 2007; Damude and Kinney, 2007, 2008). This chapter will instead focus initially on the biochemistry and biotechnology of certain non-acyl lipids found in seeds and subsequently review acyl lipid biosynthesis in seed in general, its relationship to primary metabolism with a specific focus on new findings related to measurement of photoassimilate flux to acyl lipids and the regulatory mechanisms through which acyl lipid accumulation and mobilization are coordinated with seed development. Finally a brief overview of biotechnological attempts to increase the triacylglycerol (TAG) content of seed that centers around the application of pathway genes, developmental regulators and genes associated with natural variation in TAG content in a given oil seed species will be provided.

The difference in glycerolipid metabolism between oil seeds and vegetative tissues is a beautiful example of the huge biochemical

Abbreviations: ABA – Abscisic acid; ACCase – Acetyl-CoA carboxylase; ACP – Acyl-carrier protein; CRTO – Algal β carotene ketolase; CRTZ – Bacterial β carotene hydroxylase; CRTW – Bacterial β carotene ketolase; BCCP – Biotin carboxyl carrier protein; CRTISO – Carotenoid isomerase; CoA – Coenzyme A; DGAT – Diacylglycerol:acyl-CoA acyltransferase; DHAP – Dihydroxyacetone-3-phospate; ER – Endoplasmic reticulum; FAD – Fatty acid desaturase; GC/MS – Gas chromatography/mass-spectroscopy; GGDP – Geranylgeranyl diphosphate; GAP – Glyceraldehyde-3-phospate; GPAT – Glycerolphosphate:acyl-CoA acyltransferase; HGGT – Homogentisate geranylgeranyltransferase; HMGR – Hydroxymethylglutaryl(HMG)-CoA reductase; HPPD – Hydroxyphenylpyruvate dioxygenase; IPP – Isopentenyldiphosphate; KAS – Ketoacyl synthase; LPAAT – Lysophosphatidic acid:acyl-CoA acyltransferase; MEP – Methylerythritolphosphate pathway; OPPP – Oxidative pentose phosphate pathway; PC – Phosphatidylcholine; Pi – Phosphate; PEP – Phosphoenolpyruvate; PGA – Phosphoglycerate; PDS – Phytoene desaturase; PPK – Phytol phosphate kinase; PDH – Pyruvate dehydrogenase; PK –Pyruvate kinase; QTL – Quantitative traite locus; RuBP – Ribulose-1,5-bisphosphate; RuBisCo – Ribulose-1,5-bisphosphate carboxylase/oxygenase; SAM – S-adenosyl methionine; SMT – Sterol methyltransferase; T-DNA – Transfer-DNA; TCA – Tricarboxylic acid cycle; TAG – Triacylglycerol; VLCFA – Very long chain fatty acid

plasticity generated in response to developmental reprogramming. In a typical vegetative photosynthetic cell, glycerolipids represent a few percent of cell DW and are important building blocks of the membrane systems involved in photosynthesis and oxidative phosphorylation in chloroplasts, mitochondria and other endomembrane systems, such as the vacuole, the nucleus, the ER and the plasma membranes. Acyl lipid biosynthesis in photosynthetic vegetative cells starts in the plastids and continues in the ER, followed by significant trafficking of lipid from the ER back to the plastids (Benning, 2006 et al.). This is also true of non-photosynthetic seeds. However, during oil seed development, the rates of plastidic fatty acid biosynthesis, the transfer of fatty acids from the plastid to the ER and glycerolipid assembly in the ER all increase dramatically, leading to the accumulation of large amounts (40–80% DW) of TAG in cytosolic oil bodies. One of the central objectives of this chapter is to review the current knowledge of the biochemical, and specifically the metabolic, adaptations that occur to facilitate this dramatic increase in flux of glycerolipid biosynthesis during oil seed development.

II Sterols

Phytosterols are cyclic triterpene lipids (C30) that are present in all plant tissues (Piironen et al., 2003; Ryan et al., 2007). Free (phyto)sterols (Fig. 1) have important roles in controlling plasma membrane fluidity and water permeability

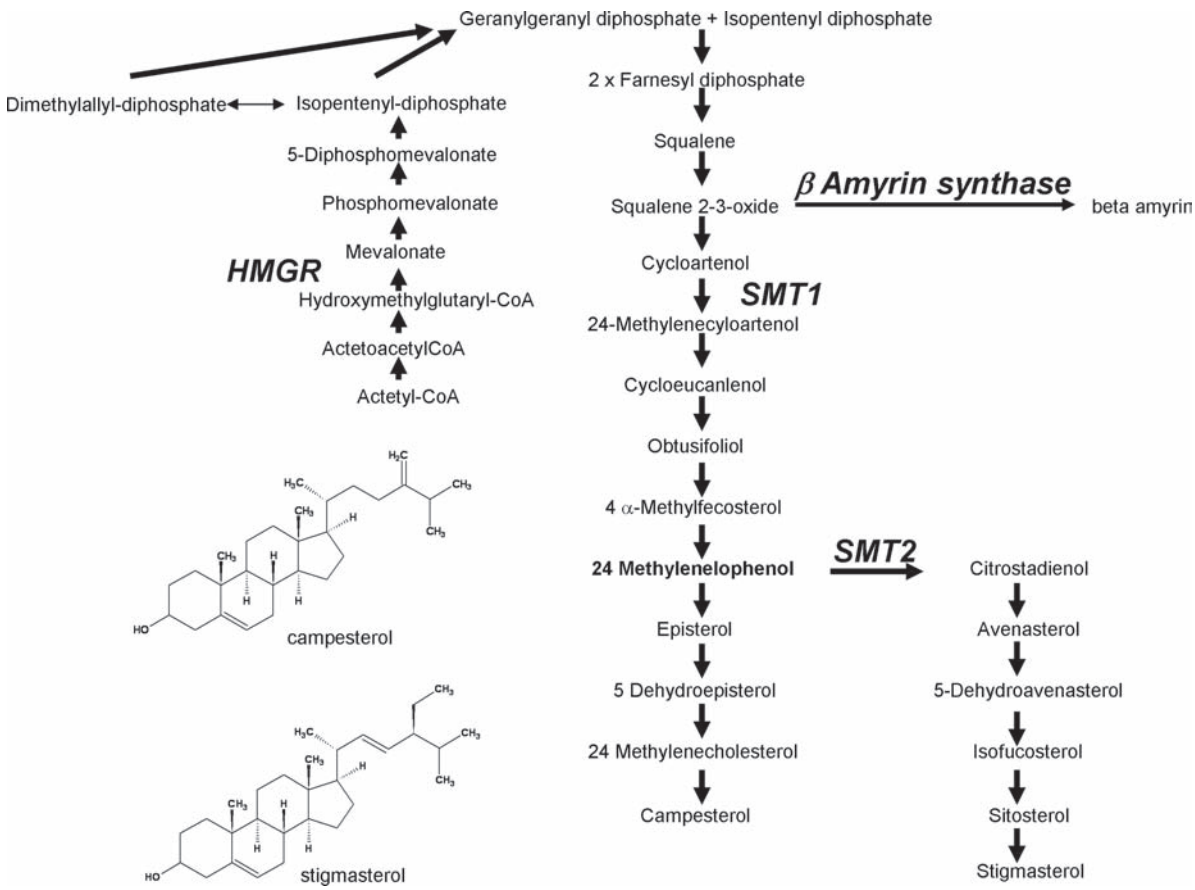

Fig. 1. Simplified pathway of phytosterol biosynthesis. Biosynthetic pathway from acetyl-CoA to the phytosterols, stigmasterol and campesterol. The pathway can be divided into the cytosolic pathway from acetyl-CoA to squalene and the sterol-specific pathway from squalene to campesterol and stigmasterol. Enzyme activities that are discussed in the text are highlighted.

in eukaryotic cells including plants (Schaller 2003, 2004). Levels of membrane-dissolved sterol are tightly regulated and excess sterols are sequestered as sterol acyl esters or other conjugates (Hartmann, 2004; Moreau, 2005). In developing seeds the phytosterol campesterol serves as precursor for the biosynthesis of brassinosteroids, a class of phytohormones that control, among other things, important steps of embryo development (Chory and Savaldi-Goldstein, 2006).

The first phytosterol intermediate, cycloartenol, is synthesized in the cytosol from two molecules of farnesyldiphosphate by a sequence of three enzyme reactions comprised of squalene synthase, squalene epoxidase and cycloartenol synthase. Phytosterol biosynthesis is mediated by a putative complex of ER bound or ER associated proteins in the cytosol. Because of the cytosolic localization of this pathway it was generally assumed that the isopentenyldiphosphate (IPP) and dimethylallyldiphosphate building blocks for phytosterol biosynthesis were mostly derived from the cytosolic mevalonate pathway of isoprenoid biosynthesis that is fed by acetyl-CoA, rather than by the plastidic methylerythritol phosphate pathway (MEP). The cytosolic pathway, and its regulation, is very well understood in yeast and mammalian systems and it is identically preserved in plants. The MEP as a source of isoprenoid precursors is a more recent discovery and this pathway appears to be restricted to higher plants, algae, streptomyces and some strains of bacteria.

It is generally believed that there is little cross talk under normal physiological conditions between the plastidic MEP and the cytosolic mevalonate pathways in the generation of C5 building blocks for phytosterol biosynthesis. Initial support for this conclusion came for early studies with inhibitors of hydroxylmethylglutaryl(HMG)-CoA reductase (HMGR), such as mevinolin, that almost completely abolish phytosterol biosynthesis in radish (Bach and Lichtenthaler, 1983) and subsequent studies in which the consequences of altered flux through cytosolic mevalonate and plastidic MEP on phytosterol biosynthesis was studied. For example, a 50-fold increase in carotenoid levels in transgenic canola seeds expressing bacterial phytoene synthase in the plastid led to reductions in plastidic MEP-derived isoprenoids, such as tocopherols and chlorophyll, but not in seed phytosterols (Shewmaker et al., 1999). However, in a recent study, application of another HMGR inhibitor, lovastatin, to *Arabidopsis* seedlings resulted only in a transient reduction in phytosterol levels and phytosterol content that was quickly recovered in the presence of the HMGR inhibitor (Laule et al., 2003). This suggests that under these non-physiological conditions the plastidic MEP can provide precursors for phytosterol biosynthesis. In support of this observation Bick and Lange (2003) characterized a plastidial proton symport system for *Arabidopsis* leaves that mediates the transport of IPP in the plastid-to-cytosol direction. Further insights into the regulation of mechanisms of IPP exchange between the plastidic and cytosolic compartments was derived from characterization of a mevinolin-resistant mutant in which a knockout of phytochrome B led to up-regulation of HMGR activity and increased transport of mevalonic acid derived precursors from the plastid (Rodriguez-Concepcion et al., 2003). No information is available at this point that demonstrates to what extent isoprenoid precursor exchange between plastid and cytosol, and its regulation, applies to phytosterol biosynthesis in developing seed.

The subsequent steps of phytosterol biosynthesis have recently been reviewed in detail elsewhere (Benveniste, 2004, 2005). Figure 1 shows a simplified, schematic view of the pathway with a focus on phytosterol classes, pathway branchpoints and enzyme reactions that have been the focus of metabolic engineering attempts. Briefly, the first product after cyclization of squalene 2,3 oxide is cycloartenol, a 4-dimethyl sterol. The subsequent pathway of six interconversions consisting of C24-methylations, removal of the two 4-methyl groups, reductions and isomerizations leads to the desmethyl sterol 24-methylenelophenol, which is a metabolic branchpoint intermediate. Methylation of this intermediate by sterol methyltransferase 2 (SMT2), leading to the creation of an alkyl group at C24, is the committed step in the pathway that ultimately leads to biosynthesis of the C24-ethyl sterols β-sitosterol and stigmasterol (Schaeffer et al., 2001). The reactions downstream of this branchpoint intermediate can also use the C24-methylated intermediate in an identical sequence of interconversions, which ultimately provides the C24-methyl sterol campesterol. Campesterol is further utilized as

precursors of phytohormones, such as brassinosteroids.

Phytosterols can accumulate in the free form (dissolved in lipid membranes), acylated to fatty acids or hydoxycinnamic acids and/or conjugated to sugars, glucose (Moreau, 2005 and references therein).

Acylation is clearly the control point that determines the concentration of phytosterol in structural membranes. Fatty acid acylation of phytosterol can be catalyzed by CoA-dependent enzymes. Recently a plant gene, acyl-CoA:sterol acyltransferase, catalyzing the acyl-CoA-dependent acylation of phytosterol was identified (Chen et al., 2007). The enzyme has a preference for palmitoyl-CoA as acyl donor and cycloartenol as acyl acceptor, however, end product phytosterols, such as sitosterol, were also acylated.

An alternative route of phytosterol acylation in which phospholipids act as acyl donors, had been identified previously (Banas et al., 2005). This phospholipid:sterol acyltransferase from *Arabidopsis*, which shares low levels of similarity (<30% sequence identity) to mammalian lecithin:cholesterol acyl transferases was recently cloned and characterized. The enzyme only uses phospholipids, namely phosphatidylethanolamine acyl donors and end product phytosterols as well as phytosterol intermediates as acyl acceptors. Loss-of-function mutations of this gene lead to a 70% reduction of phytosterol esters in vegetative tissues. Sterol metabolism in seeds of this mutant was not studied.

Phytosterols differ from the sterols found in animals and fungi, such as cholesterol, due to the presence of a methyl or ethyl group at carbon 24, which is part of the alkyl side chain (Fig. 1). It is assumed that because of this modification the molecules, when present in the diet, act as competitive inhibitors of cholesterol uptake in the small intestine of humans (Moreau, 2005). It is because of this blood cholesterol-lowering property that metabolic engineering of phytosterols has been the focus of significant efforts in academic and industrial labs.

Phytosterol concentrations in seed oils are usually low, around 0.1–1% of total oil (Ryan et al., 2007). Increasing the phytosterol content of oil seed is a compelling goal because of the existing infrastructure of oil seed processing and the opportunity of delivering bioactive phytosterols as a vegetable oil component without further processing. Initial efforts focused on over expression of plant HMGR genes using constitutive promoters (Re et al., 1995; Schaller et al., 1995). More recently, de-regulated forms of HMGR that lack phosphorylation sites (Hey et al., 2006) or n-terminal domains (Harker et al., 2003b), have been expressed in the cytosol using constitutive or seed-specific promoters.

In one noteworthy example, expression of an n-terminally truncated form of HMGR gene from *Hevea brasiliensis* lead to an 11-fold increase in seed HMGR activity and increased the total phytosterol content of tobacco seeds 2.5-fold to 3.25% of the total oil (Harker et al., 2003b). A doubling of sitosterol levels was accompanied by a fourfold increase of cycloartenol. Furthermore, several pathway intermediates of sitosterol biosynthesis, namely C4 desmethylsterols, were also increased and all the additional phytosterol was acylated. Phytosterol increases in seed have also been achieved through over-expression of SMT1, the methyl transferase at the entry point of phytosterol biosynthesis (Holmberg et al., 2002). This gene was initially cloned from *Arabidopsis* based on an SMT1 mutant (Diener et al., 2000). In this mutant, content of phytosterols downstream of cycloartenol was attenuated but not eliminated, which confirmed that SMT2, at the branchpoint of C24 methyl phytosterols and C24 ethyl phytosterols, could also methylate cycloartenol (Zhou and Nes, 2003). Seed-specific over expression of a SMT1 gene in tobacco led to a threefold increase in SMT activity and a 1.5-fold increase in sitosterol levels. SMT1 expression also caused a decrease in cycloartenol levels, which was associated with an increase of HMGR activity (Holmberg et al., 2002). In both of these successful examples of seed phytosterol engineering, changes in leaf phytosterol levels generated by constitutive expression of these genes differed dramatically from those observed in seed; namely in leaf tissue accumulation of phytosterol intermediates was more pronounced and increases in phytosterol end products were not significant. This observation indicates that developing seeds must coordinately express genes of the phytosterol pathway and are thus well suited for further phytosterol pathway engineering (Harker et al., 2003a). The studies reviewed so far support an important role for HMGR and SMT1 enzymes in

controlling flux through seed-specific phytosterol biosynthetic pathways. Co-expression of both genes using seed-specific promoters was recently reported (Holmberg et al., 2003). This approach increased total phytosterol levels slightly when compared with single gene expression experiments. More importantly, however, HMGR and SMT1 co-expression generated a profile of phytosterol end products that had the closest resemblance to that of unmodified plants. In other words, accumulation of phytosterol intermediates was reduced when compared with seed of lines carrying single gene transgenes.

Other approaches for increasing phytosterol levels in seed have centered on over expression of the acyl-CoA dependent sterol acyltransferase described above (Chen et al., 2007). This led to a 1.6-fold increase in phytosterol levels in *Arabidopsis* seeds accompanied by a reduction in free sterol as well as end products of the phytosterol pathway, such as sitosterol and campesterol, and a 20-fold increase in cycloartenol content. Although this approach was clearly not successful in increasing phytosterol end products in oil seeds, the combination of this gene with other phytosterol pathway genes, such as HMGR or SMT1, may provide a promising approach for achieving significant improvements in seed phytosterol content through increased sequestration of phytosterols.

A third approach to increase phytosterol content in seeds was recently reported in the patent literature by McGonigle et al. (2007). It consisted of suppression of certain pathways, such as saponin biosynthesis, that compete with phytosterol biosynthesis for squalene 2,3 oxide (Fig. 1). The entry point of saponin biosynthesis consists of β-amyrin synthase (Fig. 1). Seed-specific down regulation of this gene in soybean led to a twofold increase in phytosterol content in seed, which was mostly attributed to an increase in sitosterol.

It is clear that current technology of phytosterol pathway engineering can provide two- to threefold increases in the phytosterol levels of oil seeds. Further increases of seed phytosterol levels require a better understanding of the regulation of this complex pathway, which consists of more than 20 mostly membrane-bound enzymes. Although mutants for individual pathway genes are available, most of these have pleiotropic developmental defects, including embryo lethality, because of the effects on brassinosteroid biosynthesis. In contrast, over-expression of individual pathway genes using seed specific promoters did not result in developmental defects, presumably due to the fact that in the developing transgenic plant seed the additional phytosterol is sequestered by acylation and that entry points of brassinosteroid synthesis from phytosterols (campesterol) are presumably under tight control. This situation is ideally suited for the application of enhancer tag based screened for high phytosterol lines. This approach not only provides potential insight into other phytosterol pathway genes that might control flux, it may also allow identification of transcriptional regulators that exert global control over phytosterol biosynthesis in the seed, an area that is not well understood.

III Carotenoids

Carotenoids are tetraterpene lipids (C40) that accumulate in specialized plastid-derived chromoplasts or the thylakoid-membrane system of photosynthetic plastids. Figure 2 shows a simplified pathway of carotenoid biosynthesis present in plastid or plastid-derived organelles of all higher plants. Carotenoids are the second most abundant pigments in nature after chlorophyll. Plant carotenoids can be classified as non-cyclic (phytoene, lycopene) or cyclic (α and β carotene) as well as cyclic and hydroxylated molecules that are called xanthophylls (zeaxanthin, lutein). The latter class can be further classified based on the position of the double bond in two ring structures. ε,β Carotenoids have different double bond positions in the two rings, whereas β,β carotenoids have two ring structures with an identical position of the double bonds that are conjugated with the system of double bonds of the aliphatic core structure of the molecule. Hydroxylated carotenoids, such as lutein and zeaxanthin, have important roles in photosynthesis. Lutein is part of the light-harvesting complex of photosystem II, whereas the xanthophyll zeaxanthin act in non-photochemical quenching (Ruban and Horton, 1995 and references therein) and is an inhibitor of lipid peroxidation in membranes (Krinsky, 1989).

Very specialized pathways of carotenoid biosynthesis lead to high levels of carotenoid

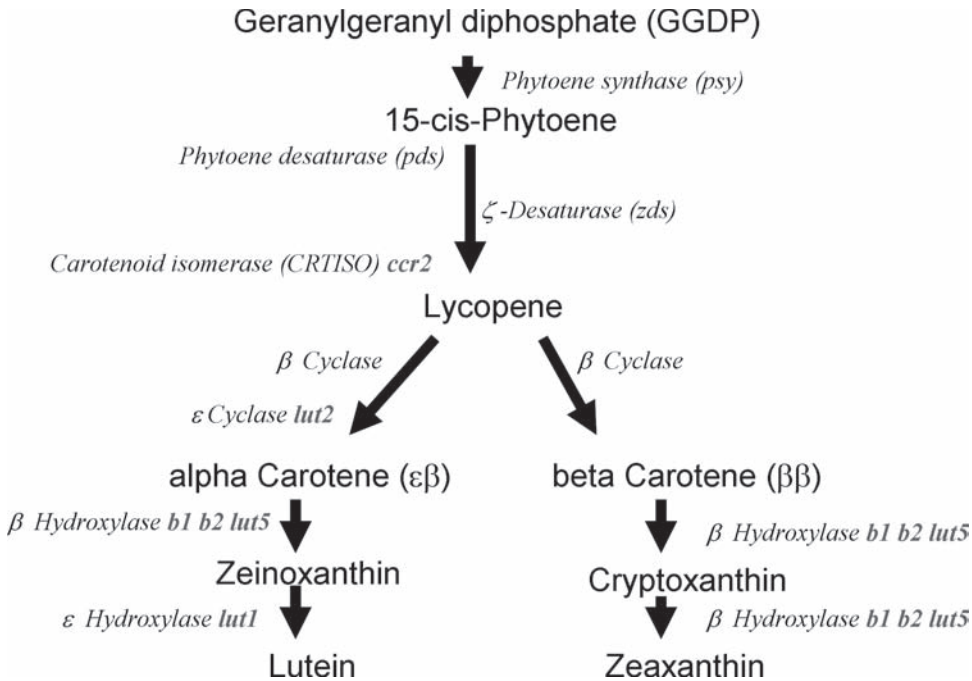

Fig. 2. Carotenoid biosynthetic pathway. Mutant alleles identified by genetic analysis of carotenoid biosynthesis in *Arabidopsis thaliana* are highlighted in red.

accumulation in plastid-derived chromoplasts of highly pigmented organs, such as petals of flowers and fruits (Camara et al., 1995). In these tissues carotenoid levels of 1.6 mg g^{-1} FW (1% DW) are not uncommon (Moehs et al., 2001). Accumulation of carotenoids to these concentrations requires specialized structures for synthesis and storage. In non-photosynthetic, plastid-derived chromoplasts carotenoids are stored in the hydrophobic core of thylakoid-associated plastoglobules (Brehelin et al., 2007; Kessler and Vidi, 2007). The plastoglobules of chromoplasts are plastidic lipid bodies containing free carotenoids, carotenoid acylesters, carotenoid-associated proteins (fibrillins) and other lipids, such as triacylglycerides and phytolesters (Brehelin et al., 2007 and references therein). Recent proteomic analysis of this structure has revealed that most biosynthetic steps of carotenoid biosynthesis are present in the proteome of this suborganellar structure (Ytterberg et al., 2006).

