

BIOLOGY OF THE PLANT CUTICLE

Annual Plant Reviews, Volume 23

*Edited by Markus Riederer
and Caroline Müller*

Biology of the Plant Cuticle

Annual Plant Reviews

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Biology of the Plant Cuticle

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Preface

During recent years the science of plant surfaces, and the cuticle in particular, has advanced at a fast pace, encompassing new fields of study along the way. Considerable progress has been made possible by the application of new concepts and techniques for investigating the biosynthesis, composition, structure and functional complexity of the plant cuticle.

We now have an increased understanding of the microscopic and sub-microscopic fine structure of the cuticular membrane as a whole, as well as of the cutin matrix and the associated wax deposits. By employing mutants and applying molecular biological techniques and advanced analytical tools, a much clearer image can now be drawn of the composition of cuticular waxes and the biosynthetic pathways leading to them. Intriguing variations can be found in the cuticular chemistry, morphology and function between and within plant species. Studies assessing the impact of UV radiation on plant life have emphasised the role of the cuticle and the underlying epidermis as optical filters for solar radiation. The field concerned with the diffusive transport of lipophilic organic non-electrolytes across the plant cuticle has reached a state of maturity, which makes it possible to quantitatively analyse and predict permeabilities based on physico-chemical predictors and to manipulate them *in vivo*. Recently, a new paradigm has been proposed for the diffusion of polar compounds and water across the cuticle. Within the context of plant ecophysiology, cuticular transpiration can now be considered in the perspective of whole-leaf water relations. New and unexpected roles have been assigned to the cuticle in plant development and in pollen–stigma interactions. Finally, much progress has been made in understanding the cuticle as a specific and extraordinary substrate for the interactions of the plant with microorganisms, fungi and insects.

Since the early 1970s, three books on the plant cuticle have been published. Only the first addressed all aspects of the subject; the other two were multi-author volumes arising from scientific meetings. Considering the progress made in this field, a book which deals comprehensively with plant surface characteristics and functions is overdue. The title, *Biology of the Plant Cuticle*, is intended to express the multidisciplinary and integrative approach to the subject. As functions are interconnected and rely heavily on the (bio)chemistry and properties of the cuticle, it is hoped that bringing together thus far disparate views of the subject will substantially advance the field of plant surface science. The book is also intended to provide a comprehensive overview and critical discussion of the current state of knowledge, paying close attention to the applied aspects of the field wherever appropriate.

Biology of the Plant Cuticle is aimed at a broad audience, ranging from biologists working on the molecular and whole-organism level to industrial agrochemists.

It is hoped that it will be of interest to phytochemists, plant (eco)physiologists, ecologists and environmental scientists, as well as to scientists and practitioners from the agricultural and horticultural sciences. In comparison with its predecessors, this book extensively considers the biological interactions occurring on plant surfaces and, therefore, is hoped to be of special appeal to scientists who, in the past, did not consider the plant surface *a priori* as a subject of prime importance. Thus, this volume is furthermore directed at phytopathologists, environmental microbiologists, entomologists and chemical ecologists.

The editors are indebted to the chapter authors for an enjoyable collaboration on this project and for timely delivery of carefully prepared manuscripts. In addition, the editors gratefully acknowledge the encouragement, advice and support continuously provided by Graeme MacKintosh and David McDade of Blackwell Publishing.

Markus Riederer
Caroline Müller

1 Introduction: biology of the plant cuticle

Markus Riederer

‘Does it make sense, and is it fun at all, to spend so much time with the outermost micrometer of a plant?’ This was the question a member of a search committee asked when the author applied for a job at a German university. As all scientists in this field know and deeply feel, it is fun indeed to study the plant cuticle and the plethora of processes related to it. The authors of this book hope that the reader will come to the conclusion that it is worthwhile to invest time, brains and funds into this endeavour.

The cuticle has often been called the ‘skin’ of the primary parts of higher plants, and in fact, the Latin word from which this term is derived (*cuticula*) means ‘thin skin’. The term cuticle has undergone a kind of evolution and profound changes in meaning during the last two centuries. At the beginning, the whole primary integument tissue or epidermis of a plant was called ‘cuticle’ stressing the convergence with animal skin, which is also cellular in nature. The modern usage of the word, meaning ‘a superficial film formed of the cutinized outer layers of the superficial walls of the epidermal cells’ (*Oxford English Dictionary Online*) of a plant, goes back to A.P. de Candolle. In 1827, he restricted the use of the French term ‘cuticule’ to the meaning in which it is used today (Wagenitz, 1996). Thus, the word cuticle is no longer used for a cellular layer but for a continuous extracellular membrane. In 1852, the word appeared in English for the first time in Henfrey’s translation of H. von Mohl’s ‘Grundzüge der Anatomie und Physiologie der vegetabilischen Zelle’ (OED Online). It is this term which will accompany us throughout this book.

1.1 The evolution of the plant cuticle

The cuticle as a structure has a very long history on the palaeobiological timescale. It is fortunate that the cuticle is a highly recalcitrant material which can easily resist decay for millions of years under favourable deposition conditions. It is fascinating that major chemical features like cutin composition are preserved over such prolonged periods of time (Ewbank *et al.*, 1996; Edwards *et al.*, 1997). Thus, essentially intact cuticles with clearly delineated epidermis cell silhouettes can be obtained from old sediments. The oldest remnants of plant cuticles date back to the boundary between the late Silurian and the early Devonian (about 400 million years ago) periods. The earliest cuticles, in the modern sense of the term which is assigned to higher plants, were found dispersed in sediments and belong to sporangia of rhyniophytoids. These specimens lack the impression of stomata while beginning with the

basal Devonian period, preserved cuticles show imprints from guard and accessory cells that are comparable to the modern stomatal apparatus. For a recent review on this subject see Edwards *et al.* (1996).

The finding that cuticles and stomata appear concomitantly in early kormophytes has profound impact on modern concepts of the evolution of vascular land plants and also on the interpretation of the selection pressure which acted on the evolution of stomata (Raven, 1977, 2002). Palaeoecophysiology has interpreted the simultaneous appearance of cuticles and stomata as evidence for the physiological adaptations to the colonisation of the land and thus for the relatively dry atmosphere by basal precursors of modern higher plants. Cuticles and stomata form a syndrome together with extended root systems, supracellular transport in vascular structures and the development of intercellular air spaces (Raven, 1977, 2002). These features are interpreted as necessary adaptations for photosynthesising homoiohydric life forms in an atmosphere with low water activity. The early cuticle probably also had additional functions equivalent to those of modern cuticles with defence against parasites, protection against ultraviolet (UV) radiation and water repellence being the most important ones.

1.2 Major functions of the plant cuticle

The cuticle is a structure that incorporates numerous functions of essential importance for plant life (Kerstiens, 1996b). This book treats the major functions in detail and, in most cases, devotes separate chapters to each of them. Nevertheless, a short synopsis is included in this introduction because it appears necessary to make one point very clear: the cuticle is a non-living though highly multifunctional structure into which numerous functions have been integrated. As will be shown later, this integration is sometimes not ideal as some physiological demands are in conflict with each other.

1.2.1 *Transpiration control*

As mentioned earlier, one of the major exigencies of the terrestrial lifestyle of higher plants is to have control over water relations. In order to stay alive, which essentially means to be more or less turgescant, the plant has to maintain the equilibrium between transpirational water loss and root water uptake. Any pronounced disequilibrium will severely compromise the viability and thus the fitness of the plant.

The control of transpiration from leaves, primary stems, flowers and fruits has two components: the stomata and the cuticle. Depending on the primary focus of scientific interest, the importance of either the stomata or the cuticle will be stressed by different authors. However, an effective control of transpiration is feasible only if the stomata *and* the cuticle act together in an optimised way. The low permeability of the cuticle makes it possible to control water loss by adjusting stomatal aperture.

But control will only work in a satisfactory way if the water loss across the cuticular surface is lower than the residual water loss through stomatal pores at optimal closure of stomata.

The cuticular permeability for water and transpiration confinement by the plant cuticle will be treated extensively in Chapters 8 and 9. This subject, of course, has met the interest of many researchers in the past. For general and early literature, the reader is referred to Stålfelt (1956), Schönherr (1982), and the textbooks by Larcher (2003) and Nobel (1991).

1.2.2 Control of loss and uptake of polar solutes

In principle, all organisms must have control over their inner milieu and therefore must have resistant integuments separating them from the environment. This is also true for terrestrial plants which would lose ions and polar organic solutes from the apoplastic solution unless they have a highly resistant cuticle that impedes the transport from the interior to the environment. Thus, the plant gains control over the loss of solutes and, at the same time, may modulate it by salt-excreting glands or hydathodes according to its specific needs. As the transport across the cuticle is symmetric, this membrane also hinders the uptake of polar substances from the outside. Control over solute loss and uptake is exerted by the same barrier properties of the cuticle as transpiration control. It might be speculated whether the need for controlling solute loss was an additional driving force in the evolution of the cuticular diffusion barrier.

A new view of the cuticular permeability of polar substances is currently evolving. There is increasing evidence that ions and small polar solutes move across the cuticle via continuous polar pathways that bypass the wax-based cuticular transport barrier. Chapter 8 (and partially also Chapter 9) will present this new view of polar solute (and water) transport across the cuticle and will put it in perspective with older work implying that such pathways may exist. For a thorough review of the older literature and for a primarily horticultural point of view on the subject of solute loss from plants (leaching), refer to the review by Tukey (1970).

1.2.3 Controlling the exchange of gases and vapours

When stomata are closed (which, on the average, is the case for approximately 12 h a day), the cuticle completely limits the loss and uptake of gases and vapours across the plant-atmosphere interface. This is true not only for water vapour as treated earlier but also for gases like carbon dioxide, oxygen, inorganic air pollutants and volatile organic compounds like terpenes (Lendzian and Kerstiens, 1991; Kerstiens *et al.*, 1992; Kerstiens, 1994). For highly lipophilic organic vapours, the cuticle is the preferred pathway of exchange even under conditions when the stomata are open (Riederer, 1995; Trapp, 1995). Exerting control over gas and vapour fluxes is, without any doubt, beneficial to the plant in most cases.

However, there is a conflict between controlling volatile exchange and photosynthesis. It has been shown experimentally in intact leaves with artificially clogged stomata that while the cuticle allows small amounts of carbon dioxide and water vapour to pass through, it markedly discriminates against the transport of carbon dioxide (Boyer *et al.*, 1997). A comparison with the properties of synthetic polymeric membranes like polyethylene, polycarbonate or polyester makes this property of the plant cuticle understandable. Woolley (1967) compared the permeabilities of plastic films to water and carbon dioxide and found that no synthetic material in existence has a higher permeability for carbon dioxide than for water. We can therefore conclude that intrinsic properties of a transport barrier against water and polar solutes confer low permeabilities to carbon dioxide and many other inorganic gases (Langowski, 2002). Evolution over the past 400 million years does not seem to have generated a membrane that can escape these physical constraints. This is the case even though a strong selective pressure acts towards a cuticle that allows photosynthesis during the light period but with the stomata closed.

1.2.4 Transport of lipophilic substances

The cuticle is the main aboveground interface for the exchange of lipophilic organic compounds between the environment and the interior of primary plant parts. All lipophilic compounds with low volatility or in solution have to cross the cuticle in order to enter or leave fruits, primary stems or leaves. The stomatal pathway is either not open to them (aqueous solutions of organic compounds) or is a very restricted route of exchange (semi-volatile compounds). The organic compounds in question may either be secondary metabolites of the plant or natural as well as anthropogenic compounds (pollutants, plant protection agents) occurring in the environment. From an applied point of view, the sorption and uptake of plant protection agents is of prime importance. Both the basic and applied aspects of this topic are discussed in Chapter 8. For publications covering the older literature, see these reviews and books: Van Overbeek (1956), Currier and Dybing (1959), Foy (1964), Sargent (1965), Bukovac (1976) and Hartley and Graham-Bryce (1980).

1.2.5 Water and particle repellence

After rains, many leaf surfaces are not covered by films of water and thus rapidly dry up. The cuticular surfaces of many plant species, at least their younger and pristine parts, are repellent to water and most water-based solutions. This is advantageous as water on the leaf surface may have several negative consequences for the plant; it (1) leads to leaching of ions and polar organic solutes from the plant's interior, and (2) creates suitable conditions for the colonisation by potentially harmful microbes like phytopathogenic bacteria or parasitic fungi. The latter aspect will be covered in Chapters 11 and 12 where the current knowledge on microbial communities and filamentous fungi on plant surfaces will be discussed in detail.

Certain plant surfaces may not only repel water and aqueous solutions but also microscopic particles like particulate aerosol, dust, spores and microbes. This is due to a self-cleaning mechanism based on the physico-chemical properties of some leaf surfaces and water droplets running off the surface taking along particles. This phenomenon has been termed Lotus effect and industrial applications have been explored (Barthlott and Neinhuis, 1997; Wagner *et al.*, 2003; Otten and Herminghaus, 2004). The fine structure of the cuticle and the chemical composition of cutin and cuticular waxes are covered in Chapters 2, 3 and 4, respectively.

1.2.6 Attenuation of photosynthetically active and UV radiation

One of the main driving forces for the colonisation of the terrestrial environment by plants is the luxuriant availability of radiation in the wavelength range from 400 to 800 nm in most cases. However, photosynthesis depends on a highly complicated and sensitive arrangement of pigments, proteins and membrane-enclosed compartments. This complex is easily damaged by excessive light. One of the protective mechanisms involves the cuticle: a dense cover of epicuticular wax crystals enhances scattering and reflection to a degree making tolerable the intensity of the radiation which reaches the photosynthetically active tissues in the interior of the leaf.

Another part of the electromagnetic spectrum hitting plant surfaces is UV radiation in the wavelength range from 280 to 400 nm. Excessive irradiation by UV results in damages in the photosynthetic apparatus and other vital parts of the plant cell. The cuticle, often together with the outer epidermal cell wall and the vacuoles of the epidermis, can contribute to an effective screening of UV radiation and thus to protecting the sensitive inner tissues. Chapter 6 covers the optical properties in the visible and UV range of the cuticle but also looks at properties of the epidermis and distinct sub-epidermal layers.

1.2.7 Mechanical containment

In a limited number of cases, the mechanical properties of plant cuticles support other structures like cell walls in maintaining the structural integrity of plant tissues. An economically important example for the mechanical importance of cuticles is fruit cracking in tomato and sweet cherry. In both cases, increasing internal pressure by uptake of water via roots or the fruit surface leads to the development of cracks. These cracks severely interfere with the economic and nutritional value of the fruits. Several studies have been performed on this issue either from an applied horticultural (Emmons and Scott, 1997; Bukovac *et al.*, 1999; Knoche *et al.*, 2002) or from a biomechanical (Wiedemann and Neinhuis, 1998; Matas *et al.*, 2004) point of view.

1.2.8 Separating agent in plant development

The cuticle plays a crucial role in plant development also and may be compared to a separating agent in developmental processes. Mutants with defective cuticles

exhibit increased water loss and, at the same time, extraordinary morphological abnormalities such as the fusion of organs. The emerging knowledge on the role played by the cuticle in cellular interactions and plant morphogenesis is extensively covered in Chapter 10.

1.2.9 Interface for biotic interactions

The cuticle-covered surface of higher plants is the main locality for major aboveground interactions with small organisms. On a microscopic scale, it is the interaction of bacteria, yeasts and fungi with the plant that may profoundly be influenced by cuticular properties. Features of the cuticle may have effects on adhesion, host recognition and mineral and carbon nutrition of the microbes as well as on the availability of liquid water. In addition, the cuticle may provide mechanical protection against the invasion by microbes. Cuticle–microbe interactions are treated in Chapters 11 and 12. For extensive reviews on these matters refer to Blakeman (1981, 1982, 1993) and Beattie and Lindow (1995).

On the macroscopic scale, the cuticle may interfere when insects or other arthropods interact with leaf surfaces. This may happen when a herbivore is searching for a suitable host for food or oviposition. Numerous cases have been reported where cuticular and leaf surface features in general influence herbivore behaviour and thus indirectly the integrity and fitness of the plant. This subject is reviewed in Chapter 13.

1.3 Convergence with other integuments

Not only plants but many other terrestrial organisms face at least some of the problems listed earlier. Very often, the main challenge is the danger of desiccation due to living in a dry atmosphere. The long-term maintenance of water balance must be solved by any terrestrial organism irrespective of its habitat. In order to hold back the water obtained from their surroundings, animals like plants typically possess an outer integument that greatly reduces the rate of water loss (Hadley, 1981, 1991).

In many species, the outer layers of the integument are covered and/or impregnated with more or less solid lipids which are primarily responsible for the observed waterproofing properties. This is especially true for plants and arthropods (insects and arachnids) both of which have a lipophilic matrix (cutin in plants, epicuticle in arthropods) with associated waxes. In both cases, these lipids are mixtures of long-chain aliphatic compounds as described for plants in detail in Chapters 3 and 4 and for insects in several reviews (Blomquist *et al.*, 1987; de Renobales *et al.*, 1991; Nelson and Blomquist, 1995). The cuticles of both arthropods and plants are continuous non-cellular membranes with multiple layers which cover the epidermis. In arthropods and in higher plants alike it is the physical structure, arrangement and composition of cuticular lipids that determines the waterproofing quality of the integument. Quantity and composition are species and age specific. For further

details on the convergence of plant and arthropod waterproofing properties and their relationship to chemical composition and physical structure of the cuticle, see the reviews by Hadley (1981, 1989, 1991).

This parallelism in the chemical, structural and physical properties of the outer layers of the integument is an outstanding example of convergent evolution in two widely divergent groups of organisms. The evolutionary success of wax-based transpiration barriers is extraordinary: if we take conservative species estimates as given by Wilson (1988, 1992), approximately 80% of all species on earth share a cuticle-like integument with low water permeability achieved by associated waxes. It may be speculated that two properties of mixtures of long-chain aliphatic molecules are responsible for this extraordinary evolutionary success: (1) waxes are plastic and can therefore follow growth and movements, and (2) they are multi-component, partly liquid or amorphous solids having self-healing properties which allow closing small defects inflicted on the integuments of plants or arthropods.

1.4 Objectives of this book

The science of the plant cuticle has received increasing attention among plant scientists as hitherto unknown functions and properties of this fraction of the epidermis have been discovered. Palaeobiologists, ecologists and especially plant evolutionary biologists become increasingly interested in cuticular remains and what can be deduced from their occurrence and structure. The volume of literature on the plant cuticle is growing at an increasing rate. A search in the BIOSIS database shows that during the last ten years (that is the time interval since the last book on the cuticle has appeared), approximately 2300 publications concerning the cuticles of plants have appeared.

The overall subject of this book has been treated in the past in several books. To the author's knowledge, the first modern experiment-based and comprehensive treatment of the cuticle and the associated waxes was provided by Frey-Wyssling (1938). Twenty years later, Martin and Juniper (1970) published a book which was the first to be exclusively devoted to the cuticles of plants. Thereafter, two volumes each compiling the proceedings of meetings devoted to the plant cuticle were edited by Cutler *et al.* (1982) and by Kerstiens (1996a), respectively. A small booklet on the surfaces of plants oriented at a general scientific audience was published by Juniper and Jeffree (1983).

Considering the progress made in this field since then, a new book covering the whole field of cuticular science appears overdue. Since Martin and Juniper (1970), the present book is the first written exclusively for this purpose and not derived from a scientific meeting. The title *Biology of the Plant Cuticle* has been chosen in order to express the multidisciplinary and integrative views of the subject. Cuticular functions are interrelated and heavily rely on the (bio)chemistry and the physical properties of the cuticle. Therefore, combining so far disparate views on this subject into a common perspective is expected to advance the field of plant surface

science substantially. Bringing the different functions together will delineate where and under which conditions they are in accordance and in conflict with each other depending on the special needs of the plant.

Obviously, the book is intended to provide a comprehensive and critical treatment of the current state of knowledge about plant surfaces and the cuticle in particular in its full depth and breadth. Recent developments having a pronounced impact on our understanding of the cuticle's fine structure, biosynthesis, composition, physical and transport properties are extensively reviewed in this book. For the first time, a comprehensive overview of cuticular functions in plant morphogenesis is given. Part of the book is devoted to the rapidly evolving field of biotic interactions taking place on plant surfaces. Where appropriate, special attention has been paid to the applied aspects of the field, especially in agricultural chemistry.

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2 The fine structure of the plant cuticle

Christopher E. Jeffree

2.1 Introduction

2.1.1 The distribution of the plant cuticle

The cuticle is a translucent film of polymeric lipids (Chapter 4) and soluble waxes (Chapter 5) located at the interface between a plant and its aerial environment. The cuticle is present on the outer surfaces of the epidermal cells at the aerial surfaces of vascular plants, and also on the sporophytes and sometimes the gametophytes of mosses, hornworts and liverworts. Only the epidermal cells of aerial organs are known to be capable of synthesising the constituents of the cuticle, and it is always absent from the root epidermis. The primary function of the cuticle is as a permeability barrier against water vapour loss from tissues (Schönherr and Mérida, 1981; Riederer, 1991; Schreiber *et al.*, 1996), one of a system of innovations including stomata, intercellular spaces in a photosynthetic mesophyll and a vascular conducting system for water (xylem) and assimilates (phloem) that enable homoiohydricity in land plants (Raven, 1977). The outer surface of the cuticle is coated with epicuticular waxes (EW) which confer water-repellency (Adam, 1963; Holloway, 1969a,b, 1970), keep the plant surface clean and dry (Barthlott and Neinhuis, 1997; Neinhuis and Barthlott, 1997), attenuate short-wave radiation (Chapter 6; Krauss *et al.*, 1997) and discourage attachment of microorganisms (Chapters 11 and 12) and climbing by insects (Chapter 13; Eigenbrode, 1996) other than those with which a plant has symbiotic relationships (Markstädter *et al.*, 2000). The cuticle may also have an important role in preventing developing organs from adhering to each other while developing in-bud (Chapter 10; Nawrath, 2002). On leaves the cuticle is present on both adaxial and abaxial surfaces, lines the stomatal apertures and covers the free inner epidermal cell surfaces of the substomatal cavity and intercellular spaces (Osborn and Taylor, 1990), but not the mesophyll. The cuticle is usually thickest above the anticlinal epidermal cell walls (CW), often forming pegs or spandrels by penetrating deeply between the anticlinal walls of adjacent epidermal cells (Figure 2.1b). The cuticular membrane (CM) thus bears an imprint of the epidermal cell pattern of the plant organs on which it was formed, which may survive as the only remaining fossil evidence of multicellular structure of the earliest land plants (Edwards *et al.*, 1996). Epidermal cells of tomato fruits are also coated on internal and external surfaces, and intercellular cutinised layers may form as permeability barriers between stem and head cells in both absorptive and secretory glands (Mahlberg and Kim, 1991; Kim and Mahlberg, 1995).

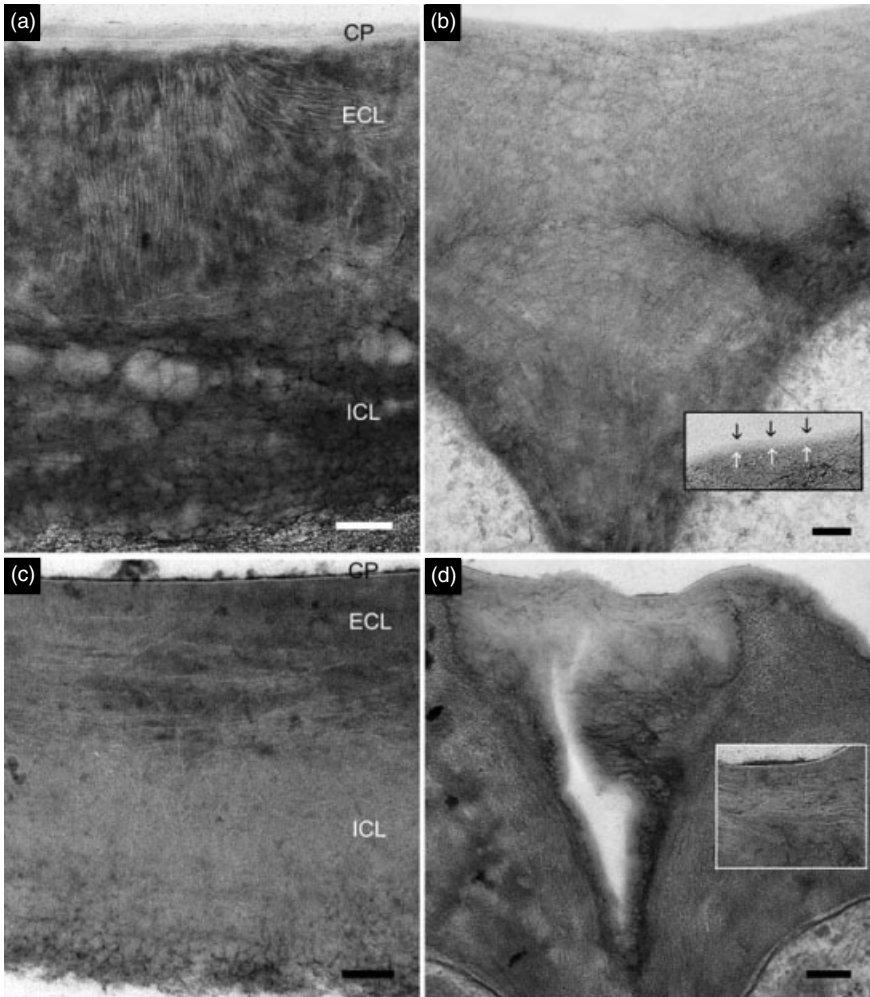


Figure 2.1 Cuticular membranes with extended cuticular layer (CL) development. (a) The adaxial leaf cuticle of pear (*Pyrus communis*), stained *en bloc* with OsO₄, section stained with Reynold's lead citrate. The cuticle proper (CP) is very electron-lucent and amorphous, and is terminated by a single outer electron-lucent lamella. Beneath the CP are two extensive CL zones, about 1 μm of chaotically lamellate material with no or few fibrillae (ECL), and an inner reticulate zone (ICL) with fewer lamellae. Note that in much of the ECL in this image, the lamellae run vertically. (b) The leaf cuticular membrane (CM) between two adjacent epidermal cells. Holloway reported this CM as Type 4, reticulate throughout, but an amorphous CP covers the fibrils of the reticulate ECL (inset), making it Type 3. (c) The adaxial leaf cuticle of apple (*Malus pumila*) following isolation and methanol extraction, showing structure analogous to that in pear (see Figure 2.1a). The CP is not clearly demarcated from the ECL, but a single continuous very electron-lucent lamella terminates the CM. (d) The outer part of a stomatal complex still in the process of cell separation in a *Phaseolus vulgaris* leaf shows heavy cuticle development. Once again, a single electron-lucent lamella terminates the CM, and the ECL layer contains extensive lamellae (see inset). (a–c) Bars = 100 nm; (d) bar = 200 nm. Figure 2.1a reproduced from Holloway (1982a), Figure 2.1b–d by P.J. Holloway.

A substomatal cuticle is unequivocally present on the surfaces of epidermal cells lining the substomatal cavity of many, if not all, species – in leaves of *Gossypium hirsutum* (Wullschlegel and Oosterhuis, 1989), *Malus pumila* (Holloway, 1982a), *Phaseolus vulgaris* (Martin and Juniper, 1970) oak, *Quercus velutina* (Osborn and Taylor, 1990) and *Cirsium horridulum* (Pesacreta and Hasenstein, 1999). This carries the implication that the surfaces of epidermal cells do not evaporate significant amounts of water into the intercellular space system. However, an internal cuticle has also sometimes been reported on the surfaces of mesophyll and palisade cells, as in the mistletoe *Phoradendron flavescens* (Calvin, 1970). Seen in transmission electron microscope (TEM) sections, the internal cuticle may extend a short distance from the underside of the epidermal cell onto the surface of the palisade and spongy mesophyll cells, before tapering off to an amorphous, slightly osmiophilic layer on the mesophyll cell surfaces that has never been isolated or characterised chemically. Pure water beads with high contact angles can be condensed on the mesophyll cell surfaces indicating that they are hydrophobic (Sheriff, 1977; Willison and Pearce, 1983; Willison *et al.*, 1984; Jeffree *et al.*, 1987), but a trace of ethanol or wetting agent will cause the beads to wet (Lewis, 1945). On the other hand, the CW microfibrils are exposed at the mesophyll cell surface in freeze-fractured rye leaves, except in the vicinity of cell junctions, where they are overlain by amorphous material on which frozen water droplets were visible (Willison and Pearce, 1983).

The aim of this chapter is to review currently available data on the structure, ontogeny and composition of the CM and the EW, and to integrate the observed variability in cuticle types into a structural and developmental model.