Carotenoid accumulation in dicotyledonous oil seeds is low, usually about a factor of 10 lower than that of photosynthetic leaf tissue. It is also a factor of 10 lower than the accumulation other non-acyl lipids, such as phytosterols and tocochromanols, in the seed (Howitt and Pogson 2006; Wang et al., 2007). Carotenoids found in dry oil seeds are mainly comprised of lutein and may at least be partly derived from the photosynthetic membranes of seed plastids. Despite its role as a minor seed constituent, seed carotenoid biosynthesis is essential for abscisic acid (ABA) biosynthesis and the associated regulation of seed dormancy. This role in growth regulator biosynthesis is illustrated by the viviparous phenotype of maize white kernel/flour mutants that has defects in the carotenoid biosynthesis pathway of the endosperm (Maluf et al., 1997).

The first committed step of carotenoid biosynthesis is catalyzed by phytoene synthase that generates a colorless C40 isoprenoid, phytoene that rarely accumulates in plant cells (Armstrong and Hearst, 1996). The conversion of phytoene to lycopene requires the introduction of four double bonds by the two closely related desaturases phytoene desaturase (PDS) (Sandmann, 1994) and ζ-desaturase (Bartley et al., 1994; Cunningham

and Gantt, 1998), followed by isomerization to the all-*trans* lycopene molecule by a carotenoid isomerase (CRTISO) (Park et al., 2002; Isaacson et al., 2002, 2004). A bifunctional cyclase (β cyclase) generates both β rings found in β-carotene and its derivatives. This enzyme and a distinct ε-cyclase, are responsible for α carotene production (Cunningham et al., 1996; Hirschberg, 2001; and references therein). A non-heme iron protein (β-hydroxylase) catalyzes both ring hydroxylations that are part of zeaxanthin biosynthesis (Sun et al., 1996). An additional distinct ε-hydroxylase (a cytochrome P450-dependent monoxygenase) and the β-hydoxylase mentioned above are required for lutein biosynthesis (Tian et al., 2004). The xanthophyll lutein is an end-product of carotenoid biosynthesis in plants and dominates the carotenoid profile of most oil and cereal seeds. Zeaxanthin is part of the xanthophyll cycle of carotenoid interconversions of non-photosynthetic quenching in photosynthesis consisting of reversible epoxidations and is also a precursor for the biosynthesis of abscisic acid (Hirschberg, 2001 and references therein).

Little genetic redundancy exists for genes of the early steps of the pathway. Consequently loss of-function mutations in these early genes may not be viable because of the essential role of carotenoids in photosynthesis. Global analysis of gene expression in *Arabidopsis* has revealed that the genes of the carotenoid biosynthetic pathway are expressed at low levels during seed development and coordinately with genes of oil biosynthesis (K. Meyer, unpublished observations).

The later steps of carotenoid biosynthesis (isomerases, cyclases and hydoxylases in particular) have been amenable to mutant analysis in *Arabidopsis*. The *lut2* mutant has no ε-cyclase activity and, therefore, does not contain any α-carotene or β,ε xanthophylls (lutein), which suggests that other β,β xanthophylls can function in photosystem II (Pogson et al., 1996). The *ccr2* mutant is defective in CRTISO activity (Park et al., 2002). This blockage has unexpected consequences on light-induced plastid maturation and carotenoid metabolism, namely a 90% reduction in β,ε xanthophyll accumulation in the light and no formation of prolamelar bodies in etioplasts, the plastids of dark-grown plants. This finding suggests a poorly understood role for CRTISO in providing precursors for the α/ε-branch of carotenoid biosynthesis in the light. This is in contrast to a situation where light-induced isomerization would provide all-*trans* lycopene, which could be used equally well by both branches of the pathway. Cuttriss et al. (2007) report reduced levels of the LUT2 transcript in the ccr2 mutant, which may indicate that absence of the CRTISO enzyme or of some of its isomerization products leads to reduced expression of genes of the β-ε branch of the pathway.

Two closely related *Arabidopsis* genes, *B1* and *B2,* are responsible for β-hydroxylation. The double mutant of T-DNA insertion alleles of *B1* and *B2* showed a 75% reduction in flux to β,β xanthophylls and 30% increase in β,ε-xanthophyll (lutein) in leaf tissue without any change in the total accumulation of carotenoids (Tian et al., 2003). In seeds, however, both α and β xanthophylls were reduced and a reduction in total carotenoids of 40% was observed. The ε-hydoxylase (LUT1) gene was isolated by map-based cloning of the *lut1* mutant gene (Tian et al., 2004; Tian and DellaPenna, 2004). A triple knockout mutant *b1/b2/lut1* surprisingly still contained hydroxylated β-β xanthophylls and, as expected, no longer contained xanthophylls with hydroxylated α groups in both leaf and seed tissues (Tian et al., 2003). The residual β-hydroxylation in the triple mutant indicates that a third β-hydroxylase gene, acting in both leaf and seed pathways of carotenoid biosynthesis, is present. This gene, LUT5, a second cytochrome P450-dependent monoxygenase of the pathway, was recently cloned and identified as a paralog of LUT1 that hydroxylates both the single β ring of β-ε carotenes and both β rings of β-β carotenes, albeit with reduced efficiency (Kim and DellaPenna, 2006). A *lut1/lut5* double mutant accumulated non-hydroxylated α-carotene and zeinoxanthin a β,ε carotenoid that only shows β ring hydroxylation (Fiore et al., 2006). This hydroxylation may be carried out by B1 or B2 or by a fifth hydroxylase of the pathway. The triple mutant *b1/b2/lut5* contained no detectable β-β xanthophylls, which indicates that these three genes comprise the total β-β specific hydroxylation of carotenoid biosynthesis (Fiore et al., 2006).

In summary, mutant analysis of the hydoxylases of xanthophyll biosynthesis in *Arabidopsis* has demonstrated that a total of four (and possibly five) hydroxylases are involved in β-ε and

β-β xanthophyll biosynthesis. Unfortunately, with one exception (Tian et al., 2003), the seed carotenoid profile of most of these mutants was not reported.

Increasing the carotenoids content of transgenic plants has led to improvements in stress response under high light conditions (Davison et al., 2002). The utility of transgenic seeds with altered and/or increased carotenoid content is mainly in the nutritional field. Studies have focused on establishing biosynthesis of pro-vitamin A related carotenoids, such as β and α-carotene, to a nutritionally important seed tissue, such as the rice endosperm (Beyer et al., 2002) and the canola embryo (Shewmaker et al., 1999; Ravanello et al., 2003). More recently attempts at seed specific expression of pathways for synthesis of other carotenoids, such as high value keto-carotenoids (asthaxanthin), with special applications in human health and as article of commerce in feed ingredients has been reported (Giuliano et al., 2008 and references therein).

A few examples of successful carotenoid pathway engineering in plants will be summarized. Carotenoid pathway engineering illustrates the advantages and limitations associated with expression of heterologous genes since many genes for carotenoid pathway engineering in plants are derived from carotenoid-producing bacteria. Over expression of the *Arabidopsis* B1 gene in leaf tissue led to a twofold increase in β-β xanthophylls, and the related epoxide intermediates antheraxanthin and violaxanthin of the xanthophyll cycle. This led to increased tolerance to high light and reduced lipid peroxidation in photosynthetic membranes (Davison et al., 2002).

Expression of a plant phytoene synthase and a bacterial gene for phytoene desaturase/isomerase (Misawa et al., 1990), which encodes an enzyme for all four desaturation and the isomerization reaction that are required for generation of all-*trans* lycopene, led to biosynthesis of non hydroxylated β-β carotene (pro vitamin A) in the endosperm of rice seed (Beyer et al., 2002). Use of phytoene synthases from rice or maize proved to be the most efficacious and allowed accumulation of 30 μg g^{-1} DW of β-carotene in rice grain, which is known as GOLDEN RICE 2 (Paine et al., 2005). Low levels of β-ε carotene indicate that the ε-cyclase was limiting in this tissue and low levels of xanthophyll accumulation indicate that in the cereal endosperm the rate of conversion of carotene to xanthophylls by hydroxylation is low, which is a distinct advantage for pro-vitamin A production.

In *Arabidopsis*, seed-specific overexpression of a plant phytoene synthase led to a more pronounced increase in the β carotene branch of intermediates, indicating that ε-cyclase is also limiting when flux is increased (Lindgren et al., 2003). In this experiment β-carotene accumulated to levels of 260 μg g^{-1} in seed. Interestingly, β-β xanthophyll epoxide levels (violaxanthin) levels were also increased in these *Arabidopsis* seed and a low germination phenotype was observed that correlated with the carotenoid increase of a given transgenic line, indicating that ABA synthesis was increased. More recently, in a related study of the expression of a bacterial phytoene synthase in flax seed, a significant increase in total carotenoids (19-fold to 160 μg g^{-1}) was observed. β-carotene, α-carotene and lutein were the major components of this increase (Fujisawa et al., 2008).

In an earlier study in canola, overexpression of a bacterial phytoene synthase led to a spectacular (50-fold) increase in carotenoid levels, mostly in the form of β and α carotenes and no increase in lutein or other xanthophylls (Shewmaker et al., 1999). There was a small build-up in phytoene in these lines, which indicates that the desaturation and isomerization steps early in the pathway were backed-up. Total carotenoid content were 1,000–1,500 μg g^{-1} seed, which is similar to those found in plant tissues specialized for carotenoid accumulation. Ultrastructural analysis of developing seeds indicated that some chloroplast had lost the thylakoid membrane system and instead contained a new inclusion body structure, surrounded by a lipid membrane that may facilitate accumulation of these carotenoids. Moreover, GGDP-derived tocopherols were reduced threefold in dry seed and transient chlorophyll in developing seed was reduced by sixfold, which indicates that MEP derived isoprenoid building bocks were limiting and that that there was no compensatory effect on IPP supply by the cytosolic mevalonate pathway (see above). In a follow-up study, combination of bacterial phytoene synthase, desaturase/isomerase and β-cyclase genes eliminated phytoene buildup and increased the ratio of β to α carotene from 2:1 to 3:1. No further increase in total

carotenoids was observed when three genes were used (Ravanello et al., 2003).

Recently, carotenoid pathway engineering in plants has focused on production of astaxanthin, a high-value xanthophyll for human health and animal feed applications. Astaxanthin is a 4,4′ keto form of zeaxanthin (Fig. 3). It is found in marine bacteria (Johnson and An, 1991; Yokoyama et al., 1994), freshwater algae, such as *Haematococcus pluvialis* (Boussiba, 2000) and *Chlorella zofingiensis* (Del Campo et al., 2004), and certain basidiomycete yeast, such as *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) (Johnson, 2003), as well as certain plants (Cunningham and Gantt, 2005). In bacteria, two genes for bacterial β carotene ketolase (CRTW) and bacterial β carotene hydroxylase (CRTZ) are responsible for introduction of 4,4′ keto groups and introduction of the β hydroxyl groups (Misawa et al., 1995a). The reactions performed by CRTZ are analogous to those of the B1 and B2 enzymes of β,β xanthophyll biosynthesis discussed above.

In *Haematococcus pluvialis*, one of the predominant hosts for commercial fermentative astaxanthin production, astaxanthin acylester accumulates in cytosolic lipid bodies at levels as high as several % DW (Grunewald et al., 2001). A β-C4 oxygenase also sometimes referred to as ketolase, responsible for astaxanthin production was first cloned and characterized by Lotan and Hirschberg (1995), Breitenbach et al. (1996) and Kajiwara et al. (1995). It is an enzyme that is unrelated in sequence and structure to the bacterial CRTW proteins involved in astaxanthin biosynthesis in marine bacteria, such as *Agrobacterium aurantiacum* (Misawa et al., 1995a, b). The algal β carotene ketolase (CRTO) gene has a strong preference for β-carotene, indicating that cantaxanthin, the di-keto version of β-carotene, is an intermediate in astaxanthin biosynthesis. It is localized in these lipid bodies, in contrast to the enzymes of the core carotenoid pathway, discussed earlier, which are found in the thylakoid membrane system of the plastid (Grunewald et al., 2001; Jin et al., 2006). β-Carotene has been identified as an intermediate exchanged between plastids and astaxanthin-containing lipid bodies (Grunewald et al., 2001; Jin and Polle, 2006). Finally a cytochrome P450 dependent enzyme that may act as a bi-functional 4-ketolase/3

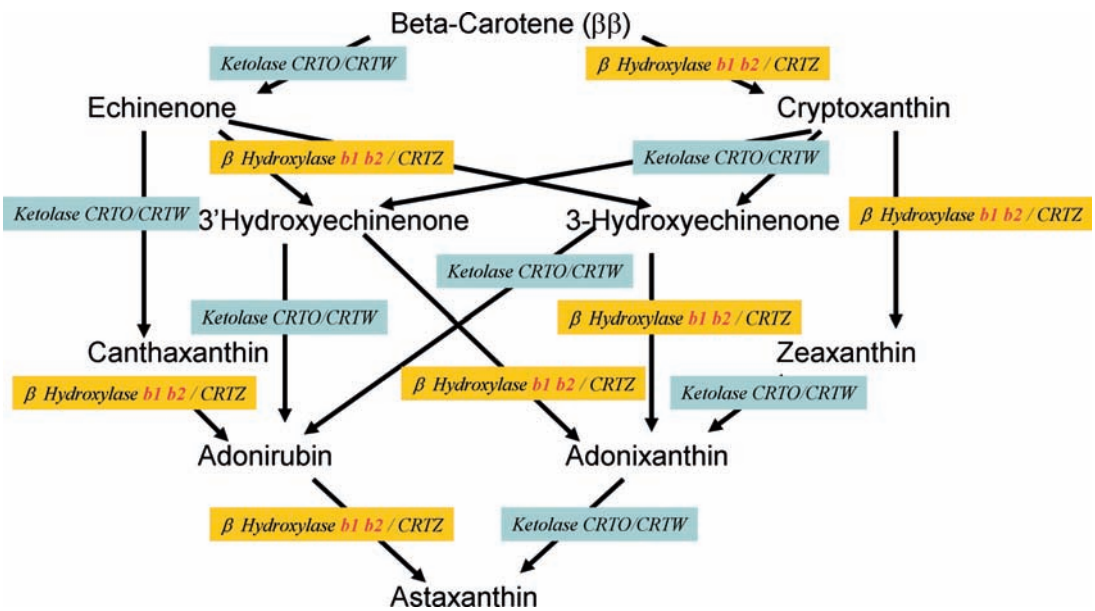

Fig. 3. Metabolic grid of ketocarotenoid biosynthesis. The metabolic grid of possible reactions that are part of astaxanthin biosynthesis is created by the combinatorial action of bacterial (CRTW) or algal (CRTO) ketolase enzymes and endogenous or recombinatly produced β-carotene hydoxylase enzymes (CRTZ, B1, B2). The figure shows intermediates/by-products that are frequently observed when ketolase genes are expressed alone or in combination with bacterial β-carotene hydoxylase enzymes (CRTZ) in transgenic plants (See Color Fig. 18 on Color Plate 15).

hydroxylase was recently cloned from *Xanthophyllomyces dendrorhous* (Alvarez et al., 2006; Ojima et al., 2006; Martin et al., 2008).

Many recent studies have focused on identification and/or design of ketolase enzymes suitable for astaxanthin pathway engineering. After the initial cloning of the first CRTO gene from eukaryotic freshwater algae, other related sequences were identified from prokaryotic sources, such as marine bacteria and cyanobacteria (Fernandez-Gonzalez et al., 1997; Tao and Cheng, 2004; Huang et al., 2006). In these gene-testing efforts ketolase enzymes were usually expressed in *E. coli* cells producing either β-carotene or zeaxanthin. CRTW and CRTO proteins efficiently convert β-carotene to the mono and di-keto forms echinenone and canthaxanthin, respectively. However, some CRTO genes show poor activity in converting zeaxanthin to astaxanthin (Choi et al., 2007). Other CRTO genes, such a recently identified CRTO homolog of *Haemotococcus pluvialis* lacking a plastid-targeting signal (Jayaraj et al., 2008) or a CRTO gene from *Chlorella zofingiensis,* show good conversion of zeaxanthin to astaxanthin (Huang et al., 2006). CRTW gene products usually have dual activity, converting both β-carotene and zeaxanthin to cantaxanthin and astaxanthin respectively (Misawa et al., 1995a, b). However, these latter enzymes have better catalytic parameters (e.g., lower Km and higher Vmax) with not hydroxylated substrates (Fraser et al., 1997).

The first demonstration of astaxanthin production in higher plants used a fusion protein of the CRTO gene from *Haemotococcus pluvialis* with a higher plant plastid-targeting signal sequence under the control of a phytoene desaturase promoter (Mann et al., 2000). Significant ketocarotenoid production was observed in flower (nectary) tissue. Conversion of carotenoids to ketocarotenoids was about 85%. Canthaxanthin and astaxanthin represented about 50% of the total keto-carotenoids. A significant amount (27%) of di-keto mono-hydroxyl and mono-keto di-hydroxyl carotenoids, such as adonixanthin and adonirubin, respectively, accumulated and esterification of ketocarotenoids was observed. Total accumulation of ketocarotenoids was in the range of 300 $\mu g\ g^{-1}$ FW.

In subsequent studies keto-carotenoid expression was targeted to other plant tissues (leaf, flower, seed) and both CRTW and CRTO genes were used (Stalberg et al., 2003; Ralley et al., 2004; Gerjets and Sandmann, 2006; Gerjets et al., 2007; Zhu et al., 2007). Conversion to the end product keto-carotenoids canthaxanthin and astaxanthin was generally very low (less that 10%). In some cases 4-keto-lutein production was observed indicating that the β ring of α-carotenoids can be the substrate of the ketolase (Stalberg et al., 2003). Conversion of endogenous carotenoids to keto-carotenoids led to moderate increases in total carotenoids. Ketolase reaction products are substrates of endogenous hydoxylase enzymes and the interaction between the heterologous ketolase and the β-hydroxylase and acylation enzymes does not allow for significant conversion of carotenoids to cantha- or astaxanthin. Finally, the source or properties of the ketolase is not an important determinant of this conversion rate in transgenic plant tissue since use of both CRTO and CRTW genes have led to about the same degree of success. One notable exception to these observations consists of the expression of a CRTO homolog with activity towards both β carotene and β xanthophylls (zeaxanthin) from *Haematococcus pluvialis* in carrot tap roots (Jayaraj et al., 2008). Constitutive, plastid-targeted expression of this gene in developing carrot tap roots led to high levels of canthaxanthin and astaxanthin production (140 $\mu g\ g^{-1}$ FW), increased expression of endogenous β carotene hydroxylases, and low levels of intermediates. This example shows that the combination of a simple ketolase substrate profile and the presence of chromoplasts for carotenoid accumulation is likely an important determinant of achieving good conversion rates to end-product keto-carotenoids.

In summary, it is clear that carotenoid pathway engineering in seed can increase pro-vitamin-related native carotenoids to levels that are nutritionally efficacious, which is most prominently illustrated by the GOLDEN RICE trait (Beyer et al., 2002; Paine et al., 2005). Production of non-native (keto-)carotenoids in seed or seed-derived oils for subsequent use in large scale ingredient and or large scale feed applications in an economically meaningful fashion is at embryonic stages and is a far more challenging task that requires additional improvements in carotenoid yield and quality. To achieve this it may be necessary to increase both ketolase activity and carotenoid

precursors by increasing flux of the plastid MEP and early steps of carotenoid biosynthesis (Rodriguez-Concepcion, 2006). It is also evident that altering (e.g., simplifying) the native carotenoid profile may be required to limit accumulation of non-desired intermediates. Analysis of *Arabidopsis* mutants with defects in genes controlling hydroxylation and cyclization reactions described above suggest that this could be achieved in the seed of relevant crop plants using tissue-specific RNAi. Perhaps most importantly, a breakthrough in the ability to engineer plastid-derived compartments for the efficient biosynthesis and storage of carotenoids in seed is required. First steps in this area have recently been made with the cloning of the *OR* gene responsible for carotenoid accumulation in non-photosynthetic tissues (Lu et al., 2006). In this mutant, variants of a plastid chaperonin-like protein caused constitutive chromoplast formation, which led to carotenoid accumulation without up-regulation of carotenoid pathway genes. Application of this technology to pathway engineering of carotenoid biosynthesis in seed may allow the production of keto-carotenoid in seed to abundances that are meaningful in the context of commodity chemical production.

IV Tocochromanols

Tocochromanols are a class of prenyl lipids found in all photosynthetic organisms. They are synthesized in the photosynthetic membrane system of blue-green algae and the inner envelop membrane (and/or the thylakoid-associated plastoglobules) of the plastids of all photosynthetic eukaryotes. Tocochromanols have a lipophilic side chain derived from a diterpene (C20) that is saturated, in case of the tocopherols, and desaturated with three double bonds in case of the tocotrienols (Fig. 4). They also have a hydrophilic head group consisting of a methylated and hyrdoxylated aromatic ring adjacent to a cyclic C5-ether structure. As for carotenoids, biosynthesis of tocochromanols depends on the activity of the MEP of isoprenoid biosynthesis, which provides IPP and dimethylallyldiphosphate for GGPP synthesis. The aromatic head group is derived from tyrosine, which is a product of aromatic amino acid biosynthesis by the plastidic shikimate pathway. There are four different classes of tocochromanols (α, β, γ, and δ) that differ in the position and number of methyl-groups on the aromatic ring.

We will briefly review our current knowledge of tocochromanol function in leaves and seed tissues, subsequently focus on a brief review of the biosynthetic pathway of this class of molecules and finally take a more detailed look at metabolic engineering attempts related to this pathway that target increased tocochromanol levels in seed.

The study of mutants of *Synechocystis* and higher plants (*Arabidopsis*) that have defects in tocochromanol biosynthesis has greatly contributed to the understanding of the function of this class of molecules in the plant cell. This body of work was recently reviewed by Doermann (2007) and Maeda and DellaPenna (2007) and will only be summarized briefly. All tocochromanols are excellent inhibitors of lipid peroxidation in vitro. Tocochromanol content of vegetative tissues, such as seedling or leaf tissue, is tightly controlled by stress signals related to temperature, high light or drought that are ultimately related to an increase in oxidative stress. This response is generally characterized by high levels of α-tocopherol accumulation (Munne-Bosch, 2005). The effect of stress on tocochromanol accumulation during seed development is not very well understood. There is a general trend for seeds to be rich in the less-methylated γ-tocochromanol species and it has been observed that seed tocochromanol contents across species correlate with seed oil content.

In support of the association of tocochromanol synthesis with stress responses, mutants of *Arabidopsis* with a complete block in tocochromanol biosynthesis show very subtle photosynthetic defects under low temperature and high light conditions that suggest that an increase in oxidative stress in the mutant is associated with lipid and chlorophyll breakdown (Havaux et al., 2005; Maeda et al., 2006, 2008). Under normal light and temperature conditions, however, no stress phenotype, or reduction in photosynthetic efficiency, was observed. This might indicate that other lipid soluble antioxidants, such as xanthophylls (see above), may substitute for the lack of tocochromanols under some conditions.

Tocochromanol-deficient *Arabidopsis*, maize and potato plants showed reduced rates of phloem loading in source tissue (Provencher et al., 2001; Hofius et al., 2004; Maeda et al., 2006), which led

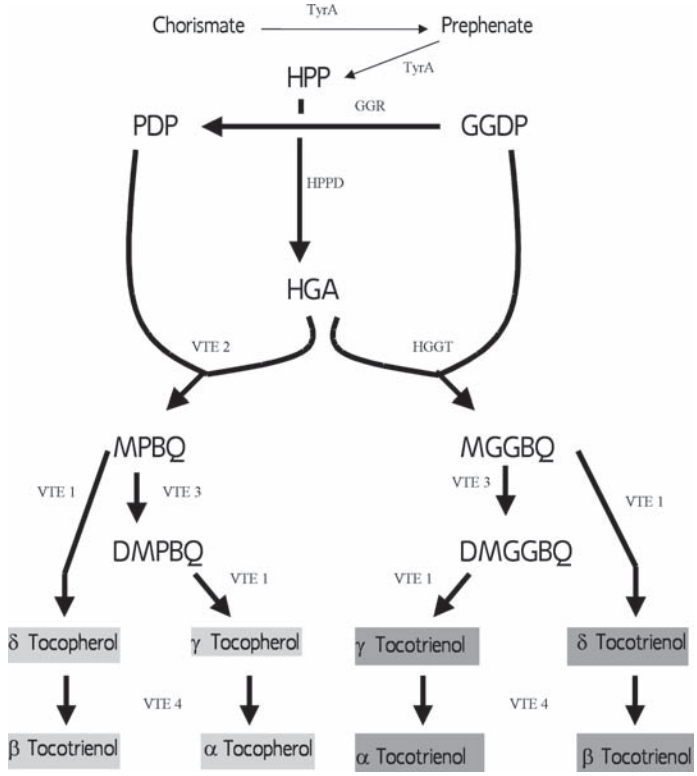

Fig. 4. Pathway of tocochromanol biosynthesis. Phytoldiphosphate (PDP) required for tocopherol biosynthesis can be generated by geranylgeranyl reductase (GGR) or can be synthesized from chlorophyll-derived phytol (P) through the action of phytol kinase (VTE5) and phytol phosphate kinase (PPK). The initial branchpoint of tocochromanol biosynthesis is catalyzed by either VTE2, which directs flux from homogentisate (HGA) to tocopherols, or by HGGT, which directs flux from HGA to tocotrienols. The products of these reactions, 2-methyl-6-phytyl-1,4-benzoquinone (MPBQ) and methylgeranylgeranylbenzoquinol (MGGBQ), can be cyclized by VTE1 to form δ- and β-tocochromanols, or methylated by VTE3 to from dimethylphytylbenzoquinol (DMPBQ) and dimethylgeranylgeranylbenzoquinol (DMGGBQ) and then cyclized by VTE1 to form γ- and α-tocochromanols. The relative abundances of δ- and β-tocochromanols and γ- and α-tocochromanols are controlled by a second methyltransferase, VTE4. HPP, 4-hydroxyphenylpyruvate; PP, phytol phosphate.

to increased levels of sucrose and transient starch in source tissue. This suggests the existence of a, poorly understood mechanism through which tocochromanol molecules (or enzymes involved in their biosynthesis) somehow control carbohydrate metabolism.

Long-term seed viability was reduced in tocochromanol-deficient mutants and non-enzymatic lipid peroxidation during germination was also increased (Sattler et al., 2004). Seed storage lipid is usually confined to specialized oil bodies that up until recently were not known to contain tocochromanols. The observation of decreased oxidative stability of seed storage lipids in vivo and of a related seed viability/germination phenotype of tocochomanol-deficient mutants suggests a site of action for tocochromanols that is outside of the plastid membrane. In keeping with this observation, Fisk et al. (2006) recently showed that purified oil bodies of sunflower seed contain a significant abundance of tocochromanols.

Mutant analysis combined with sequencing of *Synechocystis* and *Arabidopsis* genomes during the last 10 years has greatly facilitated the cloning of all genes involved in tocochromanol biosynthesis in higher plants. In most cases cross-species complementation experiments and map-based cloning of mutant loci in *Arabidopsis* have played a central role in gene identification. The availability of almost all pathway genes now allows a thorough study of the biochemical, cell-biological and physiological aspects of

this important pathway and has led to numerous attempts to increase the tocochromanol content of seed for nutritional applications.

The aromatic head group of tocochromanols is ultimately derived from tyrosine via the action of tyrosine amino transferase (Dixon and Edwards, 2006), which generates hydroxyphenyl pyruvic acid, and *p*-hydroxyphenylpyruvate dioxygenase (HPPD), which generates homogentisate. Alternatively homogentisate can be generated through the action of a heterologous prephenate dehydrogenase/isomerase from yeast or bacteria (Rippert et al., 2004; Karunanandaa et al., 2005). Homogentisate production is also an intermediate of tyrosine degradation (Dixon and Edwards, 2006). HPPD was first cloned based on the *Arabidopsis pds1* mutant (Norris et al., 1998). The related gene did not map to loci related to known genes involved in phytoene desaturation and could be rescued by addition of homogentisate to the medium. HPPD is required for generation of the aromatic head group of tocochromanols and plastoquinones (Norris et al., 1998). The defect in PDS activity in the *pds1* mutant is due to the essential nature of a plastoquinone-containing electron transport chain for phytoene desaturation in the chloroplast (Norris et al., 1995).

Geranylgeranyl reductase generates the saturated phytol side chain for both tocopherol and chlorophyll biosynthesis and plays an important role in tocopherol biosynthesis in vegetative, green tissue (Tanaka et al., 1999). Down-regulation of this gene led to reduced chlorophyll and tocopherol levels in tobacco leaves. Its role in tocopherol biosynthesis in developing seed, however, is in question because of the recent description of the *vte5* mutant of *Arabidopsis* that is defective in phytolkinase (Valentin et al., 2006). This mutant has a more pronounced reduction of tocopherol content in seed tissue compared with leaves indicating that in some tissues, including developing seed, a significant fraction of the isoprenoid side chain of seed tocopherols originates from chlorophyll degradation.

The transferase responsible for transfer of a phytol group from phytol diphosphate to homogentisate (VTE2) was isolated by complementation of a tocopherol deficient *Synechocystis* mutant using candidate genes from *Arabidopsis* (Collakova and DellaPenna, 2001). The protein catalyzes the first committed step of tocochromanol biosynthesis. It is an integral membrane protein with nine membrane-spanning domains localized in the inner envelope membrane of the chloroplast.

The subsequent pathway of tocochromanol biosynthesis is branched. Immediate cyclization of the VTE2 reaction product 2-methyl-6-phytyl-1,4-benzoquinone (MPBQ) by the tocochromanol cyclase (VTE1) is part of a pathway of δ and β tocochromanol biosynthesis whereas methylation of the VTE2 reaction product by the methyltransferase VTE3, prior to cyclization by VTE1, leads to synthesis of γ and α tocochromanols. VTE1 and VTE3 genes were identified by map based cloning using mutant loci of the respective genes (Porfirova et al., 2002; Cheng et al., 2003). VTE1 is an integral membrane protein localized in thylakoid-associated plastoglobules (Vidi et al., 2006). VTE3 is a soluble, plastidic enzyme that also acts in plastoquinone biosynthesis by methylating 2-methyl-6-solanyl-1,4-benzoquinone (MSBQ). Because of the lack of functional plastoquinones in photosynthetic membranes, this mutant (like the *pds1* mutant described above) cannot perform photosynthesis. The last methyl transferase of tocochromanol biosynthesis (VTE4) was cloned by complementation of a *Synechocystis* mutant (Shintani and DellaPenna, 1998). Like VTE3, it is a soluble, possibly membrane-associated, SAM-dependent methyltransferase.

Cloning and characterization of genes encoding all steps of tocochromanol biosynthesis in leaves and developing seed has allowed the study of the orientation of enzymes of the biosynthetic machinery in the plastid. Proteomics of plastid fractions indicates that some enzymes, such as VTE2, are selectively localized to the inner envelope membrane of the plastid whereas others, such as VTE1, are restricted to plastoglobules (Vidi et al., 2006; Austin et al., 2006). This suggests a vectorial structure organized around the phytyltransferase in the inner envelop membrane and the cyclase in plastoglobules that provides tocochromanols to the thylakoid membrane system. It is not clear at this point how the two soluble methyltransferases (VTE3 and VTE4) of the pathway are organized in this model although earlier data indicate that they are associated with the inner envelop membrane (Soll et al., 1985). Nor is it understood how the transfer of tocochromanols to the seed–storage lipid-containing oil bodies of seeds is mediated.

Leaf tocochromanol levels show a high level of plasticity, both in tocochromanol content and in composition, both can change rapidly in response to the oxidative stress status (Doermann, 2007 and references therein). Mutant analysis and global expression analysis of pathway genes indicates the same genes are used for tocochromanol synthesis in seeds as in leaf tissue (K. Meyer, unpublished observations). Notable exceptions include VTE5, the phytolkinase that may be more important for seed tocopherol biosynthesis, and VTE2-related homogentisate:geranygeranyl transferases (HGGTs) that are responsible for tocotrienol biosynthesis in the endosperm of cereals seeds, such as maize, rice, barley and wheat (Cahoon et al., 2003). The latter enzymes are only expressed in seed tissues, where their expression allows utilization of geranylgeranyl side chains for tocochromanol biosynthesis.

Significant progress has been made towards the identification of additional genes, other than pathway genes, that control tocopherol content and profile of seeds (maize, canola). In one example a total of 15 QTL loci were identified that control variation in tocopherol content and/or profile in a recombinant inbred line population of *Arabidopsis*. The mapping location of nine of these QTLs differs from those of known VTE genes (Gilliland et al., 2006).

Pathway engineering of tocopherol biosynthesis in plants can serve different objectives depending upon the intended goal. For example, increasing the tocopherol content in leaf and seed tissue may increase the oxidative stress response of the plant. In this context it would be desirable to increase the α tocopherol content of leaf and seed tissue because this most closely resembles the changes the plant makes in response to stress (Munne-Bosch, 2005).

On the other hands, the tocochromanol species that is currently generated as part of oil refinement during soybean seed crushing and processing is predominantly γ tocopherol. This is mainly used as an additive to purified plant oil in order to increase its oxidative stability. However, this application does not have special requirements related to the actual tocochromanol profile as all eight tocochromanols have fairly similar lipid antioxidant properties when used in vitro (Yoshida et al., 2007) and, therefore, simply increasing total tocochromanol content in soybean seeds would be sufficient for this end.

In contrast to these examples, engineering of tocopherol biosynthesis for nutritional uses in human food and animal feed applications needs to take into account the mechanisms of uptake, metabolism and transport of the tocochromanol molecule to the site of action in the animal (Schneider, 2005). For these uses tocochromanol profiles that are enriched in the most highly methylated α-tocochromanol species have been shown to be most efficacious. In addition these changes need to be targeted exclusively to the seeds of the plant since the nutritionally required doses of tocochromanols can most efficiently be delivered in plant oil or seed in both food and feed applications (Shintani and Dellapenna, 1998).

Numerous metabolic engineering approaches to increase total tocochromanol content or the α tocochromanol content of oil seeds have been described. For example, seed-specific over-expression of VTE4 in oil seeds, such as *Arabidopsis,* canola and soy, can be used to make the nutritionally important α-tocopherol the dominant tocopherol molecule (Shintani and Dellapenna, 1998; Van Eenennaam et al., 2003). In certain oil seeds, such as soybean, co-expression of both methyltransferases, VTE3 and VTE4, is required to direct all the flux to α tocopherol and this strategy also results in moderate increases in the total tocopherol content (Van Eenennaam et al., 2003).

Significant increases in total seed tocochromanol content have been more difficult to attain. This is due to our limited understanding of both the regulation of HPT (VTE2) activity and the source of the prenyl side chain of the tocochromanol molecule. Simple over-expression of HPT (VTE2), the entry point of tocochromanol biosynthesis, is not sufficient to increase flux at all and only moderate (twofold) increases are observed when this approach is combined with geranylgeranyl reductase and HPPD expression (Karunanandaa et al., 2005; Raclaru et al., 2006). Expression of the GGPP-specific HGGT enzymes in developing oil seeds, on the other hand, led to a fivefold increase total tocochromanol levels (Cahoon et al., 2003; Hunter and Cahoon, 2007). This indicates that neither GGPP nor HGA pool sizes per se limit tocochromanol accumulation. Instead it demonstrates that

the bottleneck in tocopherol accumulation is either in the generation or the incorporation of the saturated phytol side chain. Results similar to HGGT expression have also been achieved when homogentisate levels were increased several hundredfold through co-expression of a bifunctional prephenate dehydratase from yeast or bacteria (tyr A) and HPPD (Rippert et al., 2004; Karunanandaa et al., 2005). This result was very surprising and may indicate that a dramatic increase in homogentisate led to an allosteric change in phytol transferase activity and allowed incorporation of GGPP into tocochromanols. One notable study reported that co-expression of tyrA, HPPD and HPT in *Arabidopsis* seed led to a further increase in tocochromanol levels, which suggests that HPT (VTE2) itself may be the source of this allosteric shift (Karunanandaa et al., 2005). Using this tyrA-based approach tocochromanol levels in oil seed have been increased by four- to tenfold. This approach has been further developed by focusing on α tocotrienol accumulation. In this example, over-expression of five enzymes, tyrA, HPPD, VTE2, VTE3 and VTE4, was shown to be required to achieve high levels of α tocotrienol accumulation in oil seeds. In this strategy it was postulated that seed-specific expression of VTE3 was essential to overcome defects in seed development that are usually associated with accumulation of toxic intermediates derived from prenylated homogentisate (Karunanandaa et al., 2005). Alternatively very similar increases in α-tocotrienol content in oil seed have been achieved through the simple co-expression of HGGT with VTE4. This approach does not require manipulation of homogentisate levels and does not suffer from related toxicity issues (Meyer, 2007).

Thus it can be concluded that tocochromanols, in the form of tocotrienols, can be increased several fold in plant oils. However, combining this trait with a high rate of α tocopherol biosynthesis has been more difficult to achieve. The most promising result that may help to overcome this problem is the recent discovery of chlorophyll-derived phytol as an alternative source for tocopherol building blocks in the developing seed.

No results related to the co-expression of phytol kinases (VTE5) with VTE2, 3 and 4 have been reported in the literature to date. The identification of VTE5 as a phytolkinase also identifies phytolphosphate kinase as another potentially important step in seed tocopherol biosynthesis that could be rate-limiting for tocopherol accumulation. Finally, the identification of QTL associated with tocopherol biosynthesis will lead to a better understanding of the regulation of this pathway that may be required to assemble an efficient pathway for α-tocopherol biosynthesis in oil seeds.

V Acyl Lipids

Storage lipid (TAG) accumulation in developing seed provides a highly reduced storage compound with low osmotic potential. The selective advantage associated with accumulation of neutral seed storage lipid is likely dependent on the plant species, the mode of seed dispersal, and the conditions of over-wintering. Neutral seed storage lipid provides the major form of calories in approximately 50% of all plant species.

Fatty acid biosynthesis and TAG accumulation, part of the seed maturation process of seed development, follows cell division for formation of the embryo proper and is followed by desiccation and seed dormancy development (Ohto et al., 2007 and references therein). Our current understanding of the mechanisms of the developmental regulation that controls seed development, and specifically oil biosynthesis, in this process is reviewed later (Section V.C).

A Carbon Supply for Fatty Acid Biosynthesis in Seeds

Biosynthesis and storage of seed storage compounds occurs in a heterotrophic sink tissue from carbon skeletons that are delivered in the form of carbohydrate, organic acids or amino acids. These in turn are delivered to the developing embryo via the endosperm tissue. We will attempt to provide a summary of the current knowledge of the machinery of conversions that feed high rates of fatty acid biosynthesis in the plastids of developing seed (Fig. 5). In doing so we will mainly focus on work conducted with the model plant *Arabidopsis* that has been used to describe the transcriptome of developing oil seed. In addition we will describe steady-state labeling experiments

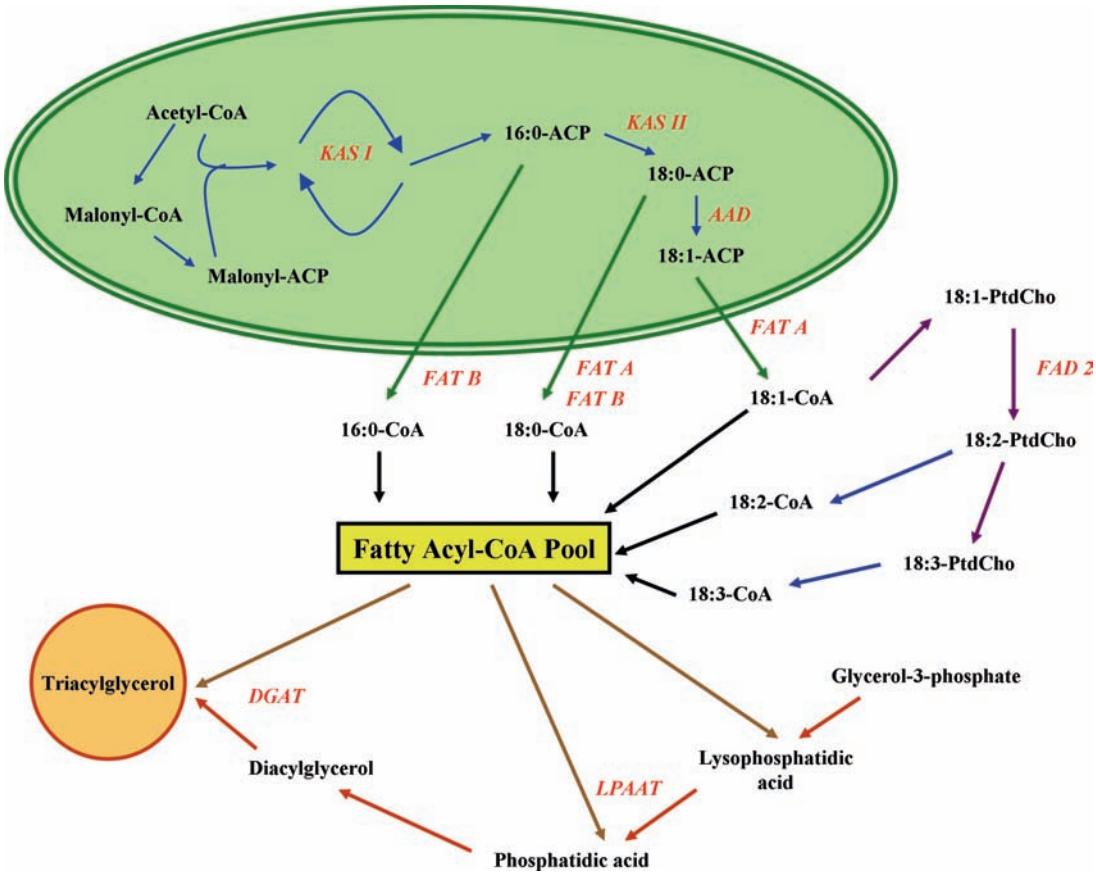

Fig. 5. Oil synthesis in seeds. Fatty acid biosynthesis originates in the plastid where 2-carbon units are extended to 16 and 18 carbons acyl chains while attached to acyl carrier protein (ACP). Acyl chains are released from the plastid by thioesterases to become part of the acyl-CoA pool. Oleoyl-CoA can be incorporated into membranes where it is further desaturated before returning to the acyl-CoA pool. The acyl-CoA pool is used as a source of acyl chains for the reactions of triacylglycerol biosynthesis. The gene families discussed in the text (KAS I, KAS II, AAD, FAT A, FAT B, FAD 2, LPAAT, DGAT) are shown in red at points in the pathway where their gene products have most effect on flux (See Color Fig. 19 on Color Plate 16).

with stable isotopes conducted with developing *Brassica napus* embryos. Both of these plants accumulate more than 40% TAG in their seeds and share closely related genome sequences. The complete annotated genome sequence of *Arabidopsis* along with saturated, sequenced-indexed T-DNA mutant collections and the simplicity of transformation protocols allow for rapid testing of hypotheses derived from global expression analyses or flux measurement experiments. The combination of these approaches provides the most complete picture available about the sources of carbon, energy and reductant that are responsible for the high rates of lipid biosynthesis in the developing seed.