2.1.2 Definition and nomenclature of the plant cuticle

This chapter uses the terminologies of Wattendorff and Holloway (1980), Holloway (1982a) and Jeffree (1996) with additions where appropriate. Brongniart (1834) gave the name ‘cuticula’ to a superficial membrane isolated from the cabbage leaf epidermis by retting in water. He recognised that this first isolated cuticle contained fatty materials because it stained with Sudan dyes. More than a century later, Norris and Bukovac (1968) defined the limits of the cuticle as ‘all of the layers that can be separated from the underlying cellulose cell wall’, thus including the so-called cuticular, cutinised and cuticularised layers (*sensu* Esau, 1953; Crafts and Foy, 1962; Sitte and Rennie, 1963). The concept of a layered cuticle is attributable to von Mohl (1847), who regarded the cuticle or CM as consisting of two main zones at maturity, as has generally been accepted since then (Lee and Priestley, 1924; von Ziegenspeck, 1928; Crisp, 1963; Sitte and Rennie, 1963; Hülsbruch, 1966a,b; Chafe and Wardrop, 1973; Jarvis and Wardrop, 1974; Sargent, 1976a,b,c; Holloway, 1982a; Jeffree, 1996). The outermost cuticle layer appears outside the primary epidermal CW very early in organ ontogeny and was called the cuticle proper (CP) by von Mohl (1847), who defined it as being composed of soluble and polymeric lipids that can be saponified without leaving a cellulosic

residue. This author accepts the positional aspect of his definition and the absence of cellulose, but the CP is not always completely saponifiable, especially in mature leaves (Schmidt and Schönherr, 1982; Domínguez and Heredia, 1999). Beneath the CP a zone of the primary cell wall (PCW) and then secondary cell wall (SCW) of variable thickness becomes progressively impregnated with cutin during organ development (Schieferstein and Loomis, 1959). Although Esau (1953) referred to this zone as the 'cuticularised layer' (see also Martin and Juniper, 1970), von Mohl (1847) called it the cuticular layer (CL) meaning the cuticular layer of the CW. This original term was preferred by Holloway (1982a), Wattendorff and Holloway (1984) and Jeffree (1996), and will also be used here, both because of its precedence and because the unspecified process and location of cuticularisation leads to ambiguity.

Late in development the CL may undergo further modification from the outside inwards, so that in the CL of some species (e.g. *Ficus elastica* and *Ilex integra*; Hülsbruch, 1966a,b) embedded cellulose may no longer be identifiable, making it hard to distinguish from the CP by von Mohl's definition (see Figure 2.2g, P.J. Holloway). Hülsbruch called this zone 'Cuticularsaum', but Jeffree (1996) referred to the internal and external zones of the CL layer as the internal cuticular layer (ICL) and external cuticular layer (ECL), respectively. Evolving concepts of the structure of the cuticle have been summarised in various diagrams (Roelofsen, 1952; Schieferstein and Loomis, 1959; Martin and Juniper, 1970; Holloway, 1971; Hadley, 1981; Holloway, 1982a; Hallam, 1982; Juniper and Jeffree, 1983; Wattendorff and Holloway, 1984; Jeffree, 1986, 1996; Schönherr *et al.*, 1991; Holloway, 1993, 1994).

2.1.3 *The pectin lamella*

During early ontogeny, the CM (CP only at this stage) is underlain by a superficial layer of the PCW that behaves like a polyanion. Cytochemically it gives positive reactions with cationic dyes (ruthenium red, Alcian blue, Coriphosphine O, hydroxylamine-ferric chloride) and periodate-Schiff consistent with the presence of pectin. No doubt influenced by the observations of Sitte and Rennie (1963) and Hülsbruch (1966a,b), Sargent (1976a,c) interpreted the CM of *Libertia elegans* as entirely external to the CW, secreted from its junction with the underlying pectic layer, not as a cutin-impregnated region of PCW. She therefore referred to the CL as the 'secondary cuticle', interpreting polysaccharide fibrils extending into the secondary cuticle as extensions from the pectin lamella.

Orgell (1954, 1955) reported the isolation of the cuticles of *Convolvulus arvensis*, *Vinca major* and *Philodendron sp.* with pectic enzymes. The pectin-rich layer beneath the cuticle of pear (Norris and Bukovac, 1968) and *Citrus* leaves (Baker and Procopiu, 1975) can be removed using pectinase, thus liberating the CM. In some species and at some stages of development, treatments with EDTA (Letham, 1958; Schneider, 1960), oxalic acid or ammonium oxalate (Huelin and Gallop, 1951) have the same effect, presumably chelating calcium ions in the egg-box pectins of the

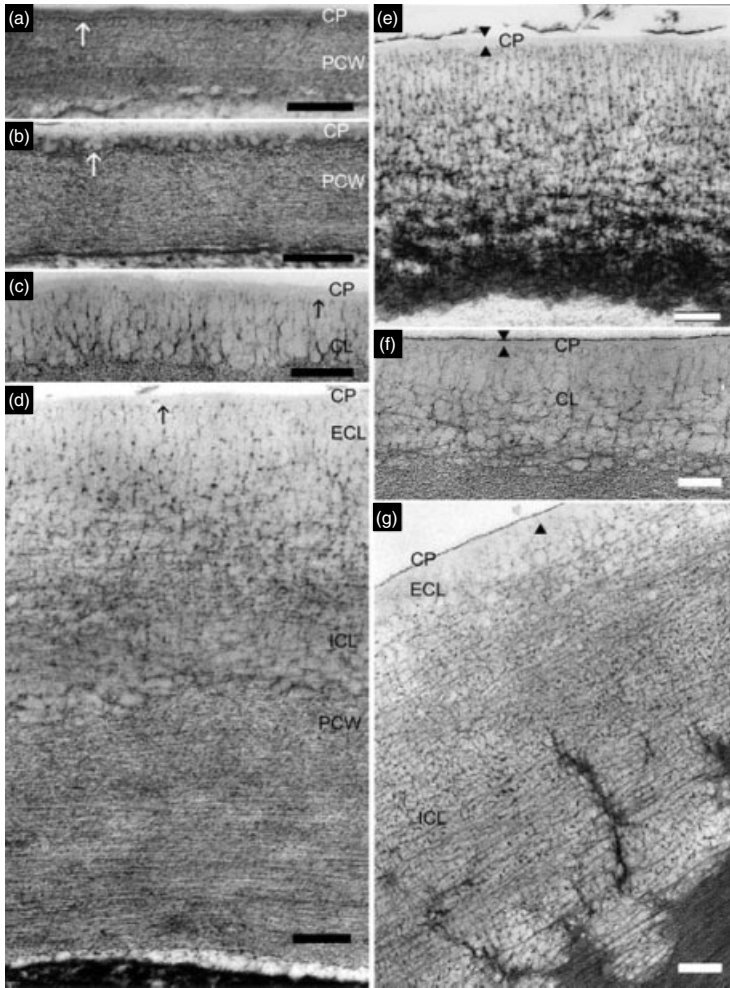


Figure 2.2 (a–e) Stages in cuticular membrane (CM) development in Norway spruce (*Picea abies*). (a) A thin (approximately 24 nm) apparently amorphous cuticle proper (CP) is formed outside the 240-nm thick PCW. Arrow marks an electron-dense superficial region of the primary cell wall (PCW). (b) Globular deposits beneath the CP initiate construction of a cuticular layer (CL) (arrow). (c) The reticulate CL now exceeds 200 nm thickness. Its fibrils terminate abruptly (arrow) beneath the amorphous CP. (d) The CL, now 1 μm thick, shows outer parallel-striated external cuticular layer (ECL) and inner cross-linked reticulate internal cuticular layer (ICL) regions above a non-cutinised secondary cell wall (SCW), now thickened to 1 μm . (e) A section stained *en bloc* with KMnO_4 , showing strong contrast between the fibrillar reticulum and the cutinous matrix of the CM. The abrupt termination of the CL fibrils beneath the CP is now very clear, but there is no trace of lamellae in the CP. (f, g) Two stages in CM development in Sitka spruce (*Picea sitchensis*). (f) A young Sitka spruce CM showing an amorphous CP (between arrowheads) demarcated by the abrupt termination of CL fibrillae, as in (c). (g) In an older leaf, the boundary between CP and the developing electron-lucent ECL layer is scarcely visible, its probable position indicated by the arrowhead. (a–g) Bars = 200 nm. Figures (a–e) from Tenberge (1992), *Canadian Journal of Botany*, **70**, 1467–1487. Figures 2.2f,g by P.J. Holloway.

pectic lamella. Norris and Bukovac (1968) report that the basal region of the isolated pear leaf cuticle (ICL as defined here) stains pink with ruthenium red, suggesting that pectins are embedded in the cutin matrix in positions protected from pectinase extraction. This point could be resolved today using immunogold labelling with antibodies against specific pectic epitopes, but there has been no attempt to characterise the location and type of pectin present beneath and embedded in the CM during a sequence of CM development. Although some cuticles are released rapidly by pectinase, those of mature leaves tend to be resistant (Lendzian *et al.*, 1986). In these cuticles the pectin lamella may become so impregnated with cutin that it is impossible to remove it using enzymes, and the CM is now attached to a region of the CW that is richer in cellulose. Combinations of cellulose and pectinase usually release such cuticles and are in routine use today (Schönherr and Riederer, 1986; Lendzian *et al.*, 1986), but more aggressive chemistry involving acids or strong oxidants may be required to liberate the CM from CW layers that are heavily cutinised or impregnated with lignin, as in conifer leaves (Silva Fernandes, 1964; Holloway and Baker, 1968).

Sargent (1976a,c) interpreted the pectin lamella as coterminous with the middle lamella. However, the middle lamella derives from the cell plate formed between daughter cells following cell division, and can only be formed internally between adjacent mother CW pairs, not at the outer periclinal wall of the epidermis. In order for the middle lamella to join a sub-cuticular lamella, a region of PCW would have to be removed (see Jeffree, 1996). The process of middle lamella formation has recently been discussed by Jarvis *et al.* (2003).

Viougeas *et al.* (1995) report that isolating the substantial (approximately 2 μm thick; Holloway, 1982a; Table 2.1) ivy cuticle with pectinase 2% and cellulase 0.2% at pH 4.5 does no damage to fine structure, and the isolated CM can therefore be considered valid for both fine structural and permeability studies. However, at the other end of the thickness spectrum, it is not yet possible to isolate the CM of e.g. *Arabidopsis thaliana* or of wheat intact, and therefore catastrophic damage is being caused to both fine structure and permeability properties. Between these extremes, a spectrum of artefactual damage must be assumed to occur, thus *caveat emptor*.

2.2 The structure of the cuticle proper

2.2.1 *The procuticle*

The CP appears on aerial plant organs very early in epidermal cell development, e.g. on unexpanded leaves still in bud (Wattendorff and Holloway, 1980; Schmidt and Schönherr, 1982; Riederer and Schönherr, 1988; Tenberge, 1992). Sargent (1976a,c) proposed that the CP be called the 'primary cuticle', by analogy with the terms primary and secondary as applied to the CW. However, it is now clear that

Table 2.1 Holloway's six cuticle structural types. The definitions are as stated by Holloway (1982a), but the examples given are selected by this author, and do not necessarily coincide either with Holloway's categorisation or the categories specified by other sources. Further examples and their source references are listed in Table 2.3

Type	Description	Examples
1	Polylamellate outer region, sharply delineated against inner mainly reticulate region	Leaves of <i>Agave americana</i> and <i>Clivia miniata</i>
2	Outer region faintly lamellate, gradually merging with inner mainly reticulate region	Leaves of <i>Hedera helix</i> and <i>Ficus elastica</i>
3	Outer region amorphous, inner region mainly reticulate	Leaves of <i>Plantago major</i> , <i>Picea abies</i> , <i>Citrus limon</i> and <i>Quercus velutina</i>
4	All regions reticulate	Leaf of <i>Hydrangea macrophylla</i> , fruit of <i>Lycopersicon esculentum</i>
5	All regions lamellate	Leaves of <i>Beta vulgaris</i> and <i>Taraxacum officinale</i>
6	Mainly amorphous	Leaves of <i>Potamogeton crispus</i> and <i>Brassica oleracea</i> var <i>gemmifera</i>

there is no secondary cuticle with the same external status as that of the CP, and the use of the terms primary and secondary cuticles now seems redundant. However the CP, as seen at maturity and in the youngest expanding leaves, is not the earliest manifestation of the cuticle. The very earliest epidermal cells in the shoot apices and leaf primordia are already covered by a highly water-repellent, osmiophilic film which forms an amorphous, electron-dense layer approximately 20 nm thick as seen in TEM sections (Figures 2.3a, 2.4a). In *Cuscuta gronovii* and *Utricularia sandersonii* an amorphous procuticle (Heide-Jørgensen, 1991) approximately 22 nm thick is present on the earliest protodermal surfaces (Figure 2.3a), increasing in thickness to approximately 30 nm, slightly reducing in electron-density to develop a globular transitional cuticle (Figure 2.3b) before transforming into a lamellate layer approximately 35–40 nm thick and containing two–three pairs of electron-dense and lucent lamellae (Figure 2.3c) identifiable as the first manifestation of the lamellate CP.

The procuticle is thus the first region of the cuticle to appear during cuticle ontogeny, quickly giving rise to the CP which is assembled externally to the PCW. In the cuticle of *Iris germanica* (Figure 2.5a, P.J. Holloway) the lamellate CP is devoid of any trace of fibrillar reticulum, which is first evident immediately beneath the basal electron-lucent lamella. Equally in cuticles with an amorphous zone representing the CP (Holloway's Cuticle Type 3) the CP is always totally devoid of fibrillar material, the fibrils terminating at a sharp boundary (Sitka spruce; Holloway, 1982a; Wattendorff and Holloway, 1984; Norway spruce; Tenberge, 1992; Figure 2.2b–e). It may be tempting to think that there is some relationship between the procuticle