1 Precursors of Fatty Acid Biosynthesis in Photosynthetic Leaf Tissues

Early experiments on fatty acid biosynthesis in leaf tissues used measurements of enzyme activities and labeling experiments of isolated chloroplast to conclude that fatty acid biosynthesis in this tissue involves large (cytosolic) acetate pools, the product of mitochondrial pyruvate dehydrogenase. However, recent isotope labeling experiments using CO_2 in intact *Arabidopsis* plants has demonstrated that the rate of fatty acid biosynthesis is more closely related to photosynthetic activity. Indeed, most of the new fatty acids in leaf tissue are apparently derived from recently

fixed CO_2, and no large acetate pools appear to be involved in the conversion of photoassimilate to oil. Carbon flux likely originates in the C3-C2 pathway and enters oil biosynthesis via pyruvate dehydrogenase in the plastid (Bao and Ohlrogge, 2000).

2 Gene Expression and Activity of Enzymes Involved in Carbon Supply for Fatty Acid Biosynthesis in Developing Seed

We now have good understanding of the network of reactions converting carbohydrate to oil in the heterotrophic, green seed of *Brassica* species and the role of the components of the photosynthetic machinery in this process, which is discussed in detail below. The first detailed picture of the molecular components of this system was derived from extensive EST sequencing of developing *Arabidopsis* seed (White et al., 2000; Mekhedov et al., 2000; Beisson et al., 2003) and global expression profiling (Girke et al., 2000; Ruuska et al., 2002). More recently Baud and Graham (2006) have used in situ staining with dyes for NAD, NADP and FAD-dependent enzyme reactions, in direct or coupled assays, to measure the enzyme activities of carbohydrate metabolism in developing *Arabidopsis* seeds.

Both activity staining of enzymes and expression analysis of genes involved in seed filling have demonstrated that oil biosynthetic enzymes in *Brassica* follow one of three general patterns during seed fill. Thus oil biosynthesis enzymes may follow a pattern of early expression and significant decline during oil accumulation, a bell-shaped pattern with high levels of expression at the peak of oil biosynthesis or a pattern of steady increase from the early to the late stages of seed filling.

Expression analysis of a sucrose/H+ transporter (SUC2) showed there were two peaks at the early stages of *Arabidopsis* embryo development and also during high rates of fatty acid biosynthesis at the embryo maturation stage. More recently Baud et al. (2005) showed that a second sucrose transporter gene (SUC5,) with endosperm localization, showed the highest expression early in seed development. Loss of function mutations in this latter gene led to a transient decrease in oil biosynthesis. Clearly, then, there is a redundancy of sucrose/H+ transporter gene activity during oil seed filling.

After uptake by the embryo, sucrose is mobilized for incorporation into seed storage products through the action of sucrose synthase, or invertase. There are six sucrose synthase genes in the *Arabidopsis* genome (Baud et al., 2004; Bieniawska et al., 2007) and at least four of these are expressed in developing seeds (Bieniawska et al., 2007). Ruuska et al. (2002) showed that sucrose synthase *SUS2* gene expression reached maximal expression in coordination with oil accumulation and not at the early stages of seed filling. More recently a detailed picture of the role of sucrose synthase in seed filling was provided by Baud and Graham (2006), who showed that sucrose synthase activity is indeed induced in seed development during oil accumulation and is the combined product of the expression of two sucrose synthase genes, *SUS2* and *SUS3*. These genes have complementary expression patterns, one is expressed early in oil biosynthesis and one later.

The relatively late expression of sucrose synthase genes in seed development supports the generally assumed role of invertase in sucrose mobilization at the very early stages of seed development in oil seeds. In keeping with this hypothesis, glucokinase activity staining is high at early stages of seed development and is absent in the *wri1* mutant that has a defect in global regulation of seed glycolysis and certain aspects of seed oil biosynthesis (Focks and Benning, 1998).

Oil and protein biosynthesis is preceded by transient starch accumulation in oilseeds. The role of transient starch as a carbon source for seed oil biosynthesis is supported by the low oil phenotype of the *Arabidopsis pgm1*mutant. This mutant has a non-functional plastidic *PGM* gene for phosphoglucomutase (Periappuram et al., 2000). Further confirmation of the role of this starch pool was observed in the developing seeds of transgenic *Brassica* lines with reduced expression of ADP-Glc pyrophosphorylase since these seeds also showed a corresponding reduction in transient TAG accumulation (Vigeolas et al., 2004). Furthermore, the pattern of gene expression and/or activity staining of other enzymes involved in plastidic starch biosynthesis or mobilization, such as plastidic phosphoglucomutase, ADP-Glc pyrophosphorylase, β amylase and isoamylase, is also early and transient.

Cytosolic and plastidic pyruvate kinase (PK) genes show distinct early and later, seed filling-

related gene expression, respectively. This suggests distinct roles for pyruvate biosynthesis by the cytosolic and plastidic glycolytic pathways of oil biosynthesis.

The early expression pattern of the plastidic glucose-6 phosphate transporter, and the seed filling/oil biosynthesis-associated, later expression of the PEP/Pi translocator, confirms the notion that early seed filling is characterized by sucrose import, sucrose mobilization and conversion to starch. Indeed, the later stages of active fatty acid biosynthesis and oil accumulation are characterized by high expression of glycolytic genes, plastidic uptake of PEP and acetyl-CoA biosynthesis by PK and PDH. In further agreement with this, it was found that expression of plastidic PDH closely follows that of plastidic PK. Properties of plastidic and mitochondrial PDH enzymes and of genes encoding subunits of this multi-enzyme complex have been reviewed recently by Tovar-Mendez et al. (2003).

Very recently, two independent studies further investigated the role of members of the *Arabidopsis PK* gene family in seed oil biosynthesis (Andre et al., 2007; Baud et al., 2007b). Plastidic PK is a heterooctamer of four α and four β subunits. Andre et al. (2007) used recombinantly produced subunits to clarify subunit composition of the plastidic PK protein and measured kinetic properties of the reconstituted heterococtamers. Three *Arabidopsis PK* genes encode subunits with plastidic localization and show expression in developing seed. They comprise one α subunit gene and two β subunit genes. A T-DNA loss-of-function mutant in the β1 subunit that was co-expressed with genes of fatty acid biosynthesis during *Arabidopsis* seed development has been characterized. There was a reduction in oil and protein content in the T-DNA mutant of 60% and 16%, respectively and a 25% reduction in seed weight. The content of sucrose and starch at late stages of seed filling are increased 2.5- and threefold, respectively and elongation (C22:1 content) and desaturation (18:3 content) of fatty acids found in seed storage lipids was also increased. This latter observation was presumably the result of a reduced fatty acid biosynthesis rate that altered the partitioning of fatty acids in ER localized desaturation, elongation or triacylglyceride assembly processes.

In summary, there is clear evidence derived from global gene expression analysis, enzyme activity measurements and genetic analysis supporting the central role of PEP uptake and the plastidic pathways of acetyl-CoA generation providing the carbon for fatty acid biosynthesis in the developing oil seed. This pathway does not provide significant precursors for seed storage protein biosynthesis. In addition, alternative pathways of acetate biosynthesis via ATP-citrate lyase from mitochondrial precursors, or pyruvate biosynthesis via cytosolic PK, cannot compensate for the absence of this pathway.

3 Sources of Carbon Energy and Reductant for Fatty Acid Biosynthesis in the Developing Seed

The general concepts of metabolism in non-photosynthetic plastids, including those from developing seed, have been reviewed by Neuhaus and Emes (2000), Rawsthorne (2002) and Tetlow et al. (2005). We will here focus exclusively on recent findings derived from metabolic flux analysis related to the contribution of plastidic, mitochondrial and cytosolic pathways in the generation of energy, reductant and carbon units for fatty acid biosynthesis in developing seed (Schwender and Ohlrogge, 2002; Schwender et al., 2003, 2004a, 2006; Sriram et al., 2004; Schwender, 2008). The experimental approaches and theoretical concepts of flux analysis and modeling have been extensively reviewed and will not be described in detail (Schwender et al., 2004b; Fernie et al., 2005; Ratcliffe and Shachar-Hill, 2006; Schwender, 2008).

Schwender and Ohlrogge (2002) designed a system of isotope labeling for developing *Brassica napus* embryos at steady-state conditions, using a medium that closely resembles the sugar, amino acid and organic acid composition of endosperm liquid. A certain ratio of fully ^{13}C labeled and unlabeled sugars was found to be preserved in both oil and protein fractions after labeling, indicating no contribution of atmospheric CO_2 fixation to storage compound accumulation. The role of the oxidative pentose phosphate pathway was investigated by feeding sugars labeled in the C1-C2 position followed by analysis of the C13 isotopomer pattern of fatty acids and pyruvate by GC/MS. This analysis showed

that during sugar breakdown 90% of the C1-C2 connectivity is preserved, indicating that only 10% of the sugar is metabolized through the oxidative pentose phosphate pathway (which would result in a breakage of the C1-C2 bond by glucose-6 phosphate dehydrogenase)

Addition of unlabeled amino acids (Asp, Gln) to uniformly labeled sugars did not dilute the label found in fatty acids (up to and including C18) that are synthesized in the plastid. However, ^{13}C label in the terminal C2 unit of C20 and C22 long chain fatty acids, which are significant constituents of *Brassica napus* and *Arabidopsis* storage lipids, was diluted by carbon derived from the added amino acids. C20:1 and C22:1 fatty acids are synthesized in the cytosol by an ER-localized fatty acid elongase complex that uses malonyl-CoA generated from cytosolic acetyl-CoA by homomeric acetyl-CoA carboxylase (ACCase) (Harwood, 2005). The authors propose a pathway in which ^{13}C labeling in acetyl-CoA derived from cytosolic pyruvate is diluted after mitochondrial uptake of the latter, is converted to acetyl-CoA by mitochondrial PDH and then used for citrate biosynthesis by mitochondrial isocitrate synthase. Citrate is used for acetyl-CoA biosynthesis by cytosolic ATP:citrate lyase. Unlabelled carbon from the amino acids Asp and Gln enters this pathway in the form of TCA cycle intermediates. Labeling studies with unlabelled malate and fully labeled sugars showed no dilution of oil or protein labeling by malate. Hence there is no evidence for a role of malate in carbon supply during seed filling.

Taken together one may conclude from these findings that fatty acid biosynthesis in oil seed is fed by independent plastidic and cytosolic acetyl-CoA pools. The former is the major contributor to plastidic fatty acid biosynthesis, which occurs via glycolytic conversion of hexoses and without significant contribution of the oxidative pentose phosphate pathway.

Schwender et al. (2003) further confirmed these findings by development of a flux network for developing *Brassica napus* seed, additional labeling experiments with stable isotopes under steady state conditions that included NMR analysis and extensive, iterative steady-state flux modeling (Schwender et al., 2003). The authors determined a glycolysis/OPPP split of 0.12 for the flux of hexoses during oil seed filling and calculated that the OPPP can only supply 25–45% of the NADPH demand of the developing seed. The authors proposed that glycolysis, PDH, the light reactions of photosynthesis and mitochondrial metabolism act as alternative sources of reductant for fatty acid biosynthesis and suggested that transhydrogenase reactions and transport mechanisms for NAD(P)H across plastid membranes generate the correct equilibrium of NADH and NADPH reductant for fatty acid biosynthesis.

What then is the contribution of photosynthesis to fatty acid biosynthesis in oil seeds? Global expression profiling have suggested high levels of expression of key enzymes of photosynthesis, such as the small subunit of RuBisCo and photosystem components, in developing *Arabidopsis* seeds (White et al., 2000; Ruuska et al., 2002). More recently Ruuska et al. (2004) have showed that developing *Brassica napus* seeds contain photosynthetic pigments that resemble the profiles found in leaves adapted to perform photosynthesis under low light conditions (shade response). Moreover, both developing *Brassica* and soybean seeds contain an active RuBisCo that is unexpectedly highly carbamylated, as well as significant levels of phosphoribulokinase activity required for the generation of the CO_2 acceptor ribulose-1,5-bisphosphate (RuBP). The high degree of carbamylation under low light conditions inside the seed pod is caused by high concentrations of CO_2 in the seed generated from active fatty acid biosynthesis (Goffman et al., 2004). In the experiments described by Ruuska et al. (2004) *Brassica* pods were partially covered and oil and carbohydrate accumulation were compared in light- or dark-exposed developing embryos. Elimination of light reduced both oil accumulation and incorporation of ^3H into fatty acids from ^3H_2O. Sucrose levels were increased in seed derived from the covered portion of the pod, indicating that elimination of light during embryo development did not reduce carbon supply for fatty acid biosynthesis and instead reduced rates of fatty acid biosynthesis from externally supplied carbohydrates. In summary, Ruuska et al. (2004) propose the following roles for photosynthesis in developing seed:

1. *Generation of oxygen, reductant and ATP*: Rates of O_2 evolution of developing *Brassica* embryos and an estimation of the intensity of light exposure of

developing *Brassica* embryos were used to calculate amounts of NADPH and ATP attributable to seed photosynthesis that were in twofold excess of the demands from fatty acid biosynthesis.

2. *Re-fixation by RuBisCo of CO_2 generated by plastidic PDH*: Previous work had suggested an important role of PEP carboxylase in this process (King et al., 1998). However, the captured carbon would only be available for storage in protein because generation of acetyl-CoA for fatty acid biosynthesis from malate by malic enzyme via pyruvate would lead to loss of CO_2. Moreover, Schwender et al. (2003) concluded from the amino acid composition of *Brassica* seeds that incorporation of oxaloacetate-derived amino acids could only capture a small portion of the released CO_2 and they did not observe export of malate or related organic acids from the embryo during seed filling. Instead Ruuska et al. (2004) propose a central role of RuBisCo in the re-fixation of CO_2 generated during acetyl-CoA biosynthesis. These authors assume that CO_2 saturation in developing seed eliminates the RuBisCo oxygenase reaction and related photorespiration and that a calculated rate of CO_2 fixation at 50% saturation of the RuBisCo active site with RuBP would still exceed the rate of CO_2 liberation by PDH at mid stage of seed filling.

This model was very elegantly validated experimentally using stable isotope labeling and flux modeling methodology in combination with mass balance and enzyme activity measurements (Schwender et al., 2004a). It was further explored and confirmed by Goffman et al. (2005). In this latter study stable isotope labeling showed that, during conversion of photosynthate to oil, the ratio of carbon incorporated to CO_2 released for developing *Brassica* embryos was 4:1. This is significantly better than the calculated ratio of <2:1 when CO_2 release by PDH, OPPP and the TCA cycle are taken into account. The CO_2 re-fixation pathway via RuBisCo was also examined in this study. Incorporation of isotope label from externally supplied CO_2 or from C1-labeled alanine, which releases labeled CO_2 internally, led to the production of only C1 labeled phosphoglycerate and a very low incorporation of label into oil. This observation is consistent with a pathway of CO_2 re-fixation that uses RuBisCo and generates ribulose bisphosphate (RuBP) via the reductive branch of the pentose phosphate pathway, without the reactions of the Calvin cycle. In this novel pathway, glyceraldehyde-3-phosphate generated by glycolysis is used for generation of RuBP that is converted to PGA after CO_2 fixation, thus bypassing the glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase reactions of glycolysis. Compared with normal glycolysis, carbon flow through this pathway would lead to an increase in the conversion of carbon from hexose to oil by 20% and a 40% reduction in CO_2 release. Further, there is a linear relationship between light intensity and carbon efficiency during *Brassica* seed filling that indicates that the operation of this pathway depends on the provision of reductant (NADPH) and ATP by photosynthetic electron transport. The discovery of this pathway provides a dramatic advance in the understanding of the carbon economy of seed storage lipid biosynthesis in oil seed.

Finally, very recently, Schwender et al. (2006) used stable isotope labeling techniques with ^{13}C and ^{15}N labeled amino acids and/or ^{13}C labeled sugars, along with steady-state flux modeling, to study the role of mitochondrial metabolism in the carbon and energy supply for oil biosynthesis. Their findings can be summarized as follows:

1. There is no cyclic, respiratory flux through the TCA cycle during seed development. The energy (ATP) generated by mitochondrial substrate oxidation and oxidative phosphorylation would provide only 22% of the total energy demand for oil and protein biosynthesis in the developing embryo.
2. There is no significant flux through a PEP carboxylase-mediated anapleurotic pathway of carbon skeleton generation for amino acid biosynthesis. Instead, because of the reversibility of the iso-citrate dehydrogenase reaction, there appears to be net consumption of glutamic acid.
3. Forty percent of mitochondrial pyruvate is generated by malic enzyme.
4. In further support of earlier models (Schwender and Ohlrogge, 2002), the central role of mitochondrial metabolism in oil biosynthesis is the generation of carbon (acetyl-CoA) for cytosolic fatty acid elongation. Acetyl-CoA for this process is generated by the cytosolic ATP:citrate lyase. Carbon for plastidic fatty acid biosynthesis is provided exclusively by the plastidic pyruvate kinase enzyme (75%) and the uptake of pyruvate generated in the cytosol (25%).

Comparison of these findings with results from steady state labeling experiments of developing

soybean embryos (Sriram et al., 2004) reveals that, in soybean, there is also a lack of evidence for an anapleurotic flux of oxaloacetic acid into the TCA cycle. In contrast to *Brassica* though there appears to be no export of acetyl-CoA via citrate and cytosolic ATP citrate lyase. This is in agreement with the low rates of cytosolic fatty acid elongation in soybean embryos that contain only trace levels of C20 and C22 fatty acids in seed storage lipid. Interestingly there is a significantly higher rate and contribution of mitochondrial substrate oxidation and oxidative phosphorylation in the provision of energy for biosynthesis of seed storage compounds in developing soybean embryos.

Most recently Alonso et al. (2007) applied similar labeling and flux modeling methods to the analysis of carbon energy and reductant metabolism during sunflower embryo development. Their findings provide the most detailed picture of the metabolic processes related to oil biosynthesis in a non-photosynthetic seed to date and can be summarized as follows:

1. Hexoses and Gln represent 80% of the amino acid and carbohydrate pools used during seed filling.
2. Greater than 90% of the carbon used for fatty acid biosynthesis is derived from hexose-derived triose phosphates that are taken up by the plastid.
3. The observed high rate of plastidic OPPP flux can provide all the NADPH required for fatty acid biosynthesis and there is no significant rate of malate uptake by the plastid or related NADPH generation by plastidic malic enzyme.
4. Carbon efficiency during seed filling in sunflower is 50%, similar to that of other heterotrophic tissues, and close to the 55% value observed by Sriram et al. (2004) in developing soybean embryos. This is significantly lower than the 80% carbon efficiency calculated for *Brassica* seed filling (Goffman et al., 2005).
5. Both sunflower and soy most likely use a more "conventional" path for carbon mobilization than *Brassica*, however, different patterns of TCA, OPPP and PDH utilization during seed filling are observed between sunflower and soybean. In sunflower, 70% of the carbon that is released as CO_2 is derived from TCA cycle activity, in contrast to 14% each for OPPP and PDH reactions. This underlines the important role of the TCA, cycle in this plant. In soybean on the other hand, 40% and 34% of carbon are released by OPPP and PDH reactions, respectively. This supports the important role of these pathways in soybean seeds to provide reductant while the energy to drive the process is likely derived from the light reactions of photosynthesis.

In summary, a breakthrough in the understanding of fluxes of pathways providing reducing power, energy and carbon during storage lipid biosynthesis in oil seed can be attributed to the recent deployment of isotope labeling and flux modeling methodology to this aspect of plant metabolism. In all plant systems investigated so far acetyl-CoA for fatty acid biosynthesis appears to be generated in the plastid. The relative contribution of plastidic and cytosolic reactions of glycolysis is not very clear in sunflower and soy. In *Brassica*, however, a modified pathway of plastidic glycolysis allows re-fixation of CO_2 released by PDH and is central to fatty acid biosynthesis. Finally, there is a negative correlation between oil and protein content of seeds, long observed by breeders selecting for these traits in common oilseed crops, such as soybean (Burton, 1985). This negative correlation has been the subject of significant QTL mapping efforts. However, no immediately obvious control point in the partitioning of carbon between oil and protein emerges from our current understanding of flux during oil seed filling. Instead it the current flux models emphasize the relative abundances of certain carbohydrates and amino acids provided by source organs as important determinants of this aspect of oil seed composition (Hernandez-Sebastia et al., 2005).

B Fatty Acid and Glycerol Lipid Biosynthesis in Seeds

Fatty acid and glycerolipid biosynthesis in seeds, and the genetic modification of seed lipid biosynthetic pathways, has been extensively reviewed in recent years (Voelker and Kinney, 2001; Thelen and Ohlrogge, 2002; Graham et al., 2007; Damude and Kinney, 2008). In this section we will focus on the essential differences in glycerolipid and fatty acid biosynthesis in seeds from that of non-seed tissue (Table 1).

The enzymology of glycerolipid synthesis in non-photosynthetic seeds (Fig. 6) is similar to that in non-seed tissues with one notable exception; many seeds synthesize large quantities of

Table 1. Selected examples of genetically modified seeds with altered lipid quality or quantity.