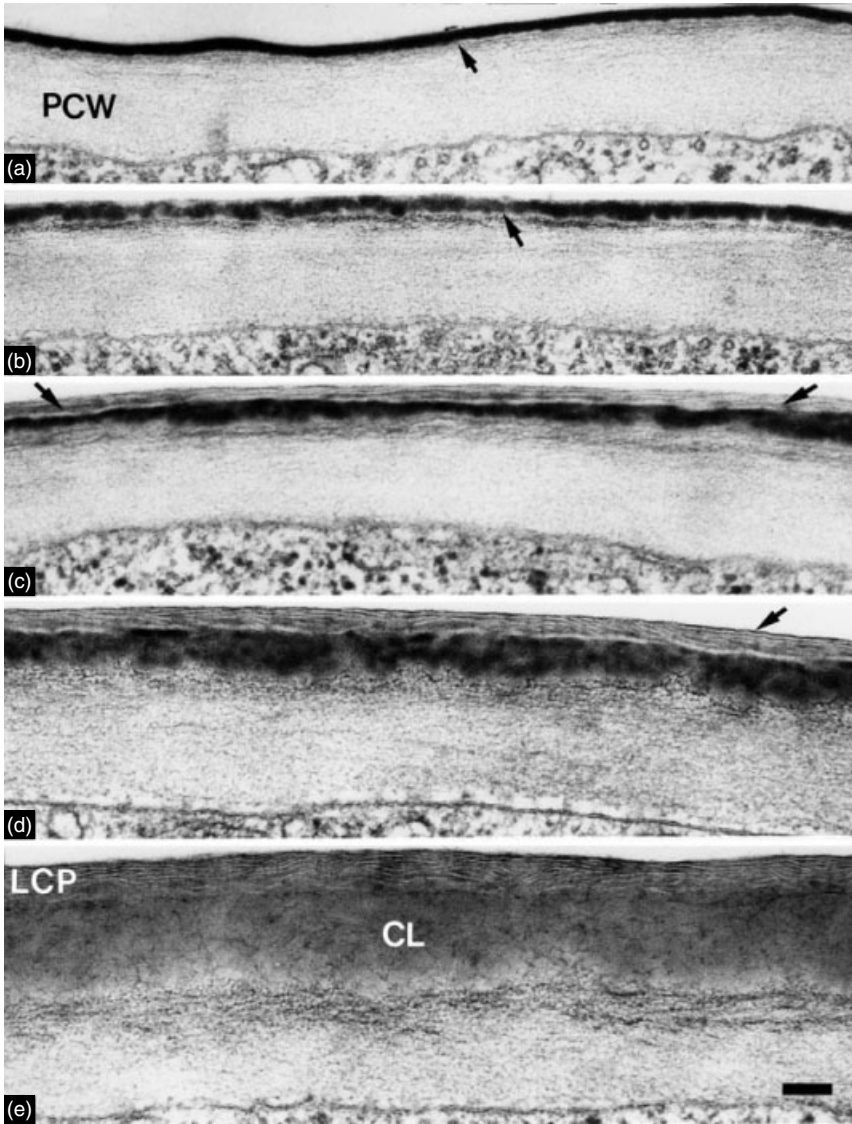


Figure 2.3 A sequence of stages in development of the cuticular membrane (CM) in the bladder primordium of *Utricularia laterifolia* (Droseraceae). (a) A uniformly electron-dense procuticle (arrow). (b) Transitional stage between procuticle and cuticle proper, showing globular, electron-dense material in a translucent matrix (arrow). The adjacent cell wall microfibrils (pectin lamella?) are electron-dense. (c) The procuticle has transformed into a lamellate cuticle proper (CP) (arrows), with an underlying electron-dense globular layer. (d) A reticulate cuticular layer (CL) is being constructed beneath the CP, which has more lamellae and is of greater thickness. (e) The reticulate CL is now as thick as the original primary cell wall (PCW), but the thickness of the uncutinised cell wall is undiminished. LCP = Lamellate cuticle proper. (a–e) Bar = 100 nm. Figures 2.3a–e reproduced with permission from Heide-Jørgensen (1991), *Planta*, **183**, 511–519.

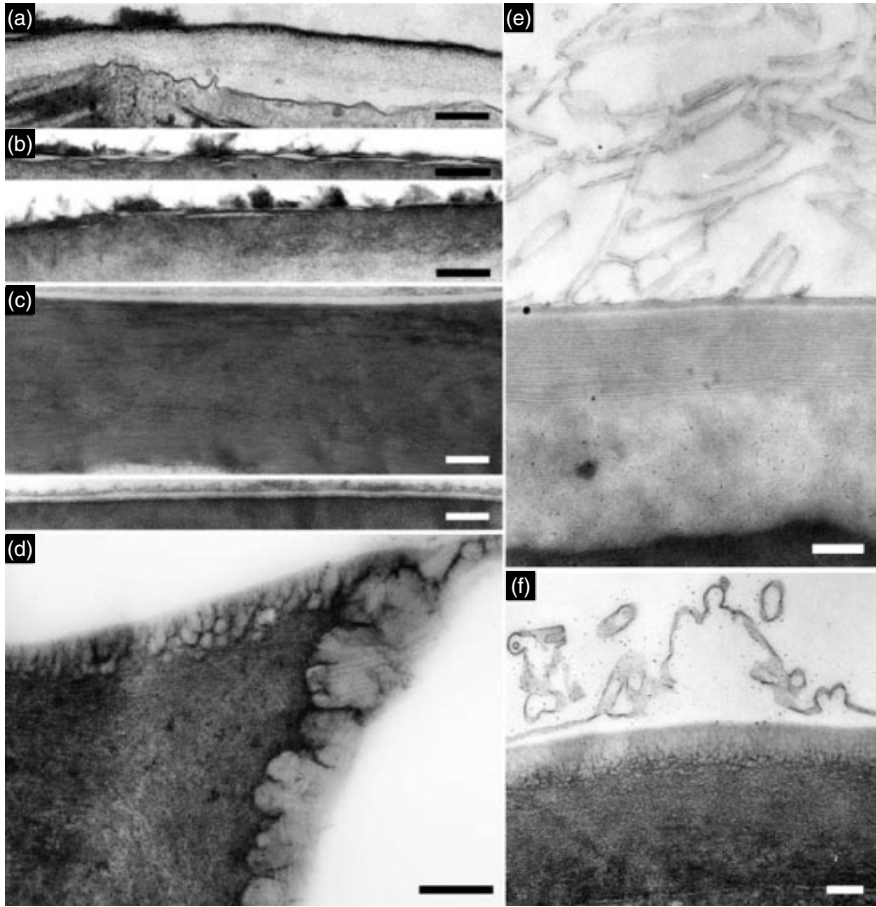


Figure 2.4 (a–d) Cuticles of mosses. (a) A three-week protonema of *Sphagnum fimbriatum* bears a superficial electron-dense layer analogous to the procuticle shown in Figure 2.3a. (b) Two sections of the cuticular membrane (CM) of ten-week protonemata of *S. fimbriatum*, showing a cuticle proper (CP) composed of lenticular electron-lucent lamellae embedded in electron-dense matrix. (c) The CM and outer epidermal cell wall of a *Marchantia polymorpha* gametophyte, fixed in GA/Os and stained with Reynolds lead citrate, shows a thin, apparently structureless CM. Staining with KMnO_4 enhances the contrast of the CM and resolves it as two equal layers equivalent to a CP and cuticular layer (CL; later). (d) The outer ledge of a guard cell in the capsule of a *Funaria hygrometrica* sporophyte shows lamellate/reticulate structure in the CM adjacent to the pore (right) but lamellae do not appear in the outer cuticle (top). The fibrils may reach the surface. (e, f) Sections of Type 1 (e) and Type 3 (f) cuticles in which the epicuticular waxes are preserved, showing their sizes relative to the CP and CL layers. (e) *Agave americana*: the lamellae of the CP are not interrupted or diverted beneath the wax crystals (f) Sitka spruce (*Picea sitchensis*). The diameter of the tube waxes is about half the thickness of the CM, but no pore of this size is resolved. Figures 2.4a–f bars = 200 nm. Figures 2.4a, b reproduced from Cook and Graham (1998), *International Journal of Plant Sciences*, **159**, 780–787. Figure 2.4c and inset by P.J. Holloway. Figures 2.4d from Sack and Paolillo (1983a) *Protoplasma*, **116**, 1–13. Figure 2.4e by J. Wattendorff, unpublished. Figure 2.4f from Holloway (1982a).

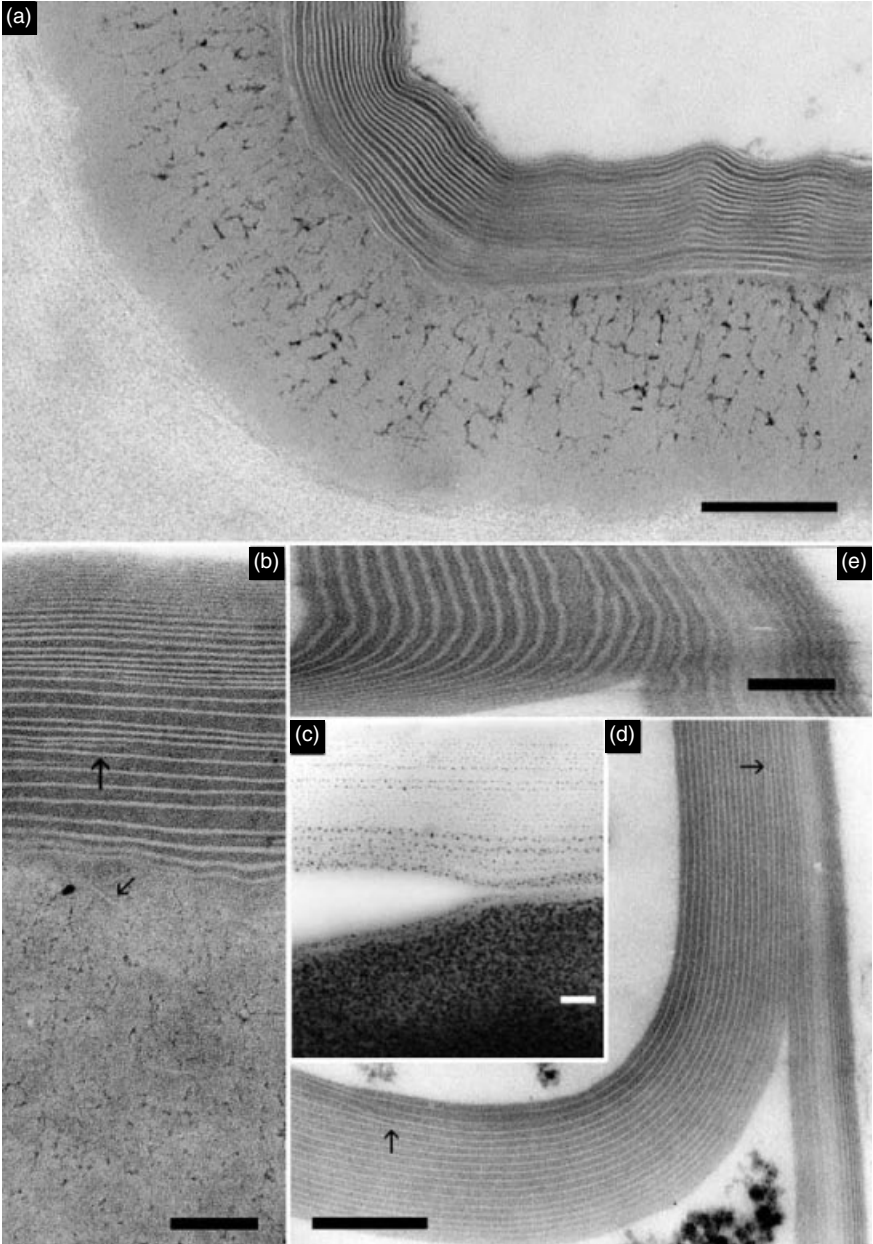


Figure 2.5 Transmission electron micrographs of Type 1 lamellate/reticulate cuticles. (a) Isolated cuticular membrane (CM) of *Iris germanica*, showing a thick lamellate cuticle proper (CP), and a coarsely reticulate cuticular layer (CL). Reticulations are mostly radial with horizontal cross-links between. (b–e) Cuticles of *Agave americana* leaves. (b) A CM fixed in Ga/Os/Ruthenium red without section stain shows enhancement of the density of electron-dense lamellae in the CP.

and the hydrophobic 'internal cuticle'. However, the presence of a CM on the surfaces of cells with a gas exchange function would be paradoxical. In the author's experience, no cuticle or procuticle *sensu* Heide-Jørgensen (1991) or Jeffree (1996) is present on mesophyll cell surfaces. It is relevant to note that the capacity to convert hydroxy-fatty acids into the polymer cutin is believed to be a specialised function of the epidermal cells alone, which is lacking in mesophyll (Kolattukudy, 1968, 1984, 1996; Hooker *et al.*, 2002).

2.2.2 *The cuticle proper*

The earliest appearance of the lamellate CP in *Phormium tenax* (Jarvis and Wardrop, 1974) is strikingly similar to that in *Clivia miniata* in which the lamellate CP is the earliest cuticular structure observed in sections taken from the youngest regions at the leaf base (Riederer and Schönherr, 1988). At the stage of transition from the procuticle (Figure 2.6), the CP consists only of two pairs of electron-lucent/electron-dense lamellae, and contains no detectable ester cutin. At 1–2 cm from the base the CP, now increased to about 70 nm thickness, has extended to 8–10 pairs of periclinal electron-dense and electron-lucent lamellae (Figure 2.6b), and contains ester cutin, although at this stage a non-saponifiable fraction is of greater mass. Leaf area increases by a factor of about 9 in the first 5 cm from the leaf base of *C. miniata* but the CP lamellae give no appearance of being stretched by this, maintaining the number of lamellae through to maturity. Indeed the thickness of the lamellae and thus of the CP is at its greatest during the period of most rapid expansion up to about 6 cm from leaf base (see also Gilly *et al.*, 1997).

During ontogeny of the CP in *U. sandersonii*, the osmiophilic procuticle begins to develop a flocculent structure prior to the appearance of lamellae on its surface (Figure 2.3b). This very electron-dense flocculent layer increases in thickness to about 100 nm while further lamellae are added to the stack, and begins to move into the outer layers of the CW (Figures 2.3c,d), representing the earliest phases of

(**Figure 2.5**) The periodicity of CP lamellae decreases towards the exterior, and lamellae may merge. Termination of an electron-lucent lamella is indicated by up-arrow. Reticulations in the external cuticular layer (ECL) extend to the base of the CP, and are interspersed with faint electron-lucent lamellae (diagonal arrow), (c) CP, ECL and part of internal cuticular layer (ICL) of a mid-aged leaf stained by the H₂SO₄-I₂/KI-Ag Proteinate reaction (Holloway *et al.*, 1981) demonstrates the distribution of epoxide groups in the electron-dense lamellae, and in ECL. The ICL is most intensely stained. As in (d and e), splitting of the CP has occurred within an electron-lucent lamella. (d) A CP detached from the CL during processing (Ga/Os fixation, U/Pb section staining) has split within an electron-lucent lamella. Arrows show terminations of electron-lucent lamellae within electron-dense lamellae. Periodicity of the lamellae decreases from inner (left) to outer (right) surfaces. The electron-dense lamellae show tripartite structure, a central electron-lucent lamella separating two denser subunits. (e) Tripartite structure of the electron-dense lamellae in Figure 2.5d is emphasised by digital foreshortening of the image. (a,d) Bar = 200 nm; (b,c) bars = 100 nm; (e) bar = 50 nm. Figure 2.5a by P.J. Holloway, unpublished, Figures 2.5b–e reproduced from Wattendorff and Holloway (1980), *Annals of Botany*, **46**, 13–28.

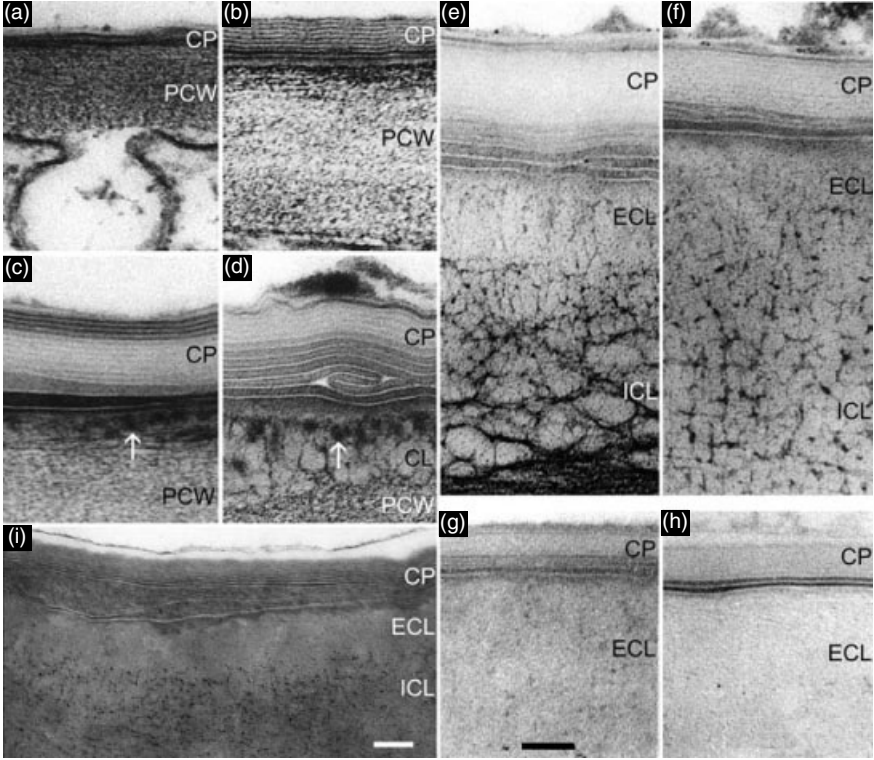


Figure 2.6 A sequence of developmental stages of the cuticular membrane (CM) of *Clivia miniata* (Amaryllidaceae) leaf (adaxial): (a) the leaf base: a simple cuticle proper (CP), with one–two pairs of lamellae. (b) The CP 1 cm from base has eight–nine pairs of lamellae, underlain by an electron-dense, probably pectinaceous, region of the outer primary cell wall. (c) The CP 2 cm from base: twelve–thirteen pairs of CP lamellae are now present, of greater and more variable thickness. The innermost and outermost lamellae are denser than in the central region. Electron-dense globules are accumulating beneath the CP, commencing the process of cuticular layer (CL) construction (arrow). (d) CP with twelve–thirteen pairs of lamellae, 3 cm from the leaf base. The electron-dense lamellae show tripartite structure. Almost 200 nm of reticulate CL layer has developed, electron-dense globules remaining in its outer layer (arrow). (e) The CP 4 cm from leaf base, in the cell-expansion phase, the CL has a finely reticulate external layer (ECL) and a coarsely reticulate inner layer (ICL). (f) The CP 5 cm from leaf base, late in the cell-expansion phase, the reticulum of the ECL extends the base of the CP. (g) The CP 6 cm from the leaf base, the reticulum in the ECL layer is still visible but with low contrast. (h) The CP 20 cm from the leaf base, the lamellae are of low contrast except at the base of the CP. Fibrils are no longer visible in the ECL, which now contains faint lamellae. Os; U/Pb, x 75,000; bar = 100 nm. (i) The CM of *C. miniata* in a leaf of unspecified age, showing parallel–periclinal lamellae in the CP, chaotically orientated lamellae at the boundary between CP and ECL, with reticulate structure visible only towards its base, and the reticulate ICL beneath. Bar = 200 nm. Figures 2.6a–h reproduced with permission from Riederer and Schönherr (1988), *Planta*, **174**, 127–138. Figure 2.6i reproduced with permission from Holloway (1982a).

construction of the CL layer by impregnation of the PCW. Additions of lamellae within the outermost parts of the PCW are often made in a less precisely periclinal orientation to those first secreted outside it in the CP (Figure 2.3d,e). Ultimately the lamellate region of the CP in *Utricularia* is about 80 nm thick, and is subtended by a reticulate CL that is more than 200 nm thick and which is now more electron lucent compared with the initial stages (Figure 2.3e).

Micrographs by H.S. Heide-Jørgensen published in Jeffree (1996) showed a thick lamellate CP under construction in *Hakea suaveolens* with densely osmiophilic basal CP lamellae. Osmiophilic globules ranging from about 20 to 75 nm in diameter are enclosed in an electron-lucent lamella about 5 nm thick, which appears to unwrap from the surface of the electron-dense globule as it coalesces with the base of the CP. Closely analogous structures are also seen in suberised layers under development in the wall of a suberised epidermal idioblast in *Cassytha pubescens* (Heide-Jørgensen, in Jeffree, 1996; see also de Vries, 1968). The lucent lamellae in the CP are widely held to contain soluble wax, as discussed later. If composed of nonpolar soluble cuticular lipids (SCL), then the position of electron-lucent lamellae outside such hydrophobic globules is incompatible with the aqueous and polar environment of the apoplast. However, a thin (approximately 2.5 nm), dense border is present that may be consistent with an outer protein shell, or the polar functional groups of long-chain fatty acids. The dynamics of the movement of these globules through the CW matrix (if indeed they do move through it) is obscure, as is the composition of the electron-dense and electron-lucent components of this system, and of the construction process itself (self-assembly or protein mediated assembly?), offering fertile ground for fundamental research.

2.2.2.1 Lamellate substructure of the CP

Probably in most species, the CP is of lamellate construction. In those species of cuticle Types 1 and 2 for which data are shown in Table 2.2 the mean thickness of the lamellate region is about 150 ± 32 nm, exceptionally more than $1 \mu\text{m}$ in thickness, as in *H. suaveolens* (Heide-Jørgensen, 1978b). For many other species, listed as Type 3 by Holloway (1982a; Table 2.2), the CP appears amorphous, as in *Picea abies* and *Picea sitchensis*. In these species the lamellate structure is either absent or composed of materials of similar electron density, and thus of low contrast.

As noted by Wattendorff (1984) the CP lamellae in *Agave americana* show substructure within the basic alternating layers of light and dark [relative to a positive print from the EM negative, this translates into electron-lucent ($\bar{\epsilon}\text{L}$) and electron-dense ($\bar{\epsilon}\text{D}$)]. Lamellar substructure is evident in many of his excellent TEM images of young and old leaves, and the reality of the observations is reinforced by the fact that images viewed both unstained and contrasted using various different staining and contrasting methods show the same pattern. The more $\bar{\epsilon}\text{D}$ lamellae are revealed as composed of three units – two outer dense lines and an inner unit of intermediate density (Figures 2.5d and e). The $\bar{\epsilon}\text{D}$ lamellae of the CL layer in *A. americana* also

Table 2.2 Summary of the distribution of plant cuticle structural types, and the dimensions of the cuticular membrane, cuticle proper and cuticular layer. Allocation to type is based on the constraints on Holloway's definitions (Holloway, 1982a; Table 2.1) discussed in this chapter, principally that 'outer region' equates strictly to the cuticle proper (CP) and 'inner region' equates to the cuticular layer (CL)

Species	Family or order	Organ	Type	CM (nm)	CP (nm)	CL (nm)	Reference
<i>Avena fatua</i>	Poaceae	St	Pc	33	~	33	Kaufman <i>et al.</i> (1970)
<i>Gossypium hirsutum</i>	Malvaceae	Fibres	Pc	20	~	~	Ryser and Holloway (1985)
<i>Gossypium hirsutum</i>	Malvaceae	SC, fringe layer	Pc	20	~	~	Ryser <i>et al.</i> (1988)
<i>Gossypium hirsutum</i>	Malvaceae	Nucellus, at anthesis	Pc	20	~	~	Ryser <i>et al.</i> (1988)
<i>Myrtophyllum verticillatum</i>	Haloragaceae	A	Pc	20	20	~	Hallam (1982)
<i>Phaseolus vulgaris</i>	Fabaceae	ssc	Pc	20	~	~	Jeffree (1996)
<i>Pisum sativum</i>	Fabaceae	A	Pc	20	~	~	Jeffree (1996)
<i>Utricularia laterifolia</i>	Lentibulariaceae	Bladder LAd	Pc	25	~	~	Heide-Jørgensen (1991)
<i>Adiantum capillus-veneris</i>	Adiantaceae	L	I	~	~	~	Wada and Staehelin (1996)
<i>Adiantum raddianum</i> var. <i>raddianum</i>	Adiantaceae	L	I	1000	~	~	Archer and Cole (1986)
<i>Agave americana</i>	Agavaceae	L	I	~	~	~	Crisp (1965)
<i>Agave americana</i>	Agavaceae	L	I	~	~	~	Wattendorff and Holloway (1980)
<i>Agave americana</i>	Agavaceae	L	I	4000	450	3550	Wattendorff and Holloway (1984)
<i>Agave lutea</i>	Agavaceae	L	I	750	238	513	Holloway, unpublished
<i>Allium cepa</i>	Liliaceae	Bs	I	760	160	600	Schönherr and Mérida (1981)
<i>Anabasis articulata</i>	Chenopodiaceae	S	I	1024	134	890	Lysheide (1977b)
<i>Apium graveolens</i>	Apiaceae	P	I	270	49	221	Chafé and Wardrop (1973)
<i>Apium graveolens</i>	Apiaceae	P	I	850	130	720	Juniper and Cox (1973)
<i>Apium sativum</i>	Apiaceae	P	I	660	85	575	Hallam and Juniper (1971)
<i>Arabidopsis thaliana</i> WT	Brassicaceae	S	I	128	64	60	Sieber <i>et al.</i> (2000)
<i>Ardisia crenata</i>	Myrsinaceae	LAb	I	800	160	640	Fisher and Bayer (1972)
<i>Ardisia crenata</i>	Myrsinaceae	LAd	I	1600	250	1350	Fisher and Bayer (1972)
<i>Beta vulgaris</i>	Chenopodiaceae	LAd	I	88	30	58	Holloway, unpublished
<i>Beta vulgaris</i>	Chenopodiaceae	LAd	I	160	120	40	Holloway, unpublished
<i>Bobartia gracilis</i>	Iridaceae	L	I	2500	500	2000	Sargent (1976a,b)
<i>Buxus sempervirens</i>	Buxaceae	L	I	0	0	0	Gouret <i>et al.</i> (1993)

<i>Clivia miniata</i>	Amariyllidaceae	L	1	210	210	~	Holloway (1982a)
<i>Clivia miniata</i>	Amariyllidaceae	L	1	4500	200	4300	Holloway (1982a)
<i>Clivia miniata</i>	Amariyllidaceae	LAd	1	6700	130	6570	Mérida <i>et al.</i> (1981)
<i>Clivia nobilis</i>	Amariyllidaceae	L	1	~	~	~	Holloway (1982a)
<i>Crocus chrysanthus</i>	Liliaceae	Stigma	1	900	100	800	Heslop-Harrison and Heslop-Harrison (1975, 1982); Heslop-Harrison (1977)
<i>Cunninghamia lanceolata</i>	Pinaceae	L	1	~	~	~	Sargent (1976b)
<i>Cuscuta campestris</i>	Convolvulaceae	S	1	70	30	40	Jeffrey (1986)
<i>Dionaea muscipula</i>	Droseraceae	T	1	560	95	465	Sievers (1968)
<i>Eryngium rostratum</i>	Apiaceae	P	1	800	130	670	Chafe and Wardrop (1973)
<i>Eucalyptus papuana</i>	Myrtaceae	L	1	100	45	45	Hallam (1970a)
<i>Eucalyptus perriniana</i>	Myrtaceae	L	1	320	177	143	Hallam (1982)
<i>Ficus elastica</i>	Moraceae	L	1	0	0	0	Gouret <i>et al.</i> (1993)
<i>Funaria hygrometrica</i>	Funariales	Capsule	1	200	130	70	Sack and Paolillo, Jr. (1983a)
<i>Gasteria planifolia</i>	Liliaceae	L	1	3000	130	2870	Holloway (1982a)
<i>Gasteria verrucosa</i>	Liliaceae	L	1	180	180	~	Heumann (1990)
<i>Gordonia axillaris</i>	Theaceae	L	1	2000	160	1840	Sargent (1976a,b)
<i>Gossypium hirsutum</i>	Malvaceae	Fibres	1	25	25	0	Ryser (1985)
<i>Hakea leucoptera</i>	Proteaceae	L	1	150	~	~	Sargent (1976b)
<i>Hakea suaveolens</i>	Proteaceae	L	1	11500	1500	10000	Heide-Jørgensen (1978b)
<i>Hakea suaveolens</i>	Proteaceae	T	1	1492	62	1430	Heide-Jørgensen (1980)
<i>Hedera helix</i>	Araliaceae	L	1	2500	100	2400	Viougeas <i>et al.</i> (1995)
<i>Hedera helix</i>	Araliaceae	L	1	0	0	0	Gouret <i>et al.</i> (1993)
<i>Hedera helix</i>	Araliaceae	Young leaf	1	40	40	0	Gilly <i>et al.</i> (1997)
<i>Hedera helix</i>	Araliaceae	Older leaf	1	210	70	140	Gilly <i>et al.</i> (1997)
<i>Hedera helix</i>	Araliaceae	Expanded leaf	1	700	80	620	Gilly <i>et al.</i> (1997)
<i>Humulus lupulus</i>	Moraceae	P	1	~	~	~	Chafe and Wardrop (1973)
<i>Iris germanica</i>	Iridaceae	L	1	800	95	700	Holloway <i>et al.</i> (1981)
<i>Lactuca sativa</i>	Asteraceae	L	1	100	50	50	Holloway <i>et al.</i> (1981)
<i>Liberita elegans</i>	Iridaceae	L	1	700	150	550	Sargent (1976b)
<i>Liberita ixiooides</i>	Iridaceae	L	1	700	150	550	Sargent (1976a,b)