Seed lipid class	Phenotype	Gene(s)	Plant species	Reference
Sterol	Increased sitosterol	HMGR	Tobacco	Harker et al., 2003b
	Increased sitosterol	SMT1	Tobacco	Holmberg et al., 2002
	Increased sitosterol	HMGR/SMT1	Tobacco	Holmberg et al 2003
	Increased total sterol, cycloartenol	ACAT	*Arabidopsis*	Chen et al., 2007
	Increased sitosterol	β-amyrin synthase	Soybean	McGonigle et al., 2007
Carotenoid	Increased total carotenoid, mainly β and α-carotene, some phytoene	PSY	Canola	Shewmaker et al., 1999
	Increased β-carotene, α-carotene, phytoene, lutein, violaxanthin	PSY	*Arabidopsis*	Lindgren et al., 2003
	See Shewmaker et al., 1999, increased β/α-carotene ratio	GGDP synthase, PSY, CRTI, β-cyclase	Canola	Ravanello et al., 2003
	Increased β-carotene	PSY,CRTI	Rice endosperm	Beyer et al., 2002
	Increased β-carotene	PSY,CRTI	Rice endosperm	Paine et al., 2005
	Increase ketocaroteinoids (ketolutein > canthxanthin > adonirubin)	PSY, CRTO	*Arabidopsis*	Stahlberg et al., 2003
	Increased total carotenoids, β and α-carotene, lutein	PSY	Flax	Fujisawa et al., 2008
Tocochromanol	Increased α- tocopherol, no increase in total tocochromanol	VTE4	*Arabidopsis*	Shintani and DellaPenna, 1998
	Increased α- tocopherol, no increase in total tocochromanol	VTE3, VTE4	Soy	Van Eenennaam et al., 2003
	Increase in total tocochromanol, mostly γ-tocotrienol	HGGT	Maize	Cahoon et al., 2003
	Increase total tocochromanol, mostly α-tocotrienol	TyrA, HPPD, VTE2, VTE3, VTE4,	*Arabidopsis*, Canola, Soy	Karunanandaa et al., 2005
	Increased tocopherol	HPPD, VTE2, VTE1	Canola	Raclaru et al., 2006
Triacylglycerol (oil)	Increase in oil content of seed	ACCase (homomeric, plastid-targeted)	Canola	Roesler et al., 1997
	Arabidopsis and canola: increase in oil content, increased VLCFA	LPAAT	*Arabidopsis*, Canola	Zou et al., 1997; Taylor et al., 2002
	Increase in oil content, reduction in sucrose	GPD	Canola	Vigeolas et al., 2007
	Increase in oil content	DGAT1	*Arabidopsis*	Jako et al., 2001
	Increase in kernel oil content, increased oleic acid content	DGAT1	Maize	Zheng et al., 2008
	Increase in oil content, reduction in insoluble carbohydrate	DGAT2	Soy	Lardizabal et al., 2008

Abbreviations: CRTI, bacterial phytoene desaturase/isomerase; GPD, glycerolphosphate dehydrogenase; PSY, phytoene synthase.

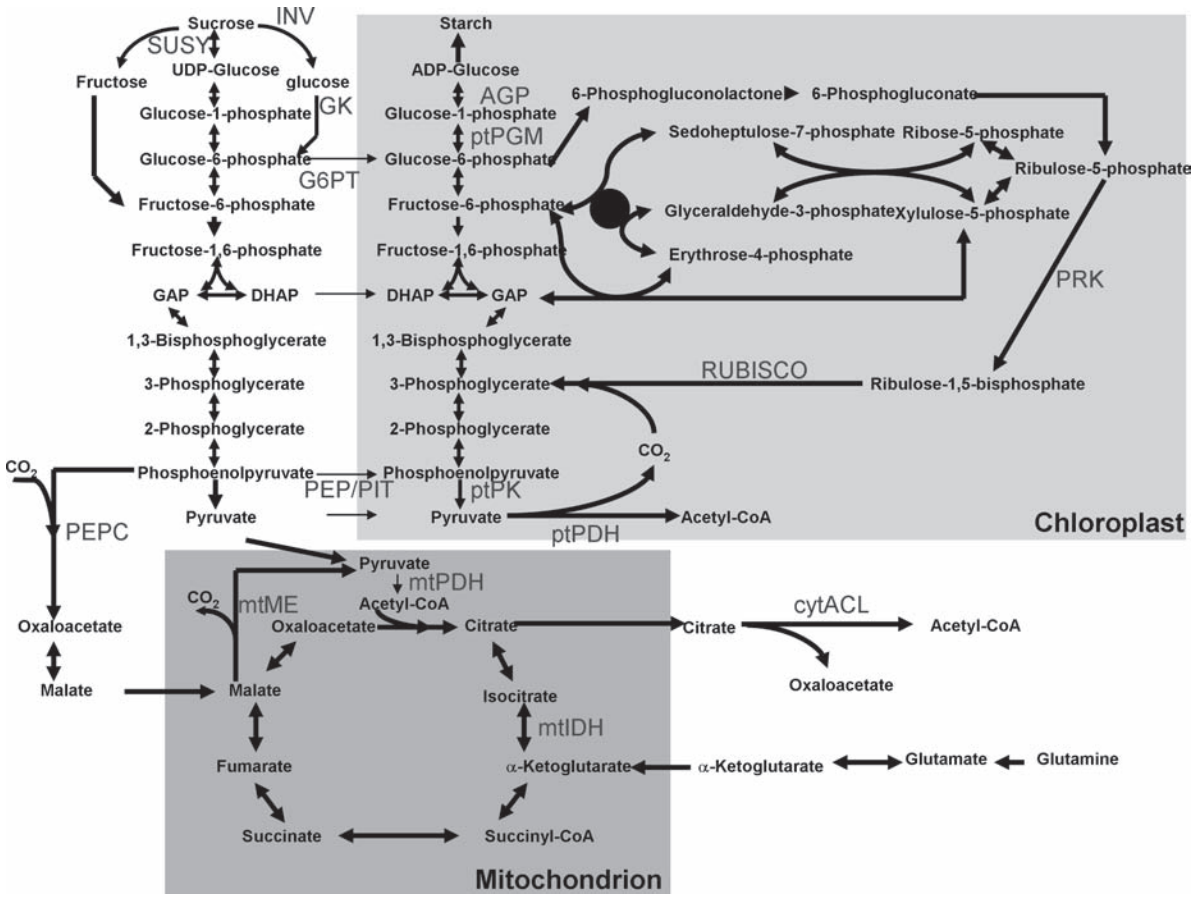

Fig. 6. Simplified pathway of primary metabolism in a developing oil seed and its relationship to production of acetyl-CoA for acyl lipid biosynthesis. Reactions discussed in Section V are highlighted in red. Abbreviations: cytACL, cytosolic ATP:citrate lyase; DHAP, dihydroxyacetone-3-phospate; G6PT, glucose-6-phosphate transporter; GAP, glyceraldehyde-3-phosphate; GK, glucokinase; INV, invertase; mtIDH, mitochondrial isocitrate dehydrogenase; mtME, mitochondrial malic enzyme; mtPDH, mitochondrial pyruvate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; PRK, phosphoribulokinase; ptPDH, plastidic pyruvatedehydrogenase; ptPGM, plastidic phosphoglucomutase; ptPK, plastidic pyruvate kinase; RUBISCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SUSY, sucrose synthase.

TAG, an important form of carbon storage, which can contribute up to 80% of the total dry matter of a seed. This triacylglycerol accumulates during the maturation phase of the seed and is used as a source of energy and carbon during germination. There is one known angiosperm that accumulates liquid waxes instead of TAG in its seeds, a desert shrub called jojoba (*Simmondsia chinensis*). These liquid waxes comprise linear esters of monounsaturated C20, C22 and C24 fatty acids and fatty alcohols (Miwa, 1971).

Triacylglycerol is synthesized by esterification of an acyl group to diacylglycerol (DAG), a reaction catalyzed by an enzyme called diacylglycerol:acyl-CoA acyltransferase (DGAT) (Lung and Weselake, 2006). Since DAG is also the substrate for membrane lipid synthesis in all tissues, the enzymatic reactions involved in oil biosynthesis, with the exception of DGAT, are analogous to those involved in glycerolipid synthesis in most tissues of the plant (Mekhedov et al., 2000).

There are two separate gene families encoding DGAT enzymes (Shockey et al., 2006). One family of DGAT (DGAT1) is part of the larger acyl CoA:cholesterol acyltransferase gene family (Cases et al., 1998; Hobbs et al., 1999) but the other DGAT family (DGAT2) is not related to any known genes except other DGAT1 members and also the monoacylglycerol acyltransferase

genes of animals (Cases et al., 2001; Lardizabal et al., 2001).

The DGAT reaction also controls flux to TAG to some extent since expression of Type 1 or Type 2 DGAT genes in plant seeds can lead to an increase to total TAG content (see Section V.D).

There is also an alternative pathway for TAG formation in plants, which involves the transfer of an acyl chain from a membrane phospholipid, phosphatidylcholine (PC), to DAG (Dahlqvist et al., 2000). This reaction is catalyzed by an enzyme called phospholipid:diacylglycerol acyltransferase. The significance of this pathway in bulk oil biosynthesis in plant seeds is still unclear.

Triacylglycerol is stored in the mature seed in the form of oil bodies. These are pools of TAG surrounded by a single monolayer phospholipid membrane, produced from the outer ER membrane by a budding process. The oil-body membrane contains oil-body specific proteins known as oleosins, which stabilize the oil body during seed desiccation (Huang, 1996).

While the most common oilseed plants contain TAG with the same five acyl groups that are found in membrane lipids (palmitate, stearate, oleate, linoleate and linolenate), many seeds contain substantial amounts of unusual fatty acyl chains in their triacylglycerol (Badami and Patil, 1981). Several hundred of these unusual fatty acids have been identified in the seeds of non-domesticated oilseeds. These non-standard fatty acids include medium (C8–14) and very long (C20–24) chain saturated fatty acids, acyl chains containing hydoxylated, epoxidated, acetylenated and methylated functional groups as well as fatty acids with conjugated double bonds. They are normally produced by variant forms of the fatty acid synthesis and modification enzymes common to all tissues (Voelker and Kinney, 2001). For example, the family of soluble, plastidial, acyl-ACP desaturases (*AAD* gene family) includes a number of which vary from the standard Δ9 18:0-ACP desaturase in their substrate specificity, region specificity or both (Shanklin and Cahoon, 1998). These diverged desaturases are expressed in the seeds of certain oilseed plants and examples include a Δ4 desaturase from carrot and coriander seeds, a Δ6 16:0-ACP desaturase from *Thunbergia alata* and a Δ9 16:0-ACP desaturases from *Doxantha* spp. and from *Asclepias syriaca*.

As a result, these species have seed oils containing monounsaturated fatty acids with double bonds in positions different from the standard 16-carbon fatty acid, palmitic acid, which has a double bond at the ninth carbon from the carboxyl group. The triacylglycerols of carrot and coriander seeds, for example, are rich in the monounsaturated fatty acid petroselenic acid (Δ6 18:1).

Perhaps the most diverged desaturase family is the FAD 2 gene family. FAD 2 genes encode membrane-associated phospholipid desaturases (Voelker and Kinney, 2001). Saturated and monounsaturated fatty acids from the plastids of seeds become esterified to coenzyme A, which are the substrates for TAG biosynthesis. Part of this 18:1-CoA pool can be transferred to the membrane phospholipid, phosphatidylcholine (PC) and further desaturated to 18:2 and 18:3 by two specialized microsomal membrane-associated desaturases, a PC oleoyl Δ12 desaturase (FAD2) and a PC linoleoyl omega-3 desaturase (FAD3). The resulting polyunsaturated fatty acids have methylene-interrupted double bonds. Diverged FAD 2-encoded desaturases, found in many oilseeds, catalyze the formation of non-methylene-interrupted bonds or other types of functional groups. These diverged desaturases include fatty acid 12-hydroxylases from *Ricinus communis* and *Lesquerella fendleri*, fatty acid 12-epoxygenases from *Vernonia galemensis* and *Crepis palaestina*, fatty acid conjugases from *Momordica charantia*, *Impatiens balsamica* and *Calendula officinalis* and a fatty acid 12-acetylenase from *Crepis alpina*. The oils of these species are rich in the corresponding fatty acid product of these diverged desaturases (Voelker and Kinney, 2001).

Heterologous expression of diverged desaturase genes in the seeds of plants that normally have a standard fatty acid profile can result in novel fatty acids accumulating in the transgenic plant oil, but usually at concentrations that are much lower than those found in the species from which the gene was isolated (Voelker and Kinney, 2001; Suh et al., 2002). This is a common observation when any diverged fatty acid synthesis or modification genes are expressed in the seeds of plants that do not normally accumulate unusual fatty acid. It reflects the fact that the entire fatty acid biosynthetic and triacylglycerol assembly mechanisms of seeds containing the unusual fatty

acids have also diverged to accommodate the change (Suh et al., 2002).

A good example of this metabolic divergence has been observed in the seeds of plants producing C8, C10 and C14 fatty acids, such as a number of *Cuphea* species (Heise and Fuhrmann, 1994). *Cuphea* species that produce medium chain fatty acids contain a diverged fatty acid condensing enzyme (Dehesh et al., 1998; Leonard et al., 1998; Schutt et al., 2002). This condensing enzyme is part of the plastidial fatty acid synthesis complex. Condensing enzymes catalyze the stepwise condensation of C2 units from malonyl-ACP to elongate the acyl-ACP chain. Each elongation step is actually a cycle of four reactions; the condensation reaction is followed by reduction, dehydration and a second reduction. The rate of the elongation, and the substrate elongated, is controlled by the initial condensation reaction of the cycle, catalyzed by a β-ketoacyl synthase (KAS) enzyme. The first condensation of the growing chain, that of acetyl-CoA with malonyl-ACP to form 3-ketobutyl-ACP and CO_2, is catalyzed by an enzyme called KAS III (Jones et al., 2003). Subsequent condensations to C16 are catalyzed by a KAS I and the final C16–C18 condensation by KAS II (von Wettstein-Knowles et al., 2000).

Plastidial KAS I and II enzymes are closely related and exist as dimers of KAS A and KAS B gene products (Voelker and Kinney, 2001). The KAS I condensing enzyme is a homodimer of KAS A polypeptides and *Cuphea* seeds that produce medium chain fatty acids contain a diverged KAS A subunit with increased specificity towards medium chain acyl-ACPs (Schutt et al., 2002). The diverged KAS A homodimer is referred to as KAS IV to distinguish it from the broader specificity C4-C14 KAS A homodimer, KAS I (Dehesh et al., 1998; Leonard et al., 1998).

Once the medium-chain acyl-ACP has been produced by KAS IV, it is transferred to the cytoplasm of the seed cell by the action of an acyl-ACP thioesterase. There are two distinct gene-families, which encode thioesterase enzymes: FAT A genes encoding enzymes that hydrolyze predominantly 18:1-ACP (with minor activities towards 18:0-ACP and 16:0-ACP) and FAT B genes, encoding enzymes that usually preferentially hydrolyze 16:0-ACP (Voelker et al., 1996).

The FAT B-type thioesterases of *Cuphea* have novel, shorter chain length specificities than the long-chain FAT B enzymes found in all plants (Dehesh et al., 1996a). Diverged FAT B genes have also been cloned from other medium-chain producing species, such as California bay laurel, coconut and elm (Voelker, 1996). The presence of a diverged FAT B gene is the main factor that determines the fatty acid composition of the seed. In support of this concept, expression of a *Cuphea* FAT B in canola, for example, resulted in substantial accumulation (up to 40%) of medium chain fatty acids in the seed oil (Dehesh et al., 1996b).

Once in the cytoplasm the medium-chain acyl-CoA is assembled into TAG by the catalytic action of acyltransferases. Again, the substrate preference of some of the acyl-transferases from species that produce medium chain fatty acids has also evolved in favor of medium chain acyl-CoAs (Knutzon et al., 1999). Expression in canola, for example, of a lysophosphatidic acid acyl transferase (LPAAT) from coconut, along with a medium chain FAT B-type thioesterase from bay laurel, can result in medium length acyl chains at position 2 of the TAG, a position normally occupied by predominantly by oleate (Knutzon et al., 1999). In the transgenic canola, lauric acid was now observed at all three positions on the TAG molecule and the total medium chain fatty acid content of the engineered canola was an impressive 60%. However, if the positional location of the new fatty acid on TAG was the only remaining factor controlling total accumulation, then the theoretical accumulation limit when this factor is removed should exceed 66%. Thus, in transgenic plants, there are clearly other factors, which prevent novel fatty acids from accumulating to relative abundances sometimes seen in native species, such as Castor (which has 90% ricinoleic acid in its seed oil).

In summary, the pathways of fatty acid and glycerolipid synthesis in seeds, while containing homologs of enzymes found in non-seed tissue, can be thought of as metabolically diverged pathways.

C Developmental Programs of Seed Maturation and their Relationship to Lipid Biosynthesis

Angiosperm seed development follows independent fertilization events leading to the generation

of the tissues of the seed coat (testa), which is maternally derived, the polyploid endosperm and the diploid embryo (Ohto et al., 2007 and references therein). Embryo development can be divided into phases of morphogenesis, characterized by cell divisions, leading to the formation of the embryo body. The embryo body itself is comprised of cotyledons, the embryonic axis (consisting of hypocotyl, root and shoot meristems) and supporting tissues. Morphogenesis is succeeded by the maturation phase and this latter phase can be divided into two stages. The first consists of cell expansion and accumulation of seed reserves, such as (transient) starch, oil, protein and soluble carbohydrates. During the second phase dehydration occurs and seed dormancy is established. Our understanding of the network of genes controlling embryo morphogenesis and seed maturation has progressed rapidly in the last 20 years, mainly due to the application of screens for mutants with defects in embryo development or seedling establishment, epistatic analysis of the resulting mutant alleles and cloning of the related genes. A detailed description of the field, including the role of sugar signaling in this process (Rolland et al., 2006), is outside of the scope of this review. Instead, we will focus on the brief description of a specific network of genes that control both embryo morphogenesis and seed maturation. This network interacts with transcription factors that provide a direct link to the control of sucrose assimilation and oil biosynthesis during seed maturation. A recent more detailed review of this field can be found in Gutierrez et al. (2007) and Santos-Mendoza et al. (2008).

During *Arabidopsis* seed development, four transcription factors *LEC1 LEC2*, *FUSCA3* and *ABI3* control almost all aspects of seed maturation (Ohto et al., 2007 and references therein). Defects in each of these genes lead to reduced storage compound accumulation, namely a severe reduction in oil and protein, and reduced establishment of dormancy as well as related defects in responsiveness to abscisic acid. *LEC1 LEC2* and *FUSCA3* mutants have also defects in seed morphogenesis: *LEC1* and *LEC2* function in early embryogenesis in specification of cotyledon organ identity and maintenance of the suspensor, a zygote-derived tissue that provides physical and physiological support for the embryo during early stages of development. Constitutive over-expression of *LEC1, LEC2* and *FUSCA3* is sufficient to induce seed storage compound accumulation (seed storage protein, TAG) in vegetative tissues, such as seedlings and leaves (Lotan et al., 1998; Luerssen et al., 1998; Stone et al., 2001; Santos-Mendoza et al., 2005; Wang et al., 2007). Expression of *LEC1* and *LEC2*, but not *FUSCA3*, is also sufficient to induce embryogenesis in vegetative tissues. The phenotype of mutations in the *ABI3* gene are restricted to embryo maturation – reduced dormancy, broad defects in seed oil and protein accumulation, and ABA insensitivity of seed germination (Finkelstein and Somerville, 1990). Reduced seed dormancy and lack of certain seed storage proteins is also observed in *Arabidopsis* mutants with defects in ABA biosynthesis (Koornneef, 1986). *ABI3* is part of a network of additional transcription factors identified by mutations related to specific defects in the seed's response to ABA, namely *ABI4* and *ABI5* (Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Brocard-Gifford et al., 2003). Defects in seed maturation are less severe in *abi4* and *abi5* mutants, which only show reduced accumulation of certain seed storage proteins.

The expression pattern of LEC and ABI genes during seed development supports the role of these genes in embryo morphogenesis and/or maturation. During embryo development, expression of *LEC* genes usually precedes that of the FUSCA/ABI gene class (Wang et al., 2007). Many recent studies have made attempts to develop a model that correctly reflects the hierarchy of LEC, FUSCA and ABI components in the control of seed maturation. These studies combine epistatic analysis of *lec*, *fusca3* and *abi* mutants, constitutive or inducible expression of individual components in mutant backgrounds and expression of reporter gene constructs in these mutant backgrounds to infer regulatory relationships between these genes. Three recent studies show that *LEC1* and *LEC2* act at least partly through *ABI3* and *FUSCA3* (Kagaya et al., 2005; To et al., 2006; Wang et al., 2007). To et al. (2006) showed that *lec2*-related defects in seed maturation can be complemented by constitutive over-expression of *FUSCA3* and *ABI3*. In addition, *FUSCA3 ABI3* expression is reduced in the *lec2* mutant.

The genes discussed so far control multiple aspects of embryo morphogenesis and maturation. While expression of these genes, in most cases,

is sufficient to induce transient accumulation of neutral seed storage lipid in non-seed tissue, they exert control of this process very likely indirectly, through additional signaling components.

Our current understanding of how this network of genes controls lipid biosynthetic pathways in seed is mostly derived from the *WRINKLED1 (wri1)* mutant of *Arabidopsis* (Focks and Benning, 1998). This mutant shows an 80% reduction of oil accumulation during seed development with no change in seed protein content but an increased accumulation of transient starch and soluble carbohydrate. The mutant also has reduced enzyme activity of glycolytic enzymes and of the enzymes of plastidic acetyl-CoA generation. Most importantly, no defects in embryo morphogenesis are observed in this mutant. Global gene expression analysis shows that a specific subset of genes involved in sugar metabolism and fatty acid biosynthesis, specifically those with the bell-shaped expression pattern during seed filling discussed above (see Section 5.1.2), are down- regulated in the *wri1* mutant. These genes include sucrose synthase (SUS2), subunits of plastidic PK and PDH enzymes together with genes of plastidic fatty acid biosynthesis, such as biotin carboxyl carrier protein (BCCP), ketoacyl synthase I (KAS I) and enoyl ACP reductase (Ruuska et al., 2002; Baud et al., 2007a).