Table 2.2 Continued

Species	Family or order	Organ	Type	CM (nm)	CP (nm)	CL (nm)	Reference
<i>Liriodendron tulipifera</i>	Magnoliaceae	L, CF air	I	200	54	146	McQuattie and Rebbeck (1994)
<i>Malus pumila</i>	Rosaceae	F	I	~	~	~	Linskens and Gellissen (1966)
<i>Malus pumila</i>	Rosaceae	F	I	~	~	~	Hilkenbäumer (1958)
<i>Malus pumila</i>	Rosaceae	L	I	380	40	340	Hoch (1979)
<i>Malus pumila</i>	Rosaceae	L, Ab	I	980	52	928	Holloway (1982a)
<i>Nicotiana glauca</i>	Solanaceae	L	I	250	46	204	Mérida and Ogura (1987)
<i>Nicotiana tabacum</i>	Solanaceae	L	I	260	35	225	Krüger <i>et al.</i> (1996)
<i>Phaseolus vulgaris</i>	Fabaceae	Outer St ridge	I	444	143	300	Holloway, unpublished
<i>Phaseolus vulgaris</i>	Fabaceae	Lower St ridge	I	20	~	~	Holloway, unpublished
<i>Phaseolus vulgaris</i>	Fabaceae	L	I	120	16	104	Holloway (1982a)
<i>Phormium tenax</i>	Agavaceae	L	I	333	133	200	Hallam (1982)
<i>Phormium tenax</i>	Agavaceae	L	I	1063	144	919	Holloway, unpublished
<i>Phormium tenax</i>	Agavaceae	L	I	635	120	515	Jarvis and Wardrop (1974)
<i>Phormium tenax</i>	Agavaceae	Papillae	I	4300	200	4100	Jarvis and Wardrop (1974)
<i>Pistacia palaestina</i>	Anacardiaceae	L	I	250	170	80	Sargent (1976b)
<i>Plantago major</i>	Plantaginaceae	L	I	230	50	180	Fisher and Bayer (1972)
<i>Pseudotsuga menziesii</i>	Pinaceae	L	I	150	150	~	Sargent (1976b)
<i>Psiloutum nudum</i>	Psilotaceae	Gametophyte	I	375	200	175	Whittier and Peterson (1995)
<i>Sarracenia psittacina</i>	Sarraceniaceae	Pitcher, inside	I	122	40	82	Joel and Heide-Jørgensen (1985)
<i>Sarracenia psittacina</i>	Sarraceniaceae	Pitcher, inside	I	43	25	18	Joel and Heide-Jørgensen (1985)
<i>Sarracenia purpurea</i>	Sarraceniaceae	Pitcher, outside	I	770	39	731	Joel and Heide-Jørgensen (1985)
<i>Secale cereale</i>	Poaceae	SP	I	26	5	21	Heslop-Harrison (1977)
<i>Silene dioica</i>	Caryophyllaceae	SP	I	330	75	255	Heslop-Harrison and Heslop-Harrison (1975, 1982); Heslop-Harrison (1977)
<i>Sisyrinchium filifolium</i>	Iridaceae	L, Cuticle < 2 µm thick	I	1500	~	~	Heslop-Harrison (1977)
<i>Spartocytisus filipes</i>	Fabaceae	S	I	~	~	~	Sargent (1976b)
<i>Symphlocos paniculata</i>	Theaceae	L, Ab	I	193	40	153	Lysheide (1978) Tegelhaar (1990)

<i>Tamarix pentandra</i>	Tamaricaceae	L	1	200	200	~	Sargent (1976b)
<i>Taraxacum officinale</i>	Asteraceae	L	1	182	30	152	Holloway (1982a)
<i>Ticoa harrisi</i>	Cycadeae	Lad	1	3270	820	2180	Archangel'sky <i>et al.</i> (1986)
<i>Utricularia laterifolia</i>	Lentibulariaceae	Bladder LAd	1	313	78	235	Heide-Jørgensen (1991)
<i>Vanilla planifolia</i>		L	1	0	0	0	Gouret <i>et al.</i> (1993)
<i>Cuscuta gronovii</i>	Convolvulaceae	S	1,5	260	50	210	Heide-Jørgensen (1991)
<i>Abutilon striatum</i>	Malvaceae	P	2	~	~	~	Chafe and Wardrop (1973)
<i>Anabasis articulata</i>	Chenopodiaceae	S	2	1120	179	941	Lysheide (1977b)
<i>Ardisia crenata</i>	Myrsinaceae	L	2	1600	250	1350	Fisher and Bayer (1972)
<i>Arum maculatum</i>	Araceae	L	2	320	200	120	Holloway (1982a)
<i>Dionaea muscipula</i>	Droseraceae	T	2	~	~	~	Sievers (1968)
<i>Eucalyptus papuana</i>	Myrtaceae	L	2	100	45	45	Hallam (1970a)
<i>Ficus elastica</i>	Moraceae	L	2	~	~	~	Holloway (1982a)
<i>Hedera helix</i>	Araliaceae	L	2	1957	260	1697	Holloway (1982a)
<i>Lactuca sativa</i>	Asteraceae	L	2	250	36	214	Srivastava <i>et al.</i> (1977)
<i>Malus pumila</i>	Rosaceae	LAd	2	~	~	~	Hoch (1975, 1979)
<i>Malus sp.</i>	Rosaceae	LAd	2	525	250	275	Holloway, unpublished
<i>Spinacia oleracea</i>	Chenopodiaceae	L	2	240	35	205	Holloway <i>et al.</i> (1981)
<i>Narcissus pseudonarcissus</i>	Amaryllidaceae	Petal	3	220	40	180	Holloway, unpublished
<i>Acacia senegal</i>	Fabaceae	L	3	2000	55	1945	Wattendorff (1974)
<i>Acer saccharum</i>	Aceraceae	LAd	3	4200	1200	3000	Gordon (1995)
<i>Acer saccharum</i>	Aceraceae	LAb	3	1875	500	1375	Gordon (1995)
<i>Arabidopsis thaliana wax2</i>	Brassicaceae	S	3	209	90	120	Sieber <i>et al.</i> (2000)
<i>Arum maculatum</i>	Araceae	LAd	3	320	68	252	Holloway, unpublished
<i>Arum maculatum</i>	Araceae	LAb	3	220	40	180	Holloway, unpublished
<i>bergenia purpurascens</i>	Saxifragaceae	L	3	175	31	144	Holloway, unpublished
<i>Berula erecta</i>	Apiaceae	Aerial L	3	161	800	810	Frost-Christensen <i>et al.</i> (2003)
<i>Cannabis sativa</i>	Moraceae	G	3	200	20	180	Mahlberg and Kim (1992)
<i>Chaetochytrium intrepidus</i>	Scrophulariaceae	L	3	~	~	~	Lehmann and Schulz (1976)
<i>Citrus limon</i>	Rutaceae	L	3	1385	30	1354	Holloway (1982a)
<i>Cuscuta odorata</i>	Convolvulaceae	S	3	~	~	~	Weinert and Barckhaus (1975)
<i>Dianthus caryophyllus</i>	Caryophyllaceae	L	3	2000	~	~	Reed (1979)

Table 2.2 Continued

Species	Family or order	Organ	Type	CM (nm)	CP (nm)	CL (nm)	Reference
<i>Dionaea muscipula</i>	Droseraceae	G	3	90	~	~	Joel and Juniper (1982)
<i>Drosophyllum lusitanica</i>	Droseraceae	G	3	300	800	220	Joel and Juniper (1982)
<i>Dryopteris filix mas</i>	Aspidiaceae	L	3	138	40	98	Holloway (1982a)
<i>Eucalyptus perriniana</i>	Myrtaceae	L	3	500	~	~	Hallam (1970a)
<i>Fagus sylvatica</i>	Fagaceae	L, Ad	3	309	43	266	Bussotti <i>et al.</i> (1998)
<i>Ficus lyrata</i>	Moraceae	L	3	2000	~	~	Davis (1987)
<i>Fragaria ananassa</i>	Rosaceae	L	3	480	160	320	Holloway (1982a)
<i>Galphimia brasiliensis</i>	Malpighiaceae	Glandular T	3	690	17	667	Castro <i>et al.</i> (2001)
<i>Gordonia axillaris</i>	Theaceae	L	3	1900	316	1560	Holloway, unpublished
<i>Gossypium hirsutum</i> cv <i>Green Lint</i>	Malvaceae	L	3	156	~	~	Ryser and Holloway (1985)
<i>Gossypium hirsutum</i> cv <i>Green Lint</i>	Malvaceae	SC	3	10055	55	10000	Ryser and Holloway (1985)
<i>Gossypium hirsutum</i> cv <i>Green Lint</i>	Malvaceae	Nucellus, post-anthesis	3	~	20	~	Ryser <i>et al.</i> (1988)
<i>Hydrangea macrophylla</i>	Saxifragaceae	L	3	727	55	672	Holloway (1982a)
<i>Ligustrum ovalifolium</i>	Oleaceae	L	3	770	~	~	Hallam and Juniper (1971)
<i>Lilium candidum</i>	Liliaceae	L	3	500	150	350	Maier (1968)
<i>Liriodendron tulipifera</i>	Magnoliaceae	L, 2xO3	3	150	54	96	McQuattie and Rebeck (1994)
<i>Lobelia dortmanna</i>	Lobeliaceae	Aerial L	3	952	220	732	Frost-Christensen <i>et al.</i> (2003)
<i>Lobelia dortmanna</i>	Lobeliaceae	Aquatic L	3	938	220	718	Frost-Christensen <i>et al.</i> (2003)
<i>Malus pumila</i>	Rosaceae	L, ab	3	980	52	928	Holloway (1982a)
<i>Malus sp.</i>	Rosaceae	L, ab	3	812	205	667	Holloway, unpublished
<i>Malus sp.</i>	Rosaceae	L, ab, Inner epidermal cuticle	3	2000	~	~	Holloway, unpublished
<i>Muscari atlanticum</i>	Liliaceae	L	3	~	~	~	Holloway (1982a)
<i>Oenothera organensis</i>	Onagraceae	SP	3	250	70	180	Heslop-Harrison and Heslop-Harrison (1982)
<i>Phaseolus vulgaris</i>	Fabaceae	Leaf	3	1357	107	~	Hallam (1982)

<i>Phyllirea latifolia</i>	Oleaceae	Mature T	3	1154	~	~	Gravano <i>et al.</i> (1998)
<i>Phyllirea latifolia</i>	Oleaceae	Young T	3	219	~	~	Gravano <i>et al.</i> (1998)
<i>Phyllirea latifolia</i>	Oleaceae	Senescent T	3	500	~	~	Gravano <i>et al.</i> (1998)
<i>Phyllitis scolopendrium</i>	Aspleniaceae	L	3	~	~	~	Holloway (1982a)
<i>Picea abies</i>	Pinaceae	L	3	3600	44	3556	Tenberge (1992)
<i>Picea rubens</i>	Pinaceae	L	3	370	45	325	Percy <i>et al.</i> (1992)
<i>Picea sitchensis</i>	Pinaceae	L	3	2200	200	2000	Holloway (1982a)
<i>Picea sitchensis</i>	Pinaceae	Young L	3	288	15-25	273-263	Holloway (1982a)
<i>Pinus sylvestris</i>	Pinaceae	Leaf, control	3	192	64	128	Laakso <i>et al.</i> (2000)
<i>Pinus sylvestris</i>	Pinaceae	Leaf UVB treated	3	412	74	338	Laakso <i>et al.</i> (2000)
<i>Pinus strobus</i>	Pinaceae	L, 2 × O ₃	3	150	54	96	McQuattie and Rebbeck (1994)
<i>Pinus strobus</i>	Pinaceae	L, CF air	3	500	54	446	McQuattie and Rebbeck (1994)
<i>Plantago major</i>	Plantaginaceae	L	3	~	~	~	Crisp (1965)
<i>Plantago major</i>	Plantaginaceae	L	3	275	38	238	Holloway (1982a)
<i>Potamogeton crispus</i>	Potamogetonaceae	L	3	95	~	~	Holloway, unpublished
<i>Prunus laurocerasus</i>	Rosaceae	L	3	188	64	124	Holloway (1982a)
<i>Prunus persica</i>	Rosaceae	L	3	~	~	~	Schneider and Dargent (1977)
<i>Pyrus communis</i>	Rosaceae	LAb	3	500	100	400	Holloway (1982a)
<i>Pyrus communis</i> cv 'Passe Crassanne'	Rosaceae	L	3	~	~	~	Gouret <i>et al.</i> (1993)
<i>Quercus velutina</i>	Fagaceae	LAb GC outer, SUN	3	871	97	774	Osborn and Taylor (1990)
<i>Quercus velutina</i>	Fagaceae	LAb GC outer, SHADE	3	661	48	613	Osborn and Taylor (1990)
<i>Quercus velutina</i>	Fagaceae	LAb GC outer, SUN	3	145	~	~	Osborn and Taylor (1990)
<i>Quercus velutina</i>	Fagaceae	LAb GC outer, SHADE	3	355	~	~	Osborn and Taylor (1990)
<i>Quercus velutina</i>	Fagaceae	LAd, SUN	3	2960	~	~	Osborn and Taylor (1990)
<i>Quercus velutina</i>	Fagaceae	LAd, SHADE	3	890	~	~	Osborn and Taylor (1990)
<i>Quercus velutina</i>	Fagaceae	LAb	3	500	~	~	Osborn and Taylor (1990)
<i>Ribes nigrum</i>	Saxifragaceae	L	3	~	~	~	Holloway (1982a)
<i>Rumex sp.</i>	Polygonaceae	Glandular T	3	350	38	313	Holloway (1982a)
<i>Rumex conglomeratus</i>	Polygonaceae	Glandular T	3	650	150	500	Chafe and Wardrop (1973)
<i>Phyllitisscolopendrium</i>	Dspleniaceae	L	3	77	21	56	Holloway, unpublished
<i>Spartocytisus filipes</i>	Fabaceae	S	3	17000	140	17000	Lysheide (1978)

Table 2.2 Continued

Species	Family or order	Organ	Type	CM (nm)	CP (nm)	CL (nm)	Reference
<i>Spinacia oleracea</i>	Chenopodiaceae	LAd	3	263	110	160	Holloway, unpublished
<i>Stellaria media</i>	Caryophyllaceae	L	3	58	15	43	Holloway (1982a)
<i>Syagrus coronata</i>	Arecaceae	L	3	~	~	~	Machado and Barros (1995)
<i>Symplocos hallensis</i>	Theaceae	L	3	2182	0	2182	Tegelaar (1990)
<i>Symplocos paniculata</i>	Theaceae	LAd	3	484	40	444	Tegelaar (1990)
<i>Tulipa gesneriana</i>	Liliaceae	L	3	240	80	160	Holloway (1982a)
<i>Vaccinium reticulatum</i>	Ericaceae	P	3	2100	0	2100	Singh and Hemmes (1978)
<i>Vicia faba</i>	Fabaceae	L	3	110	75	25	Holloway, unpublished
<i>Vicia faba</i>	Fabaceae	L	3	188	63	125	Holloway, unpublished
<i>Abies balsamea</i>	Pinaceae	L	4	1200	~	~	Chabot and Chabot (1977)
<i>Aloe arborescens</i>	Liliaceae	L	4	2200	~	2200	Kluge <i>et al.</i> (1979)
<i>Avena fatua</i>	Poaceae	C	4	1100	~	1100	O'Brien (1967)
<i>Brassica oleracea</i> var. <i>botrytis</i>	Brassicaceae	Stigma	4	125	~	~	Elleman <i>et al.</i> (1988)
<i>Caltha palustris</i>	Ranunculaceae	Petal	4	580	~	580	Whatley (1984)
<i>Cannabis sativa</i>	Moraceae	G	4	157	~	157	Kim and Mahlberg (1995)
<i>Cannabis sativa</i>	Moraceae	T	4	190	~	190	Mahlberg and Kim (1991)
<i>Cannabis sativa</i>	Moraceae	T	4	590	~	590	Hammond and Mahlberg (1978)
<i>Capsicum annuum</i>	Solanaceae	L	4	~	~	~	Gouret <i>et al.</i> (1993)
<i>Citrus paradisi</i>	Rutaceae	Peel	4	1900	~	~	Espelle <i>et al.</i> (1980)
<i>Citrus paradisi</i>	Rutaceae	SC	4	1000	~	~	Espelle <i>et al.</i> (1980)
<i>Citrus sinensis</i>	Rutaceae	Fruit	4	~	~	~	Thomson and Platt-Alloia (1976)
<i>Coffea arabica</i>	Rubiaceae	L	4	2000	~	2000	Holloway <i>et al.</i> (1981)
<i>Coffea arabica</i>	Rubiaceae	LAd	4	1000	~	1000	Holloway, unpublished
<i>Cryptomeria japonica</i>	Pinaceae	L	4	2000	~	2000	Sargent (1976b)
<i>Drosera</i> sp.	Droseraceae	G	4	130	~	~	Chafe and Wardrop (1973)
<i>Eccremocarpus scaber</i>	Bignoniaceae	Td	4	250	~	~	Junker (1977)
<i>Galium aparine</i>	L	L	4	~	~	~	Gouret <i>et al.</i> (1993)
<i>Genista aetnensis</i>	Fabaceae	L	4	850	~	850	Lysheide (1982)

<i>Hakea leucoptera</i>	Proteaceae	T	4	~	~	~	Sargent (1976b)
<i>Ilex aquifolium</i>	Aquifoliaceae	LAd	4	1880	~	~	Holloway (1982a)
<i>Ilex aquifolium</i>	Aquifoliaceae	LAb	4	28	~	~	Holloway (1982a)
<i>Ilex aquifolium</i>	Aquifoliaceae	L	4	~	~	~	Holloway (1982a)
<i>Ilex aquifolium</i>	Aquifoliaceae	L	4	10000	~	~	Sargent (1976b)
<i>Ilex integra</i>	Aquifoliaceae	L	4	~	~	~	Hiltsbruch (1966a,b)
<i>Lycopersicon esculentum</i>	Solanaceae	L	4	~	~	~	Gourat, Rohr, and Chamel (1993)
<i>Lycopersicon esculentum</i>	Solanaceae	F	4	9000	~	9000	Wilson and Sterling (1976)
<i>Lycopersicon esculentum</i>	Solanaceae	F	4	2000	~	2000	Rijkenberg <i>et al.</i> (1980)
<i>Magnolia grandiflora</i>	Magnoliaceae	L Ad.	4	1800	~	~	Postek (1981)
<i>Malus pumila</i>	Rosaceae	F	4	10000	~	10000	de Vries (1968a)
<i>Nicotiana tabacum</i>	Solanaceae	T	4	300	~	300	Akers <i>et al.</i> (1978)
<i>Phaseolus vulgaris</i>	Fabaceae	Inner epidermal cuticle	4	6	~	~	Holloway, unpublished
<i>Pinus nigra</i>	Pinaceae	L	4	4700	~	~	Campbell (1972)
<i>Pinus sylvestris</i>	Pinaceae	L	4	~	~	~	Wallis <i>et al.</i> (1973)
<i>Prunus laurocerasus</i>	Rosaceae	L	4	~	~	~	Gourat <i>et al.</i> (1993)
<i>Sisyrinchium filifolium</i>	Iridaceae	L, cuticle > 2 µm thick	4	2500	~	~	Sargent (1976b)
<i>Spartocytisus filipes</i>	Fabaceae	L, Mucilaginous cell	4	110	~	110	Lyshede (1977a)
<i>Spartocytisus filipes</i>	Fabaceae	Mucilaginous cell	4	~	~	~	Lyshede (1977a)
<i>Tamarix pentandra</i>	Tamaricaceae	Papillae	4	~	~	~	Sargent (1976b)
<i>Tradescantia virginiana</i>	Commelinaceae	Petal	4	156	~	~	Holloway, unpublished
<i>Triticum aestivum</i>	Poaceae	Ovule	4	870	~	870	Morrison (1975)
<i>Tropaeolum majus</i>	Tropaeolaceae	Stigma	4	240	~	240	Shayk <i>et al.</i> (1977)
<i>Utricularia sandersonii</i>	Lentibulariaceae	Stolon, wart	4	520	~	520	Heide-Jørgensen (1991)
<i>Vicia faba</i>	Fabaceae	L	4	100	~	100	McKeen (1974)
<i>Arabidopsis thaliana</i>	Brassicaceae	Cutinase-expressing L	5	240	~	~	Sieber <i>et al.</i> (2000)
<i>Atriplex semibaccata</i>	Chenopodiaceae	L	5	100	100	0	Kolattukudy (1980)
<i>Beta vulgaris</i>	Chenopodiaceae	Epidermis, internal	5	80	67	15	Holloway, unpublished
<i>Beta vulgaris</i>	Chenopodiaceae	L	5	56	56	0	Holloway (1982a)
<i>Eucalyptus cinerea</i>	Myrtaceae	L	5	590	100	490	Hallam (1964)
<i>Gordonia axillaris</i>	Theaceae	L	5	2000	~	~	Sargent (1976a,b)
<i>Plantago major</i>	Plantaginaceae	Stomatal lip	5	1000	~	~	Holloway, unpublished

Table 2.2 Continued

Species	Family or order	Organ	Type	CM (nm)	CP (nm)	CL (nm)	Reference
<i>Sarracenia purpurea</i>	Sarraceniaceae	Young Pitcher, outside	5	642	75	567	Joel and Heide-Jørgensen (1985)
<i>Abutilon venosum</i>	Malvaceae	N	6	400	~	~	Findlay and Mercer (1971)
<i>Arabidopsis thaliana</i>	Brassicaceae	Leaf	6	91	~	~	Sieber <i>et al.</i> (2000)
<i>Avena fatua</i>	Poaceae	L	6	~	~	~	Holloway (1982a)
<i>Berula erecta</i>	Apiaceae	Aquatic L	6	25	~	~	Frost-Christensen <i>et al.</i> (2003)
<i>Brassica napus</i>	Brassicaceae	L	6	~	~	~	Armstrong and Whitecross (1976)
<i>Brassica oleracea</i> var. <i>gemmifera</i>	Brassicaceae	L	6	~	~	~	Reed (1979)
<i>Brassica oleracea</i> var. <i>botrytis</i>	Brassicaceae	L	6	~	~	~	Holloway (1982a)
<i>Cannabis sativa</i>	Moraceae	T	6	~	~	~	Hammond and Mahlberg (1978)
<i>Elodea canadensis</i>	Hydrocharitaceae	L	6	36	~	~	Holloway, unpublished
<i>Elodea canadensis</i>	Hydrocharitaceae	L	6	~	~	~	Crisp (1965)
<i>Fragaria ananassa</i>	Rosaceae	L	6	~	~	~	Holloway <i>et al.</i> (1981)
<i>Hordeum vulgare</i>	Poaceae	L	6	~	~	~	Sargent and Gay (1977)
<i>Hygrophila corymbosa</i>	Acanthaceae	Aerial L	6	52	~	~	Frost-Christensen <i>et al.</i> (2003)
<i>Hygrophila corymbosa</i>	Acanthaceae	Aquatic L	6	3	~	~	Frost-Christensen <i>et al.</i> (2003)
<i>Lemna minor</i>	Lemnaceae	L	6	~	~	~	Holloway (1982a)
<i>Malus pumila</i>	Rosaceae	Lab	6	~	~	~	Holloway <i>et al.</i> (1981)
<i>Marchantia</i> sp.	Marchantiales	gametophyte	6	25	25	0	Holloway, unpublished
<i>Mentha aquatica</i>	Lamiaceae	Aerial L	6	71	~	~	Frost-Christensen <i>et al.</i> (2003)
<i>Mentha aquatica</i>	Lamiaceae	Aquatic L	6	62	~	~	Frost-Christensen <i>et al.</i> (2003)
<i>Posidonia australis</i>	Posidoniaceae	L	6	~	~	~	Kuo (1978)

<i>Potamogeton crispus</i>	Potamogetonaceae	L	6	42	~	~	Holloway (1982a)
<i>Potamogeton ssp.</i>	Potamogetonaceae	Aquatic L	6	35	~	~	Frost-Christensen <i>et al.</i> (2003)
<i>Potamogeton ssp.</i>	Potamogetonaceae	Aquatic LAd	6	45	~	~	Frost-Christensen <i>et al.</i> (2003)
<i>Ribes nigrum</i>	Saxifragaceae	LAb	6	~	~	~	Holloway <i>et al.</i> (1981)
<i>Ribes nigrum</i>	Saxifragaceae	LAd	6	~	~	~	Holloway <i>et al.</i> (1981)
<i>Ricinus communis</i>	Euphorbiaceae	L	6	~	~	~	Holloway <i>et al.</i> (1981)
<i>Saxifraga granulata</i>	Saxifragaceae	L	6	~	~	~	Lehmann and Schulz (1976)
<i>Spartocytisus filipes</i>	Fabaceae	T	6	~	~	~	Lysheide (1976)
<i>Tulipa gesneriana</i>	Liliaceae	L	6	~	~	~	Holloway <i>et al.</i> (1981)
<i>Utricularia monanthos</i>	Lentibulariaceae	Bladder T	6	71	~	~	Fineran and Lee (1975)
<i>Veronica anagallis-aquatica</i>	Scrophulariaceae	Aerial L	6	91	~	~	Frost-Christensen <i>et al.</i> (2003)
<i>Veronica anagallis-aquatica</i>	Scrophulariaceae	Aquatic L	6	28	~	~	Frost-Christensen <i>et al.</i> (2003)
<i>Vicia faba</i>	Fabaceae	GC	6	72	~	~	Pallas and Mollenhauer (1972)
<i>Zea mays</i>	Poaceae	L	6	~	~	~	Holloway (1982a)
<i>Zostera capensis</i>	Zosteraceae	L	6	570	~	~	Barnabus <i>et al.</i> (1977)
<i>Pyrus communis</i>	Rosaceae	LAd	7	1200	88	1112	Holloway, unpublished
<i>Pyrus communis</i>	Rosaceae	LAd	7	1100	42	1058	Holloway (1982a)
<i>Malus sp.</i>	Rosaceae	LAb, Outer epidermal cuticle	7	2400	~	~	Holloway, unpublished
<i>Cicer arietinum</i>	Fabaceae	H	?	~	~	~	Schnepf (1969)
<i>Citrus paradisi</i>	Rutaceae	Juice sac	?	100	~	~	Espelie <i>et al.</i> (1980)
<i>Lonicera japonica</i>	Caprifoliaceae	N	?	400	~	~	Fahn and Rachmilevitz (1970)
<i>Lonicera japonica</i>	Caprifoliaceae	N	?	3000	~	~	Fahn and Rachmilevitz (1970)

Abbreviations used: A = shoot apex, Ab = abaxial, Ad = adaxial, Bs = bulb scale, C = coleoptile, Ch = chambered CM, CM = cuticular membrane, CP = cuticle proper, CL = cuticular layer, ECL = external CL, ICL = internal CL, PL = pectin lamella, GC = guard cell, D = detached CM, EP = epidermal papillae, F = fruit, G = gland, H = hydathode, L = leaf, Mc = mucilaginous cell, O = ovule, N = nectary, P = petiole, Pc = prouticle, po = porous CM, S = stem, ssc = sub-stomatal cavity, SC = seed coat, SP = stigma papillae, St = stoma, T = trichome, Td = tendril, wr = wrinkled CM, ~ = no data available.

show tripartite structure in sections stained with KMnO_4 (Figure 2.5b, Holloway, 1982a; Figure 2.7b, Wattendorff and Holloway, 1984). CP and CL lamellae are of similar structure indicating that they are of similar composition. The thickness of the $\bar{\text{e}}\text{L}$ lamellae is approximately 5–8 nm (Chafe and Wardrop, 1973), generally rather greater than for the $\bar{\text{e}}\text{L}$ lamellae of suberin (3–6 nm; Heide-Jørgensen, 1980) although the ranges overlap. The $\bar{\text{e}}\text{L}$ lamellae of cuticles are often reported to be more uniform in thickness at about 5 nm than the $\bar{\text{e}}\text{D}$ lamellae (see e.g. Domínguez and Heredia, 1999). In youngest leaves of *Hedera helix* the CP has 3–5 lamellar units increasing to 14 with mean thickness of a lamellar unit (one $\bar{\text{e}}\text{D}$ and one $\bar{\text{e}}\text{L}$ layer) of 5.2 nm. Later in the expanded leaf the number of lamellar units reduces again to 9, and the thickness of the lamellar unit increases to 9.4 nm (Gilly *et al.*, 1997). Graded periodicity in the thickness of the lamellae is commonly observed, with the closest spacing at the external surface as in Wattendorff's images of *A. americana* (Figures 2.4e and 2.5b,d). Graded thickness of the CP lamellae in *P. tenax* is most pronounced in early CM development (Jarvis and Wardrop, 1974). The same can be said of many other species, including *Gasteria planifolia*, *C. miniata*, and so on (Riederer and Schönherr, 1988; Figures 2.6c–f). In general, the $\bar{\text{e}}\text{L}$ lamellae are of more uniform thickness, while $\bar{\text{e}}\text{D}$ lamellae towards the bases of the stacks are often particularly variable in thickness, especially where there is high convex curvature of the CM. However, there is considerable variability, making it hard to make consistent generalisations. In a set of Holloway's negatives from the same *Iris* leaf it is possible to find locations where all these generalisations are contradicted, so that electron-lucent lamellae may in places be larger and more variable. Clearly there may be changes in apparent thickness with local tilt relative to the beam (as demonstrated by Heide-Jørgensen, in his figure 2.8 in Jeffree, 1996). However, the following points lead me to conclude that lamellar thicknesses are intrinsically variable over a limited range: first, the commonly observed tapering from base to surface of the stack cannot be explained by variation in tilt angle, and second the contrast and edge sharpness of regions which demonstrate variability often both remain high, while tilting out of their plane of alignment with the beam degrades both.