The *WRI1* gene was identified by map-based cloning (Cernac and Benning, 2004) and is a transcription factor with an expression pattern that reaches maximal abundance prior to seed maturation. The phenotypes observed in this mutant, along with the expression patterns of the gene itself, support its role as a global regulator of glycolysis and certain aspects of fatty acid biosynthesis during oil seed filling. Indeed, constitutive expression of the *WRI1* gene leads to increased oil content in seeds. Interestingly, seedling establishment on media containing sucrose is impaired and only improves when metabolizable sugars are omitted from the medium. This supports the notion that presence of sucrose and WRI1 over-expression induces and maintains a physiological status of seed filling in vegetative tissues. In keeping with this hypothesis, *WRI1* expression in the presence of sucrose is sufficient to induce seed storage lipid biosynthesis in *Arabidopsis* seedlings. How does WRI1 interact with the previously identified network of genes controlling embryo development and maturation? Baud et al. (2007a) have shown that, in the *lec2* mutant background, *WRI1* expression in the developing embryo is reduced and is restricted to the hypocotyl. This observation provided the first indication that WRI1 is part of the signaling network through which *LEC2* controls oil accumulation in developing cotyledons.

Clearly, identification of WRI1 greatly refines our understanding of how increased flux of fatty acid biosynthesis and supply of precursors is regulated during embryo maturation.

D Approaches to Increased Oil Accumulation in Seed

Metabolic engineering approaches to increase flux to pathway end products, similar to those applied to non-acyl lipid biosynthetic pathways in seed (discussed in Sections II–IV), have also been applied in attempts to increase seed storage lipid biosynthesis in developing oil seed. These approaches can be grouped into three classes. The first two of these classes consist of metabolic engineering attempts to either "push" flux down a pathway or, conversely, "pull" it down (Kinney, 1998). Push approaches comprise expression of genes at the entry point of (or early in) the pathway, pull approaches attempt to increase flux through a specific pathway by increasing the sequestration of pathway end products (Kinney, 1998). The third class consists of approaches that target the global regulators of carbon supply and the biosynthetic activities of a pathway (Cernac and Benning, 2004). We will discuss specific examples related to the application of these three approaches to the transgenic modification of seed storage lipid quantity in oil seed.

Seed oil content in a given plant is a highly variable trait that responds strongly to plant growth conditions and genetic background. Reliable determination of an oil increase associated with a particular metabolic engineering strategy is further complicated by different methodologies used for oil quantitation that include spectroscopic methods, such as NMR, near infrared spectroscopy and quantitative gas chromatography. Taking into account the above parameters, it is important that an increase in oil content associated with a particular strategy is observed in multiple environments, in different genetic

backgrounds and under conditions that allow for maximal oil accumulation by isogenic control lines. *Arabidopsis* is usually the preferred model for testing of transgenic approaches to increase seed oil content. Fortunately, a recent study by Li et al. (2006) provides a comprehensive framework describing oil distribution in different seed tissues and the environmental effects on oil content in *Arabidopsis* seed. This study also demonstrates the reliability of GC analysis to measure small differences in oil content of different *Arabidopsis* ecotypes. The study showed, for example, that oil content in *Arabidopsis* seed increased from 36% to 44% when the light intensity during seed set was increased.

All transgenic approaches to increase oil content by transgenic means were developed before a full picture of the interaction of pathways, and the related fluxes that provide energy reductant and carbon for storage lipid biosynthesis described in Section V.A was available. Work with suspension cultures from a number of oil seeds indicates that significant control of storage lipid accumulation is exerted by both plastidic fatty acid biosynthesis and cytosolic triacylglycerol assembly (Ramli et al., 2002). Thus, in theory, both "push" and "pull" approaches should be applicable to engineer an increase in oil.

On the "push" side, Roesler et al. (1997) generated a fusion protein of homomeric, cytosolic acetyl-CoA carboxylase (ACCase) to a signal sequence for plastid targeting. The gene was expressed in developing *Brassica* seed. This resulted in a heritable oil increase of 5%, which was directly associated with the resulting increased ACCase activity in the transgenic developing seed.

Most subsequent attempts at increasing oil content have used the "pull" approach by targeting enzymes of cytosolic glycerolipid assembly to the developing oil seed. One of the first examples of a successful application of this "pull" strategy used seed-specific over expression of a variant of a lysophosphatidic acid:acyl-CoA acyltransferase (LPAAT) *sphingolipid-compensation 1* (*SLC1-1)* from *Saccharomyces cerevisiae* in *Brassica* (Zou et al., 1997). The mutant form of this LPAAT has increased specificity for long-chain acyl CoAs substrates and is associated with suppression of a sphingolipid-defect that is compensated by *SLC1-1* mediated incorporation of long chain fatty acids into phosphatidylinositol. An increase in *Arabidopsis* seed oil content of 25%, from 36% DW to 45% DW, was observed together with increased synthesis and incorporation of long chain ≥C20 fatty acids into the sn-2 position of triacylglycerol. Oil content in *Brassica* seed was increased from 41% to 45% DW when seed were generated under field conditions (Taylor et al., 2002). Interestingly there was no significant reduction in seed storage protein content that could account for the increase in oil content. In a related approach Vigeolas et al. (2007) targeted a baker's yeast glycerol 3-phosphate dehydrogenase to the cytosol of developing *Brassica* seed. The role of this gene is the conversion of glycolysis-derived dihydroxyacetone phosphate to glycerol 3-phosphate one of the substrates of cytosolic glycerol 3-phosphate:acyl-CoA acyl transferase (GPAT) the fist step of glycerolipid assembly. Expression of this gene led to an increase in oil content of 40% under controlled growth conditions, no reduction of protein and a 25% reduction in sucrose content of dry seed. Transient starch and sucrose levels were not affected.

A lot of activity has recently centered on the use of diacylglycerol:acyl-CoA acyltransferase (DGAT) genes to increase oil content in seed. The first plant gene of the DGAT1 gene family that shows similarity to mammalian cholesterol:acyl-CoA acyl transferase was cloned using the AS11 low oil mutant of *Arabidopsis* (Katavic et al., 1995; Routaboul et al., 1999; Zou et al., 1999). The *as11* mutant shows a 55% reduction in oil content. Clearly, in *Arabidopsis* and related oil seed, DGAT1 enzyme activity is a major factor required for seed storage lipid synthesis. Plant DGAT genes have been reviewed in detail recently by Lung and Weselake (2006). Transgenic targeting of DGAT1 expression to developing *Arabidopsis* seeds increased oil content from 27% to 35% DW (Jako et al., 2001). This clearly shows that DGAT1 over-expression can increase the oil content of *Arabidopsis* seeds under sub-optimal growing conditions. Similar results were obtained very recently with a closely related DGAT1 gene from garden nasturtium (*Tropeolum majus*) (Xu et al., 2008). It remains to be seen, though, to what extent DGAT1 expression can increase oil content in *Arabidopsis* seed under ideal conditions for oil biosynthesis, when untransformed control plants can produce over 40% oil

(Li et al., 2006). Very recently Zheng et al. (2008) identified an ancestral maize DGAT1 allele encoding a protein variant with higher specific activity than that encoded by the allele that is more common in current maize germplasm. The gene was identified by map-based cloning of a quantitative trait locus (QTL6) affecting embryo oil content. Transgenic targeting of the maize DGAT1 variant to the developing maize embryo increased oil content by 25%.

A second class of eukaryotic DGAT enzymes, referred to as DGAT2, has also been described (Lardizabal et al., 2001). The first member of this family of enzymes was purified from an oleaginous fungus (*Umbelopsis ramanniana*) that contains two closely related DGAT2 genes. Discovery of these DGAT2 genes led to the identification of related orthologous sequences in other eukaryotes including yeast, mammalian organisms and plants. Analysis of a DGAT2 mutant (*dga1*) of *Saccharomyces cerevisiae* revealed that the DGAT2 enzyme of is a central contributor of triacylglyceride accumulation in this organism (Oelkers et al., 2002). In plants, the tissue-specific expression pattern of DGAT2 genes, along with a lack of DGAT2 mutants with an oil defect, do not allow for a clear role of these genes in seed storage lipid accumulation to be assigned. Instead, recent findings suggest that they may play a role during seed development in the selective transfer of unusual fatty acids from acyl-CoA and membrane lipids to triacylglycerides (Shockey et al., 2006; Cahoon et al., 2007). Lardizabal et al. (2008) reported transgenic seed-specific expression of one of the *Umbelopsis ramanniana* DGAT2 genes in soybean. They observed a heritable oil increase of 7.5% associated with a 20-fold increase in specific DGAT activity in developing seed in multiple environments over several growing seasons. Interestingly, there was no decrease in protein or soluble carbohydrates associated with this transgenic oil trait. Instead, a 15% reduction in neutral digestive fiber in the transgenic seed suggested that redirection of carbon from insoluble carbohydrates may at least partially account for the observed oil increase.

In summary, there appears to be a distinct advantage in the use of heterologous genes to increase flux to storage lipids in developing seed. These enzymes may not be subject to the normal regulatory environment and thus may allow for higher specific activity than can be achieved by over-expression of native genes. Most importantly there is, at this point, no evidence to support the hypothesis that increased oil biosynthesis by transgenesis leads to a reduced availability of carbon skeletons for amino acid and protein biosynthesis, even in a high (40% DW) protein seed like soybean. This lends further support to the hypothesis that the pathways providing carbon for amino acid and oil biosynthesis in developing seed are separated. The carbon partitioning between oil and protein in developing oil seeds is most likely controlled by the ratio of amino acids and carbohydrates that are supplied to, and/or assimilated by, the developing seed.

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Chapter 20

Advanced Mass Spectrometry Methods for Analysis of Lipids from Photosynthetic Organisms

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Summary

The analysis of lipids is technically very challenging, not only due to the large number of various classes of compounds provided by the cell, but also due to the enormous heterogeneity of these various classes. Especially plants with their large pigment content and their high degree of polyunsaturated acyl residues make lipid analysis in this system even more complicated. Due to this high complexity, more and more sophisticated large-scale lipid-analysis systems have been developed, intended for the analysis of the so-called "lipidome", that is the entire lipid composition of a cell or a whole organism. These methods often consist of various building blocks, which show interlaced modularity. The two basic elements of a lipidomic analysis are an efficient extraction procedure and a sensitive detection system. In addition to these two basic elements, depending on the analytical question, additional separation and sample purification procedures can be introduced. Mass spectrometry (MS)-based techniques are at the forefront of lipidomic analysis. These extremely sensitive and accurate detection methods are applied to investigate all kinds of lipid. Their application, in combination with various plant-specific techniques of extraction and separation, including solid-phase extraction, thin-layer chromatography and high-performance liquid chromatography are presented in this chapter. Most of the MS-based technologies for lipid profiling of partially polar lipids rely on electrospray ionization, while more apolar lipids, like sterols, can be

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ionized by techniques, such as atmospheric pressure chemical ionization. A number of various applications employing these mass spectrometric methods will be discussed. Finally combinations of various separation and detection technologies, so-called hyphenated approaches, such as high-performance liquid chromatography coupled to mass spectrometry or gas chromatography coupled to time of flight or quadrupole mass spectrometry, are introduced and their suitability for lipid analysis are discussed.

I Introduction

Lipids, as a class of compounds, still await a precise and generally accepted definition. One way to define these compounds could be to summarize them on the basis of their solubility, meaning that compounds either insoluble or immiscible with water, but soluble in organic solvents, such as chloroform, ether, benzene and acetone, would belong to this chemical class. This definition would cover a wide range of substances ranging from polar to non-polar compounds, which contain moieties chemically characterized as hydrocarbons, alcohols, aldehydes, acids, amines and glycosides (Hauser and Poupart, 2003). One problem with this definition based solely on compound solubility is that compounds, such as glycosylated phospho-ceramides, due to their good solubility in water, would be excluded thereby contradicting widespread and accepted convention in the lipidomic field (Markham et al., 2006). This example indicates just how complex and potentially controversial, but also how dynamic and continually evolving, the field remains.

The analysis of lipids is quite complicated due to the high degree of complexity and the heterogeneity of their components. The analysis of plant lipids, compared to that of animal or prokaryotic organisms, is technically more demanding since plants contain, in addition to a high proportion of pigments, a large number of polyunsaturated acyl residues, thereby introducing a higher degree of diversity (Vieler et al., 2007). The most important lipid groups of plants are glycerolipids (phospholipids, galactolipids, and sulfolipids), sphingolipids (ceramides, glycosylceramides, and phosphosphingolipids), phytosterols (sterols, sterol ester, sterol glycosides, and acylated steryl glycoside), carotenoids and prenyllipids. Thus far, no single analytical system is capable of simultaneously identifying and quantifying all these lipid species without combining various analytical technologies. These technologies can include diverse stepwise extraction methods, but also the combination of various chromatographic separation techniques like one- or two-dimensional thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) using various stationary phases. These extraction, purification and separation methods are usually followed by a wide array of various qualitative and quantitative detection systems. Therefore, to enable a real lipidomic analysis of a photosynthetic organism, including pigments, phospholipids, glycolipids and non-polar lipids, requires the combination of various, complimentary technologies.

For example, carotenoids and prenyllipids are usually measured by high-performance liquid chromatography with fluorescence or UV/VIS detection. For this purpose the most comprehensive technique covered the separation of nine carotenoids together with all *trans* retinol, capsaicin, dehydrocapsaicin, chlorophyll *a*, chlorophyll *b* and tocopherol analogs by two monomeric C18

Abbreviations: ASG – Acetylated steryl glycosides; APCI – Atmospheric pressure chemical ionization; APPI – Atmospheric pressure photoionization; CAD – Charged aerosol detector; Cer – Ceramide; DGTA – Diacylglyceryl-hydroxymethyltrimethylalanine; DGTS – Diacylglyceryltrimethylhomoserine; DGDG – Digalactosyl diacylglycerol; ESI – Electrospray ionization; ELSD – Evaporative light scattering detector; FID – Flame ionization detection; GC – Gas chromatography; GlcCer – Glycosyl ceramide; GIPC – Glycosyl inositol phosphatidyl ceramide; HPLC – High-performance liquid chromatography; LCB – Long-chain base; MS – Mass spectrometry; MALDI – Matrix assisted laser desorption ionization; MGDG – Monogalactosyl diacylglycerol; NPLC – Normal phase liquid chromatography; PA – Phosphatidic acid; PC – Phosphatidylcholine; PE – Phosphatidylethanolamine; PG – Phosphatidylglycerol; PI – Phosphatidylinositol; PS – Phosphatidylserine; RPLC – Reversed-phase liquid chromatography; SPE – Solid phase extraction; SG – Steryl glycosides; SQDG – Sulfoquinovosyl diacylglycerol; TLC – Thin-layer chromatography; UV – Ultraviolet; X:Y – X number of carbon atoms and Y number of double bonds

columns with a column-switching technique (Lee et al., 2004). Quantification was achieved by UV and fluorescence detection and peak integration. Thus far there are no mass-spectrometric methods with a comparable sensitivity for carotenoids and prenyllipids.

Nevertheless, mass spectrometry is the method of choice for all the other lipid analyses. In this chapter the advantages of mass spectrometry are highlighted, as it is the single technology that enables the most comprehensive form of lipid analysis, allowing the detection and distinguishing of several lipid classes within a single run. Direct infusion approaches, without any lipid preseparation are compared to sophisticated hyphenated methods and the advantages and disadvantages of each technique are discussed.

II Lipid Extraction

The aim of a lipid extraction procedure is, like with other extraction protocols, to quantitatively isolate all lipids in their native state and separate them from the other constituents, such as proteins, polysaccharides, and small water soluble non-lipid contaminants. One crucial point in the extraction of lipids is to minimize processes of oxidation of the unsaturated fatty acids and lipid hydrolysis. This is facilitated by extracting lipids at low temperature at 4°C, as rapidly as possible, and using antioxidant agents in every extraction solvent. For example, butylated hydroxytoluene (BHT), which is the preferred compound for lipid extractions, is added in a concentration of 0.005%.

The purpose of the extraction solution is not only to overcome the interaction between the lipid and the tissue matrix, but also to readily solubilize all the lipids present. For such a complex mixture of compounds, this task cannot be achieved by a pure solvent, but instead ternary solvent systems are used. The most popular extraction procedure is the so-called Folch extraction (Folch et al., 1957), which was developed for the extraction of lipids from animal systems. Here a mixture of chloroform and methanol (2:1) is added in a ratio of 1:20 to the sample tissue and agitated at room temperature (although 4°C has proven preferable). Subsequently 0.2 volumes 1 M KCl or NaCl solution is added and a phase separation is performed. The lower chloroform phase that contains lipids is washed several times with original upper phase buffer.

For analysis of lipids from plant tissues, one further problem was encountered during the extraction procedure. High activities of lipases, which hydrolyze phospholipids and glycolipids rapidly, have to be inactivated to avoid loss of these lipids and introduction of artifacts. To overcome this problem, Nichols (1963) developed the most widely used extraction procedure for plant tissues. Here, an initial enzyme-deactivating incubation step in hot isopropanol at 60°C is applied prior to performing a Folch extraction.

Further improvements in lipid extraction have been made through the introduction of new solvents. For example, using methyl-*tert*-butyl ether (MTBE), instead of chloroform, allows a faster and cleaner lipid recovery. Since MTBE's have a low density, the lipid-containing organic phase forms the upper layer during phase separation, which simplifies its collection and minimizes losses (Matyash et al., 2008). Another modification to clean up the lipid phase makes use of Sephadex impregnated with "Folch-upper phase", which can be simply filtered off after vortexing the extract (Christie et al., 1998).

Additional care has to be taken during the extraction if near-water-soluble lipids, such as polyphosphoinositols and sphingolipids, are to be extracted. For these lipid classes, specialized extraction methods exist. Due to their highly amphiphilic nature, plant sphingolipids are extracted most efficiently into an isopropanol/hexane/water mixture (Markham et al., 2006). The standard Folch-based extraction procedure does not allow the efficient extraction of GIPCs because they are only poorly soluble in chloroform (Markham and Jaworski, 2007).

III Chromatographic Methods for Lipid-Class Separation

Although a large proportion of lipids can be measured from crude extracts by direct infusion mass spectrometry (see Section IV.A), for many lipid classes it is desirable to chromatographically separate them prior to analysis. So it is clear that direct infusion does not cover the nonpolar lipids like, for example, sterols, sterol ester (SE), and steryl glucosides (SG). Due to fragmentation during

the APCI ionization process all these compounds lead to the same dehydrated molecular ions of the sterols and can be only distinguished by retention times. The main problem with crude lipid extracts in mass spectrometric analysis, particularly at least at higher concentrations, is ion suppression (Koivusalo et al., 2001). This might be due to a limited amount of excess charge available on ESI droplets or to saturation of the ESI droplets with analyte at their surface at high analyte concentrations, thus inhibiting ejection of ions trapped inside the droplets. This is especially true for phospholipids. It can be observed, that phospholipids have a high propensity to suppress the ionization of other lipid molecules when introduced simultaneously into the mass spectrometer. The ionization efficiency of these compounds is strongly dependent on their saturation and acyl chain length (Brügger et al., 1997). In highly diluted samples this ion-suppression effect can be strongly reduced (Schwudke et al., 2007), but for plant extracts dilution is not a practicable solution since here there are huge differences in concentration. Several MGDG and PC species are, in *Arabidopsis thaliana* samples, highly abundant and their concentrations have to be adjusted to prevent dimer formation, which makes quantification impossible. By diluting the sample other minor lipid compounds are easily diluted below detection thresholds. Therefore, to avoid or overcome ionization suppression a separation of the lipid classes is frequently desirable. Compared to the analysis of lipids from animals, the separation of plant lipids requires improved selectivity of the chromatographic system to separate the highly abundant pigments and various glycolipids.

A Thin-Layer Chromatography

Traditionally, thin-layer chromatography (TLC) has been used to isolate and quantify complex lipids. Plant lipids elute in the order of pigments, MGDG, PE, PG, DGDG + PC, PS + SQDG, PI with chloroform: methanol: acetic acid: water (75:13:9:3) on silica gel 60 A plates (Bavaro et al., 2007). I_2 vapor can be used as a general stain to visualize these substances (Palumbo and Zullo, 1987). Neutral lipids are separated by a mixture of petroleum ether: ethyl ether: acetic acid (80:35:1), while chloroform: methanol: acetic acid can be used to separate both glycolipids (using a ratio of 65:25:4) and phospholipids (using a ratio of 65:25:8) (Uemura et al., 1995). Primuline staining and video densitometry measurements can be used for visualization (White et al., 1998), while a solution containing 33% phosphoric acid, 0.5% acetic acid, sulfuric acid and copper sulfate can be used as spray reagent, which, followed by heating and scanning with a grey image scanner, allows quantification of various lipid classes (Oku et al., 2003).

Individual lipids are identified by co-chromatography with authentic standards and the use of specific spray reagents (Lynch and Steponkus, 1987). Molybdenum blue in 0.5% sulfuric acid is specific for phospholipids (Dittmer and Lester, 1964), while betaine lipids can be visualized with Dragendorffs reagent resulting in an orange color (Kunzler and Eichenberger, 1997). Some, but not all, of the stains are compatible with mass spectrometric analysis, which can at times make the combination of TLC and MS problematic. A solution to detect and analyze TLC separated lipids can be to run two batches of sample in parallel, one of which is stained the second is used for the MS analysis.

A commonly used method to increase resolution and separation relies on the successive use of two development zones using various solvent mixtures, allowing the separation of first the polar and afterwards the nonpolar lipids (Kupke and Zeugner, 1978). Additionally, if two-dimensional TLC procedures are used with complex lipids, even higher resolution is possible, exceeding even the results obtained in a single HPLC run. If necessary, it is possible to separate and detect almost every lipid in the sample using these multidimensional TLC techniques, whereas some compounds may not elute from an HPLC. So if suitable detection is achieved, TLC in combination with MS analysis is useful, but also quite laborious method (Bavaro et al., 2007). An additional disadvantage is that TLC separation may destroy the phospholipid structure owing to oxidation or hydrolysis, while the plate is exposed to the oxygen atmosphere (Pulfer and Murphy, 2003). Furthermore, TLC has a relatively poor reproducibility compared to HPLC or GC (Breton et al., 1989) and it is not a system that lends itself well to high throughput and automation. Last but not least, quantification is time-consuming, tedious and labor-intensive.

B Solid-Phase Extraction

Solid-phase extraction (SPE) methods are being used especially as a rapid means of isolating particular components of interest for further analysis. The crude lipid extracts are passed through a column containing a lipophilic stationary phase, which retains, depending on its chemical properties, the various lipid classes. These lipids are subsequently recovered by elution with various organic solvents. This type of method allows for a rough separation of lipids according to the polarity of the components. One example is the procedure of Lynch and Steponkus (1987), which found application in more recent publications (Uemura and Steponkus, 1997). In this method Sep-Pak Silica cartridges are used to separate the lipid extract into neutral lipids [eluted with chloroform: acetic acid (100:1)], glycolipids [eluted with acetone and acetone: acetic acid (100:1)] and phospholipids [eluted with a mixture of methanol: chloroform: water (100:50:40)]. A number of various types of packing material are available. That enables a variety of different short and fast chromatographic separations to be performed, including adsorption-, reversed-phase-, silver-ion- and ion-exchange chromatography. The complete resolution of all glycerolipids from a plant lipid extract is a complicated task because glyceroglycolipids tend to co-elute with glycerophospholipids (Rizov and Doulis, 2000). Additional difficulties arise from the presence of chlorophyll and other pigments which often interfere with the analysis of less abundant lipid classes. Therefore it must be borne in mind that SPE separations with their low-resolving power are not comparable to those obtainable with TLC or HPLC (Christie, 1992). This is perhaps a major reason why only a limited number of publications exist, which deals with SPE separation of plant-lipid classes. However, when various SPE columns are combined as, for example, silica gel, aminopropyl cartridges and a weak anion exchanger, both capacity and selectivity of the separation can be increased and, together with a stepwise change of the elution solvent, the main plant lipid classes can be at least partially separated (Rizov and Doulis, 2001).

Still it can be concluded that in many cases the rough pre-separation of a sample prior to a mass spectrometric analysis might be of value for the subsequent analysis (see following sections).

C High-Performance Liquid Chromatography

The use of HPLC offers the possibility of full automation and higher speed, resolution, sensitivity and specificity as compared to SPE or TLC systems (Lima and Abdalla, 2002). Another benefit of HPLC is that fractions containing single lipid classes can easily be collected for detailed MS-based analysis or identification of molecular species.

Since total plant lipid extracts are highly diverse, gradient elution procedures are necessary to achieve effective separations. To separate simple lipids, glycolipids, and phospholipids, ternary gradient systems composed of mixtures of hexane (or heptane), chloroform, isopropanol or methyl-*tert*-butyl ether, acetonitrile or methanol and water have been used (Christie and Urwin, 1995). If water is used as a part of the mobile phase, 1 mM ammonium sulfate can be added according to a protocol of Guan et al. (2001). This supplement prevents column degeneration and improves reproducibility.

There are several alternative polar stationary phases available in addition to the pure silica phases. These include bonded phases, which are preferred because they equilibrate more rapidly with the mobile phase, especially in gradient applications. These phases also tend to provide more reproducible separations as was shown by Christie and Urwin (1995) for the lipid class separation of plant extracts. Diol phases, consisting of 1,2-dihydroxypropyl moieties linked covalently to silica gel, give excellent separations of phospholipids but the reproducibility of neutral lipids is not as good (Heemskerk et al., 1986). Aminopropyl-bonded phases are well suited for the analysis of zwitterionic lipids, while they are less useful for acidic lipids (Shimbo, 1986). PVA-Sil is prepared by bonding a layer of polymerized vinyl alcohol to silica, which greatly improves the reproducibility from run to run (Christie and Urwin, 1995; Deschamps et al., 2001).

D Detection Methods for Lipids in High-Performance Liquid-Chromatographic Systems

Most lipids lack a chromophore or do not contain at least one carbon–carbon double bond, which precludes their detection by UV/VIS or fluorescence

spectroscopy. Derivatization could overcome this problem, but such chemical modifications are likely to add complexity and additional preparative steps to the analysis. A further problem in the analysis of lipids with UV or fluorescence-based detection arises from the fact that gradient elution procedures are necessary, and these might themselves introduce difficulties due to various absorption maxima of the solvents used, thereby leading to complication of accurate quantitative and qualitative analyses. For this reason, evaporative light scattering detectors (ELSD), which are largely unaffected by mobile phase composition, have become a standard technique for quantitative analysis of lipids by HPLC systems. An ELSD generates particles from the eluent of the HPLC column via heated nebulization and produces a signal based on light scattered by the analyte. The ELSD responds only to the number of particles present, and thus does not discriminate based on physical characteristics of the molecule being measured. Thus ELSD does not provide selectivity in terms of mass or other compound-specific information. Additionally ELSD produces a nonlinear response, which has to be taken into account for the quantitative analysis of the data.

ELSD is known to have acceptable reproducibility (signal-to-baseline distance ±5%) enabling reasonable quantification, regardless of the absorption properties of eluents and sample molecules (Picchioni et al., 1996). The detector response intensities of the distinct lipid classes are markedly different (Silversand and Haux, 1997). The acidic phospholipids (PI and PS) give an especially poor response, while sterols, TAGs, and other phospholipids produce strong signals, meaning that only class-specific quantifications are possible. An example for the separation of the various lipid classes from a crude extract from leaves of *Arabidopsis thaliana* is shown in Fig. 1. The separation time of 30 min is optimized to separate even the PIs from the PAs and the PGs from the DGDG. In this example it can be seen that the highly abundant pigments are clearly separated from the sterols and MGDGs.

A disadvantage of the ELSD is its narrow working range for a given gain factor. But still quantification of compounds with different

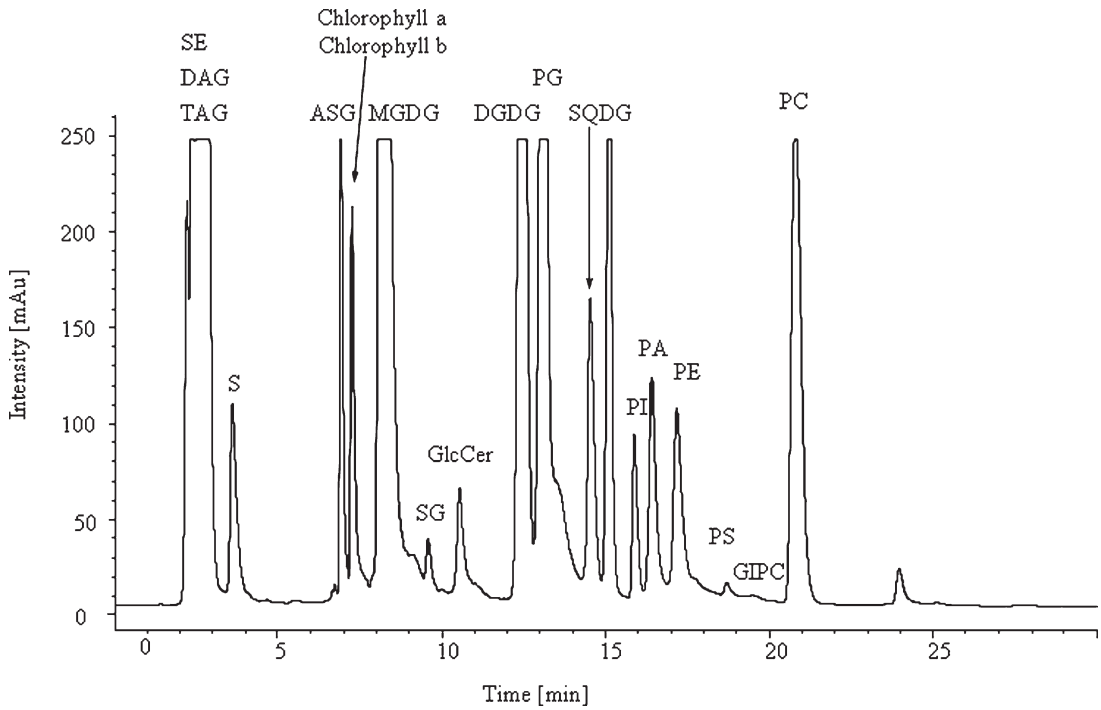

Fig. 1. Separation by HPLC-ELSD on a YMC-PVA-SIL column (250 × 46 mm, 5 µm) of lipid classes of *Arabidopsis thaliana* by a binary gradient system consisting of *tert*-butyl-methylether (A) and methanol (B). The elution profile was 100% A for 2 min, a linear gradient to 100% B over 23 and 5 min with 100% B.

concentration ranges is simplified by making use of a dual-channel modification of the ELSD (Nordbäck et al., 1998). The detection limits for the various lipid classes are in the range of 12 ng for hydrocarbons, 85 ng for MGDG and up to 820 ng for SQDG.

An alternative technique to the ELSD, which allows detection of all lipid classes, utilizes a charged aerosol detector (CAD), which involves nebulization of the HPLC column effluent, evaporation of the solvents, charging the aerosol particles by charge transfer from charged nitrogen gas, before measuring the current from the charged aerosol flux. The major advantage of the CAD is its low minimum limit of detection (down to 1 ng) and its relationship of nearly linear mass to peak area and its broad applicability to many types of lipid. However, one serious shortcoming of this system is its high background when the classical HPLC solvents, such as methanol and acetonitrile, are used (Moreau, 2006).

IV Mass Spectrometry-Based Lipid Analysis

With the introduction of atmospheric pressure ionization methods like ESI, APCI, or APPI, the analysis of many analytes, including most lipid classes, has been facilitated. Small volumes of samples are sufficient for mass spectrometry compared to other detection techniques. Electrospray ionization (ESI) is an ionization method used with polar compounds. The solution is passed through a metal capillary, which is biased at high voltage (4–5 kV). The electric field causes the solution to disperse into small droplets. These small droplets undergo a process of solvent evaporization and/or Rayleigh disintegration until gas-phase ions are produced. Through formation of adduct ions, ESI can be used for molecules that do not possess any intrinsic ionizable site but have a sufficient dipole potential.

APCI is fundamentally a gas-phase ionization, which contrasts to the liquid phase ESI process. A sample solution flows through a heated tube from where it is volatilized and sprayed into a corona discharge with the aid of nitrogen nebulization. Solvent ions are produced in the discharge and charge transfer reactions subsequently take place with the analytes. As an alternative ionization technique to APCI-MS for non-polar compounds, atmospheric pressure photo-ionization (APPI) has been introduced (Robb et al., 2000). The APPI interface can be considered as a modified APCI source, with the corona discharge being replaced by a gas discharge lamp, which emits photons in the vacuum UV region of the electromagnetic spectrum. When the energy of the photons is higher than the first ionization potential of a species in solution, their absorbance leads to single-photon ionization. With APPI and APCI relatively apolar compounds can be ionized.

Electron impact (EI) ionization is another way to ionize non-polar compounds. It can be often found coupled to gas chromatography–mass spectrometry (GC/MS) systems. Because ionization takes place in a beam of electrons of 70eV, EI is a relatively harsh ionization method, producing a wide range of molecular fragments that can be complex and sometimes difficult to interpret.

MALDI-MS, which is often combined with a time of flight analyzer, is an ionization method that requires the co-crystallization of the analyte in a matrix of organic acids. Pulsed laser beams of different wavelengths are then utilized to evaporate the organic acid matrix and to transfer the analytes into the gas phase, where they are ionized by electron transfer processes (Hillenkamp et al., 1991). Unfortunately, even though MALDI is a relatively mild and, most importantly, a fast ionization method, enabling high sample throughput, there are relatively few publications describing the use of MALDI-MS for plant lipid analysis (Schiller et al., 2004).

A Direct Infusion-Based Lipid Profiling

In recent years two main directions in the large scale, separation free analysis of animal lipids have been developed. One is based on a method of Brügger et al. (1997) where the precursor and neutral-loss ion scanning capabilities of triple quadrupole mass spectrometers were employed. The first quadrupole (q1) of the mass spectrometer is set to scan various precursor mass ranges of interest and the second quadupole (q2) is set to fragment these ions, thus generating characteristic fragmentation patterns. The third quadrupole (q3) is set to specifically detect a group of fragments representative for a specific substance class (Table 1). For the neutral-loss, q1 and q2 are again

scanning and fragmenting, but instead of measuring specific fragments in q3, the third quadrupole is then scanned with a fixed offset to q1.

The second method that was mainly used for direct infusion-based lipid analysis is the method developed by Han and Gross (2003). Here, in contrast to the method of Brügger et al. (1997) lipids are not only profiled by precursor- and neutral loss ion scans, but additionally the various lipid classes are resolved through their differential ionization properties in the ionization source (Han and Gross, 2003). This ionization selectivity is based on the differential propensity of each lipid class to acquire either positive or negative charges and their preference to form special adducts with LiOH (Han and Gross, 2003). An advantage of the selective ionization method is that quantification is done in MS rather than tandem MS, so that the signal of each compound can be related directly to the amount of each lipid.

In the field of plant lipidomics the group of Welti et al. (2003) adapted the method of Brügger et al. (1997) for the plant field. The method involves the direct infusion of crude lipid extracts, dissolved in chloroform/methanol (2:1), into a triple quadrupole mass spectrometer equipped with an ESI source. For measurements in a positive-ion mode the addition of ammonium acetate can increase the ionization efficiency, while the alternative addition of methylamine results in an improvement of the ionization efficiency of ceramides, PE, PI and PC (Ejsing, 2006). The singly charged molecular ions of phospholipids can usually be identified and quantified by precursor and neutral-loss scanning, while the sample is continuously infused into the mass spectrometer. Fragment analysis in negative-ion mode allows identification of fatty-acyl groups and the tentative assignment of these groups to the *sn*-1 and *sn*-2 positions. The *sn*-2 position produces more abundant ions for many phospholipids (Welti et al., 2002). In the case of MGDG and DGDG this is valid for the *sn*-1 position (Esch et al., 2007). Welti et al. (2002) profiled more than 80 molecular species of PC, PE, PG, PI, PA, MGDG, DGDG, lysoPC and lysoPE within a 43 min analysis. The detection limit of phospholipids with ESI-MS was around 10 nM (Welti et al., 2002). The same group expanded their own method further to approximately 200 polar plant lipid molecular species, including SQDG, lysoPG, PI and PS (Devaiah et al., 2006; Wang et al., 2006) requiring a total analysis time of 55 min. Recently also SM, Cer, HexCer and CerPE were included (Welti et al., 2007).

PC, SM, PE, MGDG, DGDG and all kinds of ceramides are best detected in positive ion mode where common head-group specific fragments are formed by the various lipid classes (Table 1). Precursor ion scanning for m/z 184 selectively detects the $[M + H]^+$ ions of SM, PC and lysoPC, whereas the sodium and potassium adduct ions lose the phosphocholine head group with a mass of 183 amu, which is a neutral fragment.

A product ion spectrum of 34:1 PC in the positive and negative ion mode is shown in Fig. 2. PC and SM can be easily discriminated, since PC appears at even m/z values, whereas SM appears at odd m/z values (Table 1). PE, LysoPE and CerPE lose the ethanolaminephosphate head group as a neutral fragment of 141 amu. Precursor-ion scanning of m/z 264 in positive ion mode detects Cer and HexCer. The specificity of the precursor-ion scan of 264 amu is confirmed by using the neutral loss of hexoses (162 amu) for HexCer. MGDG and DGDG form negative molecular ions and negatively charged acetate complexes. But a superior mass spectrum with a strong signal of MGDG and DGDG is obtained by forming positively charged sodium ($[M + Na]^+$) (Welti et al., 2003) or ammonium adducts ($[M + NH_4]^+$). Precursor-ion scanning in positive-ion mode, using the m/z 243 fragment ($C_9H_{16}O_6Na$), can, therefore, be used for the analysis of MGDG and DGDG. SQDG is more intensively detected as a deprotonated molecule $[M - H]^-$ in the negative-ion mode than as protonated molecule $[M + H]^+$ or sodiated molecule $[M + Na]^+$ in the positive ion mode. Precursor ion scanning of m/z 225 ($C_6H_9O_7S$) was proved to be useful to identify SQDGs. PI is detected by precursor-ion scanning of m/z 241 and PS by neutral-loss scanning of 87 amu in the negative-ion mode. The major phospholipid class in chloroplasts is PG, which can be detected in the negative-ion mode as deprotonated ions $[M - H]^-$. The most sensitive method is the unspecific precursor-ion scanning of m/z 153, which detects unfortunately all kinds of glycerophospholipid. In contrast, scanning for the precursors of m/z 227 (glycerophosphate glycerol minus 1 H_2O) provides a more specific mode to detect PG in plant samples.

Table 1. Summary of the lipid class-specific scan modes.

Lipid class	Polarity	Detected ion	Even or odd m/z value	Precursor	Neutral loss (amu)	Chemical composition of the fragment	Reference
PC	Positive	$[M + H]^+$	Even	m/z 184		$C_5H_{15}O_4PN$	Brügger et al., 1997
		$[M + Na]^+$	Even		183	$C_5H_{14}O_4PN$	Welti et al., 2002
Lyso PC	Positive	$[M + H]^+$	Even	m/z 184		$C_5H_{15}O_4PN$	Welti et al., 2002
PE	Positive	$[M + H]^+$	Even		141	$C_2H_8O_4PN$	Brügger et al., 1997
							Welti et al., 2002
Lyso PE	Positive	$[M + H]^+$	Even		141	$C_2H_8O_4PN$	Welti et al., 2002
MGDG	Positive	$[M + Na]^+$	Odd	m/z 243		$C_9H_{16}O_6Na$	Welti et al., 2003
SQDG	Negative	$[M - H]^-$	Odd	m/z 225		$C_6H_9O_7S$	Welti et al., 2003
DGDG	Positive	$[M + Na]^+$	Odd	m/z 243		$C_9H_{16}O_6Na$	Welti et al., 2003
PS	Positive	$[M + H]^+$	Even		87	$C_3H_5O_2N$	Brügger et al., 1997
	Negative	$[M - H]^-$	Even		185	$C_3H_8O_6PN$	Welti et al., 2002
PI	Negative	$[M - H]^-$	Odd	m/z 241		$C_6H_{10}O_8P$	Brügger et al., 1997
							Welti et al., 2002
PA	Negative	$[M - H]^-$	Odd	m/z 153		$C_3H_6O_5P$	Brügger et al., 1997
							Welti et al., 2002
PG	Negative	$[M - H]^-$	Odd	m/z 153		$C_3H_6O_5P$	Welti et al., 2003
				m/z 227		$C_6H_{12}O_7P$	
Lyso PG	Negative	$[M - H]^-$	Odd	m/z 153		$C_3H_6O_5P$	Welti et al., 2007
Cer	Positive	$[M + H]^+$	Even	m/z 264		$C_{18}H_{34}N$	Markham and Jaworski, 2007
Hex-Cer	Positive	$[M + H]^+$	Even	m/z 264		$C_{18}H_{34}N$	Welti et al., 2007
					162	$C_6H_{10}O_5$	Markham and Jaworski, 2007

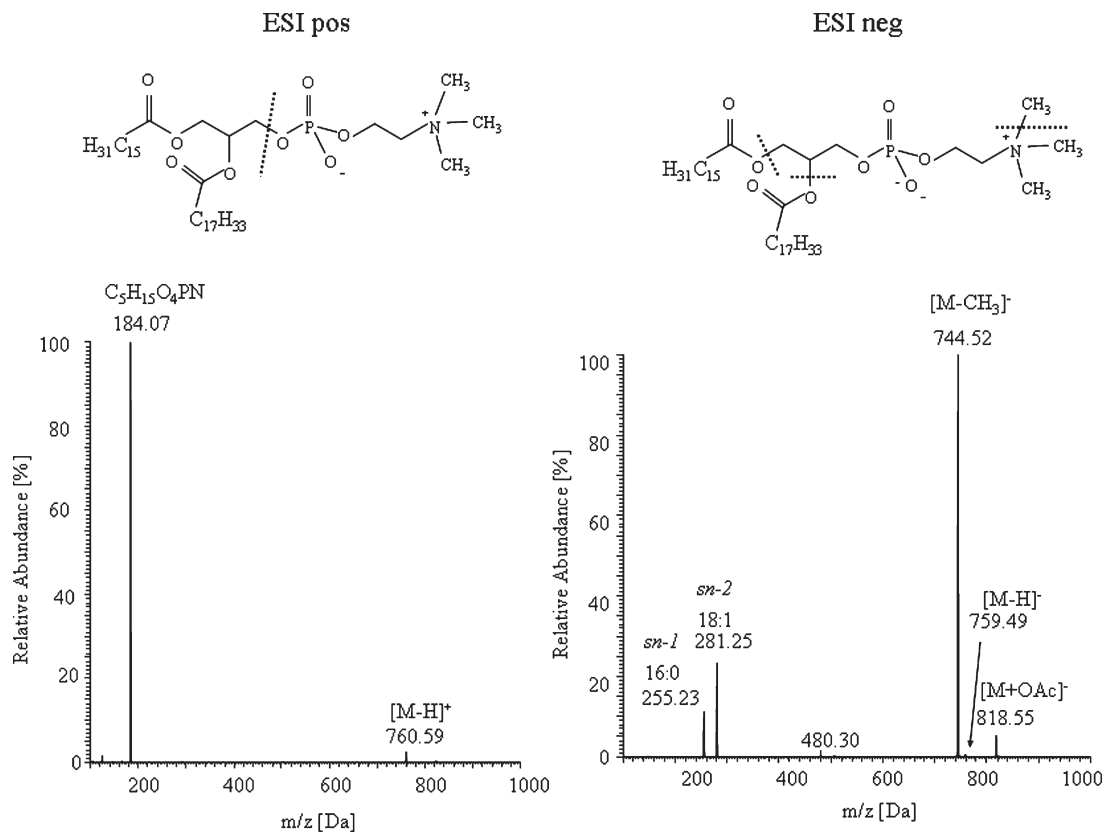

Fig. 2. Product ion spectra of the $[M + H]^+$ ion of 34:1 PC in *Arabidopsis thaliana* (**a**) and product ion spectra of the $[M - H]^-$ ion (**b**) detected by an LTQ-Orbitrap mass spectrometer (Thermo Fisher) with higher collision decomposition (HCD) at 25 V.

Tandem MS experiments using, for example, neutral-loss experiments, offer the possibility to scan for distinct fatty acids, such as oxylipins or 3-trans-hexadecenoyl (Hsu et al., 2007). In addition to their advantageous precursor- and neutral-loss scanning capabilities, triple quadrupole mass spectrometers are also known to show extremely high sensitivity and good signal-to-noise for the analyzed ions.

B Hyphenated Technologies for Lipid Analysis

Hyphenated technologies comprise different separation and detection systems including the combination of chromatographic methods, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC), with spectrometric methods, such as mass spectrometry (MS), UV/VIS, light scattering detection (ELSD) and charged aerosol detection (CAD). The combination of these technologies permits making use of the advantages of both worlds, leading to automated and sensitive analytical platforms.

Procedures for separation of plant lipids into molecular species by reversed-phase HPLC are now well established and robust (Markham and Jaworski, 2007; Shui et al., 2007). An advantage of RPLC is the possibility to separate even structural isomers. Lipids can then be detected by unspecific [ELSD (Larsen et al., 2002), CAD (Moreau, 2006)] or more specific (MS) detection methods. But still the most reliable and most sophisticated methods use MS detection.

1 High-Performance Liquid Chromatography/ Electrospray Ionization Mass Spectrometry

Measuring the sphingolipids (Cer, hydroxyCer, GlcCer, GIPC) in plants is a more demanding task than it is for other organisms due to higher degree of heterogeneity found within the class of lipids that contain long-chain bases. For example, *Arabidopsis thaliana* contains four different long-chain bases compared to only two in yeasts and animals. Direct infusion mass spectrometry cannot be applied because identification is difficult by mass spectrometry alone due to identical precursor and product masses and overlapping of the isotopic pattern of different compounds. This problem can be overcome by using RP-HPLC separation prior to MS analysis (Markham and Jaworski, 2007), but it necessitates the removal of as many of the other lipid classes as possible before analysis. This reduction in lipid complexity can be achieved by hydrolysis of lipid classes and due to the fact that sphingolipids are resistant to mild-base hydrolysis it can be applied to remove phospholipids, for example. The addition of methylamine, as a hydrolyzing reagent, provides a very efficient way to fulfill this purpose (Clarke and Dawson, 1981).

The LC/MS/MS method for the analysis of plant sphingolipids uses mobile phases consisting of methanol, tetrahydropyran and ammonium formate and allows the separation and detection of 168 compounds belonging to four different sphingolipid classes with an analysis time of 1 h. In addition to identification of pure lipids, this method also allows the differentiation of the LCB/fatty acid combinations of each sphingolipid.

Phospholipids (PG, PE, PC, PA, PS, PI) and glycolipids (MGDG, DGDG, SQDG) can be efficiently separated and identified using C8 ultra-performance liquid chromatography (UPLC) columns in combination with various MS technologies, such as triple quadrupole MS, or high resolution MS, such as the Orbitrap (Thermo Electron). The Orbitrap is a high resolution MS which offers mass resolution of up to 100,000 and mass accuracy of better than 3 ppm, thereby allowing large numbers of lipids to be distinguished and often identified by mass alone (Schwudke et al., 2007). The UPLC technology uses columns with packing in the particle size range of 1.7 µm, leading to a significant gain in efficiency and resolution When an UPLC separation is combined with high resolution MS it provides a very powerful and efficient lipid analysis platform. Using a mobile phase consisting of ammonium acetate (pH 5) and acetonitrile works very well for the separation of these compound classes and the addition of piperidine to the mobile phase has been shown to increase sensitivity by two- to ten-fold of all polar lipids in negative-ion mode (Shui et al., 2007).

Taking a systematic look at the retention-time behavior of phospholipids and glycolipids, it can be observed that retention time increases with the number of carbon atoms in the fatty-acyl chains, as shown in Fig. 3a for the MGDG 34:6 (t_R = 10.45 min), which has a longer retention time

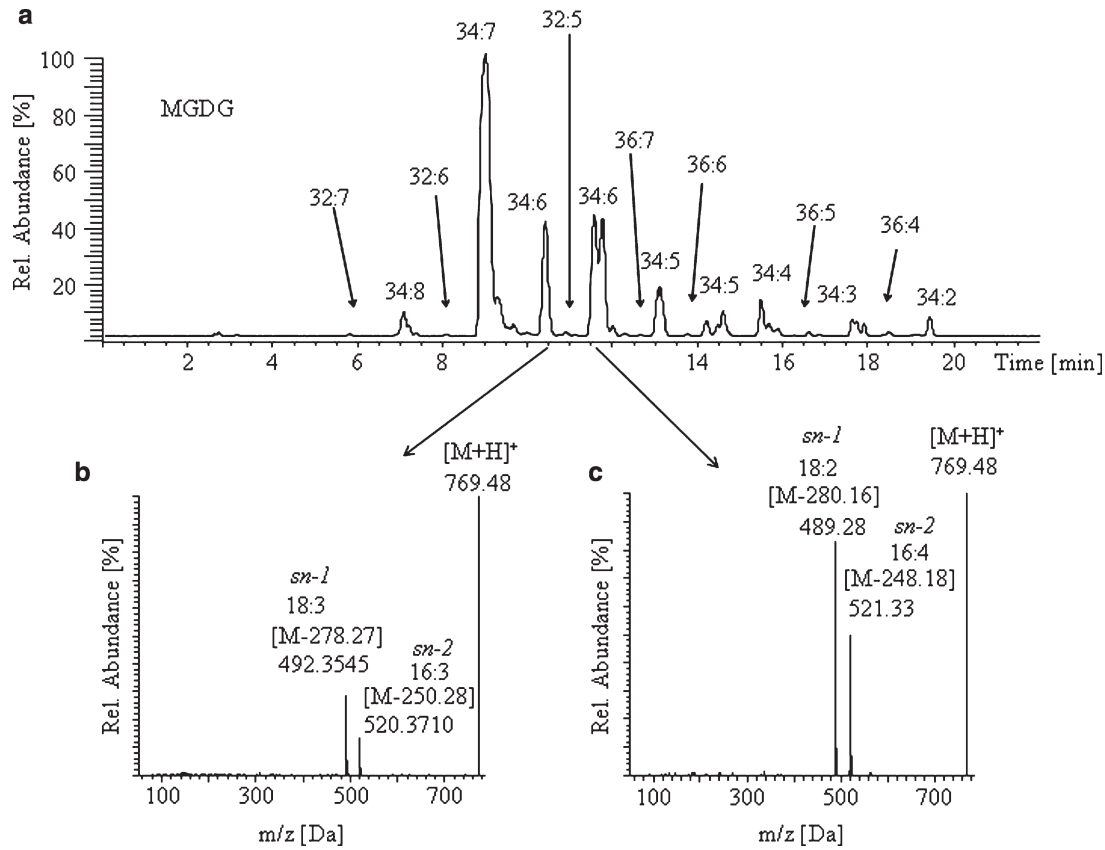

Fig. 3. (**a**) Total ion chromatogram of the MGDG fraction of *Chlamydomonas reinhardtii* measured by UPLC-ESI/MS. Column, C8 column; mobile phase, acetonitrile/ammonium acetate (10 mM, pH 5); (**b**) and (**c**) MS/MS spectrum of the isomers 34:6 at retention time 10.45 min (**b**) and 11.72 min (**c**) detected by an LTQ-Orbitrap mass spectrometer (Thermo Fisher) with higher collision decomposition (HCD) at 25 V.

than MGDG 32:6 (t_R = 8.10 min). Conversely unsaturation leads to an increase in polarity and consequently a decrease in retention time; so for instance, MGDG 34:6 (t_R = 10.45 min) elutes before the MGDG 34:5 (t_R = 13.15 min).

One of the main advantages of the combination of an LC and a MS system lies in the separation of structural isomers. In the example shown in Fig. 3, the MGDG 34:6 shows a number of distinct chromatographic peaks, which contain a different combination of fatty acid side chains. The composition of fatty acid side chains of these differently eluting peaks can be easily assigned using MS/MS experiments as shown (Fig. 3b and c). The distinction of these different, but nevertheless similar, molecules would have proven very complicated if direct infusion, that is, without chromatographic separation, were used.

Finally it should be mentioned that even a less than perfect chromatographic separation of certain compound classes might anyway provide useful information, and therefore lead to a positive identification. PA and PS species, for example, elute as broad peaks on LC systems, providing inferior, but reproducible and sufficiently well resolved signals. Interestingly, this broad-peak shape is, in a certain way, a characteristic of PA-species and helps therefore to detect and identify them. However, this undesired peak tailing can be circumvented by using either a special C30 column (Larsen et al., 2002) or a mobile phase containing a low concentration of phosphoric acid (5 µM) and a high percentage of water (Ogiso et al., 2008). Unfortunately the use of phosphoric acid is not fully compatible with common mass spectrometers due to the low volatility of the phosphate.

2 High-Performance Liquid Chromatography/ Atmospheric Pressure Chemical Ionization and High-Performance Liquid Chromatography/ Atmospheric Pressure Photoionization

Because sterols are highly lipophilic with only a few polar functional groups, they are difficult to ionize using electrospray ionization thereby, leading to an undesirably high detection limit. APCI is able to ionize sterols as $[M + H - H_2O]^+$. Several papers claim that APPI improves the detection limits compared to APCI but the ionization efficiency is furthermore dependent on the geometry of the ion source and can differ between different mass spectrometer suppliers. The analytical sensitivity of HPLC-APPI/MS can be up to 150-fold higher compared to common GC/MS methods (Lembcke et al., 2005). Additionally the total analysis time for sample pretreatment is reduced, since no sample derivatization is needed prior to HPLC/MS. The detection limit for these apolar molecules depends in a certain way on the sterol structure: being lower for unsaturated sterols with one double bond in the ring structure than for saturated sterols.

A separation of the sterols of *Arabidopsis thaliana* detected by UPLC-APCI/MS is shown in Fig. 4. A C18 column was used for these relatively apolar compounds. Sitosterol is the predominant sterol in *Arabidosis thaliana* and d5-, d7-avenesterol, campesterol, episterol, 24-methylene cholesterol, stigmasterol and cholesterol could also be detected. Steryl glycoside can be separated and analyzed using the same instrumental set up. Using HPLC, the SG eluted earlier than the corresponding free sterols but the elution sequence of the different sterols remained the same as for the free sterols: Interestingly with APCI-MS detection, only the aglycone of the sterols is detected as $[M + H - H_2O$ - glycosyl group$]^+$. An assignment of the full structure is possible by ESI-MS, but with this mild-ionization method the detection limit is, due to diminished ionization efficiency, considerably lower. Using ESI, the SG are detected as $[M + NH_4]^+$. Acetylated steryl glycosides (ASGs) are detected as $[M + H - H_2O$ - glycosyl group-FA$]^+$ and a small $[M + NH_4]^+$ ion is formed with APCI-MS, which makes assignment possible. The ASGs can be separated on C18 columns with a mobile phase consisting of acetonitrile and isopro-

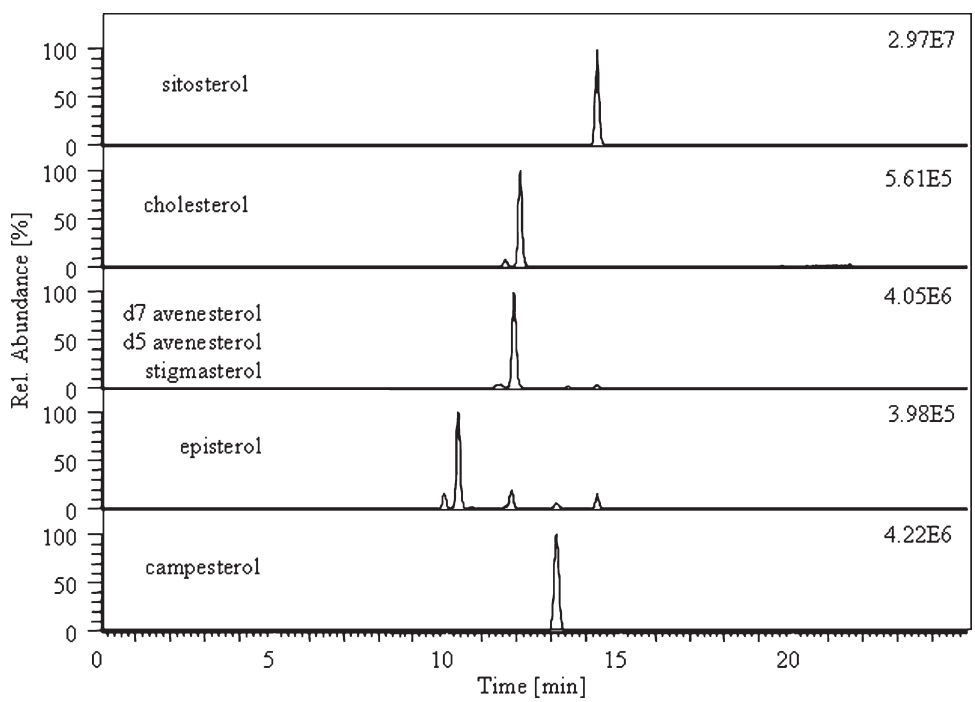

Fig. 4. Extracted ion chromatogram of the sterol fraction of *Arabidopsis thaliana* measured by UPLC-APCI/MS. Column, C18 column; mobile phase, acetonitrile/0.1% formic acid.

panol. Direct infusion measurements of phytosterols are not possible because sterols, SGs and ASGs are measured with the same mass due to fragmentations during the APCI/APPI ionization process.

3 Gas Chromatography–Mass Spectrometry

Many lipids, such as phospholipids, have a boiling point that is too high to be compatible with GC systems. A possibility to overcome these limitations is to convert the phospholipids into the corresponding diacylglycerols by treatment with phospholipase C. The identification in GC/MS is based purely on the comparison of the retention times from a sample to previously analyzed standard compounds (Kates, 1986). Another possibility to lower the boiling point of lipids can be transmethylation. After fractionation of lipid classes, sample methylation, using acetylchloride, can be performed for an immediate analysis of fatty acid methyl ester (FAME) by GC/FID, according to Lepage and Roy (1984). Injection onto the GC column is performed with a cold split/splitless injector to prevent fatty acid discrimination (Kohn et al., 1995). Using specific amide and ester derivatives structural analysis (double bonds, branchpoints or ring structures) of fatty acids is possible. If mass spectrometry alone is considered, picolinyl (3-hydroxymethylpyridine) esters are the most useful derivatives, but 2-alkenyl-4,4-dimethyloxazoline (DMOX) has superior gas chromatographic properties (Destaillats et al., 2005).

Therefore, even though the analysis of most complex lipid classes is not possible with GC/MS, fatty acid composition of the total lipids can be determined very efficiently and sensitively using this platform (Orhan et al., 2003) or GC/FID (Guil et al., 1996) after transmethylation. Even the structurally labile plant oxylipins can be profiled as derivatives of pentafluorobenzyl hydroxylamine by GC/MS in negative ionization mode with methane as reactant gas (Schulze et al., 2006).

Furthermore sterols are routinely analyzed in GC/MS systems as the respective trimethylsilyl (TMS) ethers (Pelillo et al., 2003). Nevertheless, lipid analysis by GC is not fully straightforward since relatively large sample quantities are required and because high temperatures are used, which carries a risk of isomerization of unsaturated fatty acids.

V Qualification and Quantification

Mass spectrometric identification of lipids relies on comparing experimental spectra with reference spectra in databases. Over the past years, different methods and programs have been developed for processing and identifying lipids from MS and MS/MS data. Unfortunately, none of them was developed especially for plant lipids and therefore galactolipids and sulfolipids are not included. But some of them are expandable.

An example for software, which is available for purchase is the Lipid Profiler Software (Schwudke et al., 2006), which was developed for the QTOF/MS of Applied Biosystems. The software implements algorithms for isotope correction, identification and quantification of lipid species detected by precursor ion scans.

One of an open source program is LipidNavigator (Yokoi et al., 2005), which is applicable to several mass spectrometers. This program is a high throughput web tool and enables automated phospholipid identification. The identification is either based on the comparison of fragmentation pattern to previously collected reference spectra stored in a database [Product Ion Survey (PIS)], head group [Head Group Survey (HGS)] or fatty acid assignment [Fatty Acid Survey (FAS)]. It is possible to use raw files of QTRAP4000™, a Q-Tof micro™ or a Finnigan™ LTQ and to evaluate direct infusion data and LC/MS data.

Another example is LIMSA together with SECD, which have been developed by Haimi et al. (2006). LIMSA is based on Microsoft Excel and perform peak finding, integration, assignment, isotope correction and quantification based on internal standards. It is applicable to direct infusion data and compares the calculated masses of lipids to an internal library, which is open to use and extension. SECD was developed for the extraction of LC/MS separated lipids and displays the result in a pseudo-3D map where the retention time, m/z and the intensity is displayed on x, y and z, respectively.

For the Orbitrap-based lipid analysis Lipid X (Herzog et al., 2008) was developed. Lipid X uses the high mass resolving power of this kind of mass spectrometer. The software calculates, based on the elemental composition of lipid classes, possible molecular masses, which are compared to MS spectra. Known fragmentation pathways of

lipid molecules are described by the molecular fragmentation language, which is used for probing each individual MS/MS spectrum. By that it is expandable to the plant specific lipids as well.

For quantification internal standards are required. Not all lipid classes have equal ionization efficiencies. Therefore, quantitative measurements require an addition of a mixture of internal standards. Normally standards are chosen that either absent from the analyzed sample or only present in negligible amounts. For this purpose the 14:0/14:0 and 22:0/22:0 phospholipids (Brügger et al., 1997) and hydrogenated galactolipid standards (Welti et al., 2002) are suitable. Furthermore, the optimal collision energy increases with increasing chain length. It is recommended to use at least two internal standards per lipid class to calculate an appropriate calibration function (Brügger et al., 1997). A combination of surrogate standards is likely the most accurate method of quantification. Surrogate standards are deuterated analogs added to the sample prior to extraction and carried through the extraction process with the endogenous compounds. These standards allow corrections for any losses due to incomplete transfer, oxidation and degradation of the sample. Unfortunately, for most of the plant lipid classes no deuterated standards are available. For direct infusion approaches, isotopic corrections of peak intensities have been implemented (Welti et al., 2002).

VI Outlook

Separation-free, direct infusion-based lipid profiling, in combination with MS/MS is a very promising technology for lipidomics. Based on short times of analysis a comprehensive and sensitive analysis of phospholipids and glycolipids is possible. However, more comprehensive computational approaches for the study of plant lipids that include galactolipids and sulfolipids are needed. Furthermore, the availability of deuterated and nondeuterated standards has to be improved. The main disadvantages of direct infusion approaches concern ion suppression and, therefore, exclusion of minor lipid species. Avoidance of ion suppression is difficult because of the large concentration differences between various lipid classes and species. Furthermore, it is not possible to detect and quantify, for example, phytosterols, because ASG, SG and sterols yield the same ions by APCI ionization. One possibility is the fractionation of lipid classes in combination with direct infusion. Still, a true lipidomic approach would have to cover all possible lipid classes and should be able to analyze even the lipid-like pigments.

The aim for future lipidomic analysis, therefore, is to combine several separation and extraction techniques and detection methods into a workflow, which is not only sensitive and comprehensive, but also automatable. The extraction should combine various solvents, such as isopropanol, hexane–isopropanol–water, chloroform–methanol and butanol, to solubilize all lipids classes. In our opinion, separation of lipid classes before qualitative and quantitative analysis is a necessity for the analysis of plant lipids. TLC and SPE are still performed in most laboratories but HPLC will most likely become the workhorse in the future since it allows higher throughput and automation. One possible way for a comprehensive lipidomic analysis might be based on the use of normal phase HPLC separation of the lipid classes combined with UV, FL and ELSD detection. The combination of the three detection techniques, on one hand, will enable the detection of carotenoids and prenyllipids, while, on the other, permitting detection of phospholipids, glycolipids and neutral lipids. Additionally continuous fraction collection using NPLC will provide the basis for class-specific MS analysis of the separated lipids. Direct infusion or HPLC separation-based MS analysis using high resolution MS or/and MS/MS will enable the identification of the lipid species within each fraction. Whether chromatographic separation is needed or not depends on the lipid class and on the complexity of the fraction. The direct infusion approach in combination with a lipid class separation by NPLC is, nevertheless, favored because the analysis time is shorter and is often adequate. But, chromatographic separation might be necessary for the quantification of structural isomers. The choice of the ionization method (APCI, APPI, ESI, EI) ultimately depends on the nature of the lipid and is optimized for each fraction.

Applying these comprehensive analysis schemes to the analysis of different plants including various mutants, developmental stages, or plants under various stress conditions might lead us to

an even better understanding of the processes that happen in the complex plant system.

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