2.2.2.2 Lamella position and orientation

Usually the cuticular lamellae are superficial, located in the CP or outermost ICL, but many examples exist of lamellae extending throughout the CM. In some species with heavy cuticles such as *A. americana* (Figure 2.7a), the lamellate region extends fully to the base of the ICL. Most cuticles demonstrating lamellae in the CL layer also have a lamellate CP. Very occasionally, examples arise of CMs with lamellae only in the CL layer, and not in the CP, as in the adaxial leaf cuticles of pear (*Pyrus communis*, Figure 2.1a) and apple (*M. pumila*; Figure 2.1c; Holloway, 1982a), and there is no reason to suppose these are unique. Lamella orientation is strictly parallel to the epidermal cell surface in the CP layer of most species, although Hallam (1967) described the CP lamellae of *Eucalyptus cinerea* as anastomosing. The orientation of lamellae in the CL layer generally becomes increasingly chaotic

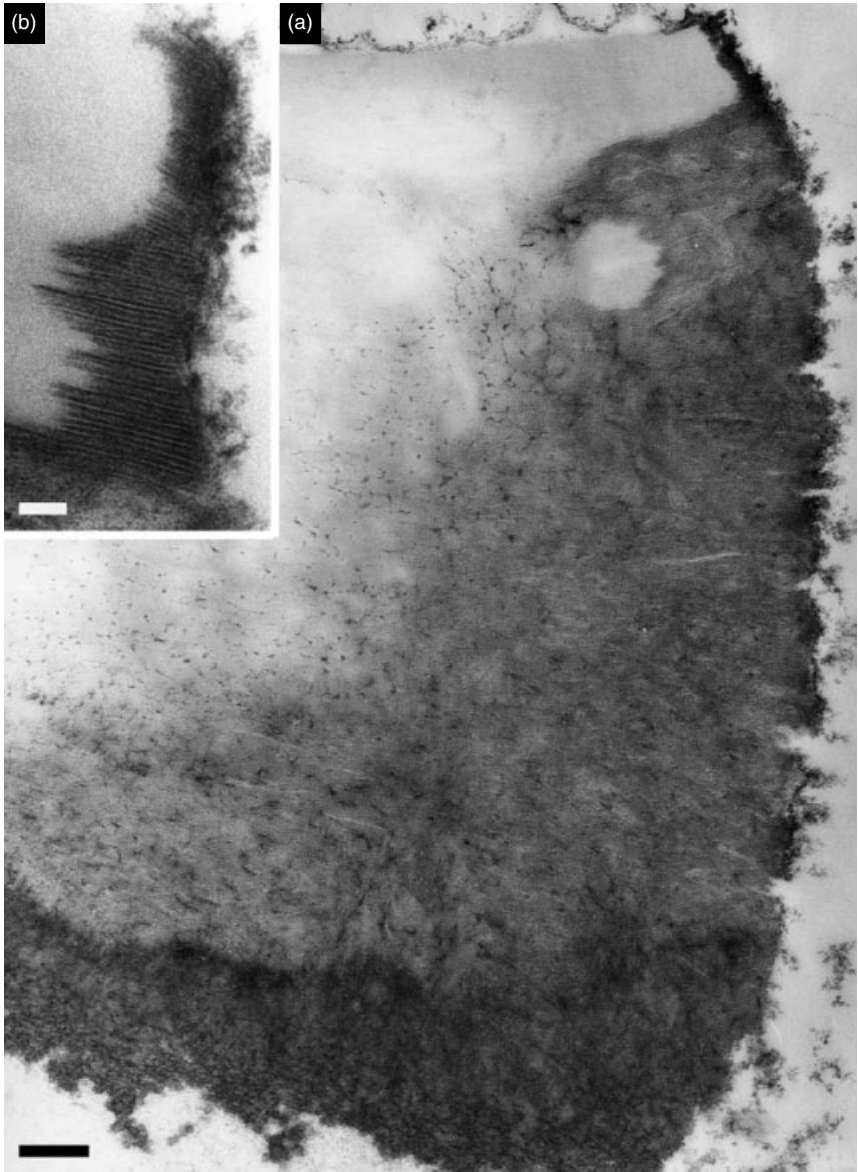


Figure 2.7 The cuticular membrane (CM) of *Agave americana* following exposure of the cut end of the membrane to KMnO_4 , simultaneously fixing and staining *en bloc*. (a) The distance of penetration of the CM by KMnO_4 from right to left of this image gives a graphical representation of the permeability to the salt in aqueous solution. The cuticle proper (CP) (top right) is scarcely penetrated. In the cuticular layer (CL) the polysaccharide reticulum appears to provide fast-track access to deeply located sites. The salt also moves more rapidly in the electron-dense lamellae of the CP than in the electron-lucent lamellae (b). The entire CL of this Type 1 CM is lamellate to its base. (a) Bar = 200 nm; (b) bar = 50 nm. Figures 2.7a,b from Wattendorff and Holloway (1984), *Planta*, **161**, 1–11.

with depth in the CL. In Holloway's image of *P. communis* adaxial leaf cuticle (Figure 2.1a; Holloway, 1982a) the CL lamellae are sometimes almost vertical, and traverse most of the thickness of the CL. If the permeability properties of the CM depend on orientation of the lamellae transverse to the diffusion pathway for water vapour then these are in a relatively unfavourable configuration.

2.2.2.3 What are the CP lamellae?

The $\bar{e}D$ lamellae in the CP and CL appear to be a fast pathway for periclinal penetration of $KMnO_4$ (Wattendorff and Holloway, 1984). Their contrast is increased by section staining with $KMnO_4$, and they are also positive for the KI/H_2SO_4 silver-protein reaction that localises epoxide groups in the cutin (see later). These findings are consistent with a polar cutin domain, while the $\bar{e}L$ lamellae appear impermeable and unreactive to most EM stains, including osmium tetroxide, consistent with a non-polar hydrocarbon. It has been suggested that the $\bar{e}L$ lamellae represent the location of SCL that have been extracted during specimen preparation and that these may constitute the main cuticular permeability barrier (Wattendorff, 1992). Gilly *et al.* (1997) discuss the concept that the $\bar{e}L$ lamellae consist of soluble lipids and the $\bar{e}D$ lamellae of cutin, citing Fisher and Bayer (1972), Heide-Jørgensen (1978b) and Wattendorff and Holloway (1980) (see also Hallam, 1964; Sitte, 1975; Schönherr and Mérida, 1981; Domínguez and Heredia, 1999). Fisher and Bayer (1972) clearly regarded the $\bar{e}L$ lamellae of *Plantago major* and *Ardisia crenata* as equivalent to EW plates lying in horizontal orientation. The extraction of SCL from the $\bar{e}L$ lamellae would account for their almost total non-reactivity to electron-stains, but the presence of non-polar saturated hydrocarbons would give the same result. The CP of *A. americana* cuticles is prone to splitting along the $\bar{e}L$ layer during exposure to the electron beam indicating that this layer is both mechanically weak and thermolabile (Wattendorff and Holloway, 1980; Figure 2.5c). The extraction of waxes from the $\bar{e}L$ lamellae may account for this mechanical weakness, but again the same result may be obtained with SCL present. The polylamellate structure of suberin, which closely parallels that of cutin, is likewise thought to result from the self-assembly of alternating layers of a polyester with phenolic constituents and soluble waxes, predominantly long-chain alkanes and alcohols (Scott, 1994).

There are a number of good reasons to doubt that the $\bar{e}L$ lamellae are composed *only* of soluble, crystalline intracuticular waxes. The existence of CP lamellae is only known from EM treatments which normally expose them both to solvent extraction, and to potentially damaging temperatures close to the melting points of the SCL. If the $\bar{e}L$ lamellae consisted entirely of SCL they would be extracted by many standard EM procedures, resulting in the abolition or more chaotic spacing and appearance of the lamellae. Wattendorff reported that after extraction in hot chloroform/methanol, the *A. americana* cuticle was penetrated equally by $KMnO_4$ in anticlinal and periclinal directions (Wattendorff, 1984, 1992), but that the lamellar structure remained. CP lamellae may appear more prominent

following extraction in chloroform (Méri­da *et al.*, 1981), consistent with the exposure of polar material to staining by the removal of obscuring SCL, but Viougeas *et al.* (1995) found that lamellae of *H. helix* CP were abolished by treatment with lipid solvents. Domínguez and Heredia (1999) state that 'wax extraction from isolated cuticles followed by cutin depolymerisation still yields a solid residue with this bilayered pattern' (see also Schmidt and Schönherr 1982 and Figures 2.8d,e).

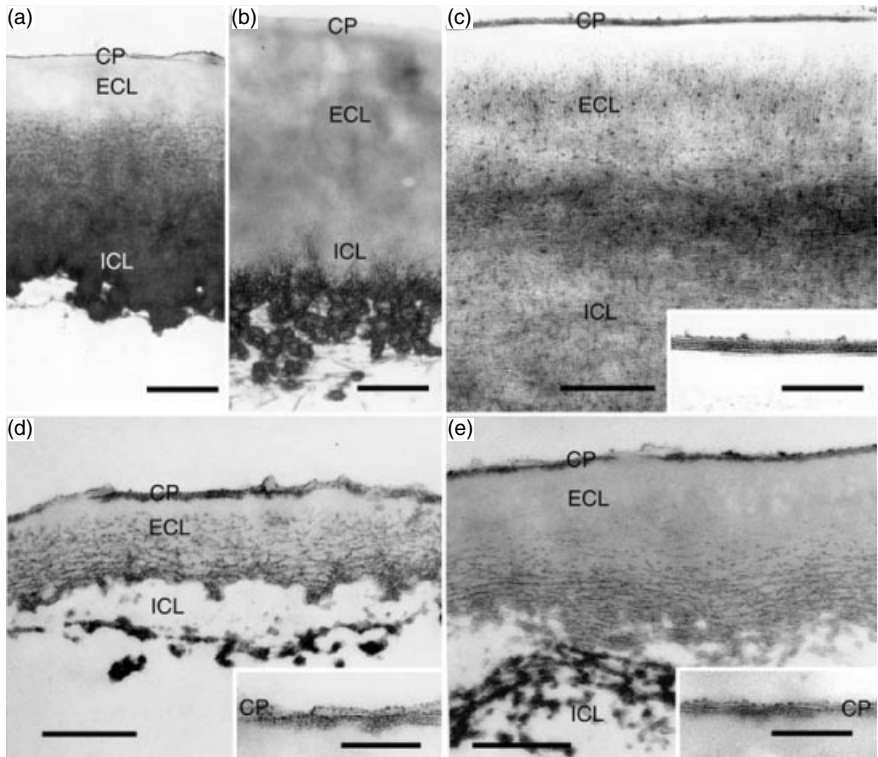


Figure 2.8 *Clivia miniata* leaf (adaxial): sections of enzyme-isolated cuticular membranes (CM) and polymer matrix membranes (PMX) that have been CHCl_3 /methanol-extracted and acid hydrolysed (6 M HCl, 120°C , 24 h) (Tissue fixed and stained *en bloc* with GA/KMnO₄, sections stained with U/Pb). (a) CM of young leaf, 6.5 cm from base, showing a dense (polar) internal cuticular layer (ICL) and electron-lucent (non-polar) external cuticular layer (ECL). (b) CM of a mature leaf, 12.5 cm from base, with polar cutinised cystoliths in the inner ICL. (c) PMX, showing lamellate CP (inset). Cuticular layer (CL) are both reticulate and polylamellate. (d,e) PMX membranes de-esterified in BF_3 -methanol. (d) 6.5 cm from base: the ECL is alkali-resistant (= non-ester cutin), but the ICL has been extracted (= ester cutin). Reticulate fibrils occur in the inner two-thirds of the ECL. (e) 12.5 cm from base, the non-ester cutin layer of the ECL has doubled in thickness. The outer two-thirds is now non-reticulate or weakly reticulate. The ICL has been extracted, but a polar, globular residue remains at its base, probably polysaccharide-rich. Insets to Figures 2.8d,e show that the lamellate structure of the cuticle proper (CP) survives the combination of solvent extraction, acid and alkaline hydrolysis. (a,b) Bars = 1 μm ; (c–e) bars = 500 nm; insets, bars = 200 nm. Figures 2.8a–e reproduced from Schmidt and Schönherr (1982), *Planta*, **156**, 380–384.

These observations indicate significant variability in response of cuticles to extraction treatments, which may either be attributable to differences in cuticle age and development or to intrinsic differences between species. While they are consistent with the involvement of SCL in the lamellate structure, a telling observation is that the CP of *Allium cepa* contains only $1 \mu\text{g cm}^{-2}$ of wax, equivalent at best to about 25% of the amount required to fit the alternating polymer/wax model (Schönherr and Mérida, 1981). This does not, however, negate the possibility that equivalent chemical species are involved in this lamellar structure. Simply that they are no longer soluble. A possible model would be that the waxes involved in the $\bar{e}L$ lamellae of the CP are primary alcohols esterified to an ester cutin lamella, with their hydrocarbon chains co-aligned transverse to the plane of the lamella. Set against this is the key observation of the extreme sensitivity of cuticular permeability to solvent extraction (Schönherr and Mérida, 1981) which indicates that the permeability barrier is soluble. The lamellar structure is known to survive in the fossil record into the Eocene (Tegelaar, 1990) and early Cretaceous (Archangelsky *et al.*, 1986). It is inconsistent at one point in a paper to be talking about the possible mobility of this class of compounds in epicuticular crystal self-assembly (see later), and at another to suppose that similar waxes will remain immobile during 200 million years of burial in sediment. This apparent paradox could be resolved if the $\bar{e}L$ layer were composed of a crystalline array of carbon chains covalently bonded to a cutinous $\bar{e}D$ lamella, by e.g. esterification of primary alcohols with cutin acids. Domínguez and Heredia (1999) argue that it may be sufficient for partial bonding to occur by cross-linking between fatty acids of the $\bar{e}D$ and alcohols of the $\bar{e}L$ lamella, to form a framework into which SCL (mostly alkanes) may self-assemble. In *C. miniata*, the main SCL constituents are approximately 38% C16 and C18 fatty acids and approximately 41% C19–C26 alkanes with C22 dominant. Their molecular model predicts the self-assembly of these constituents by hydrophobic interactions between the tails of the molecules to form into a molecular bilayer unit 4.9 nm thick, corresponding closely to the commonly reported $\bar{e}L$ lamella dimension of about 5 nm (Riederer and Schönherr, 1988). This model seems to encompass the objections of resistance of the lamellae to extraction and the simultaneous vulnerability of their permeability barrier properties to solvents. The remaining challenge is to explain how it comes about that this framework is resistant to depolymerisation.

2.3 Cuticle polymers

2.3.1 Chemical types in Angiosperm and Gymnosperm cuticles

10,16-Dihydroxyhexadecanoic acid and its positional isomers are generally the major constituents of the cutins of modern Angiosperms and Gymnosperms (Holloway, 1982b) as in leaves and fruits of the Solanaceae and *Citrus* species, in leaves of *Magnolia grandiflora*, *Liriodendron tulipifera* and *Prunus lusitanica*,

and notably as the only cuticle type in Gymnosperms. Mixed cuticles composed of C16 dihydroxy acids and C18 trihydroxy acids are very common in Angiosperms, e.g. in *Vitis vinifera*, *A. cepa* and *I. germanica*. C18 trihydroxy acids are rarely major constituents but occur in *A. americana*, *Hyacinthoides non-scripta* (Caldicott, 1973) and *Spinacia oleracea* (Holloway, 1974). Widely separated taxa often have similar cutin composition, and a single species may have different cutin compositions on different organs. *Citrus* leaf cuticles have a C16 type cutin, whereas in the cuticle of the inner seed coat of *Citrus paradisi* C18 acids predominate (Espelie *et al.*, 1980).

A completely new cuticle polymer 'cutan' is now known to occur in species as diverse as *C. miniata* (Amaryllidaceae), which contains both cutin and cutan, and sugar beet *Beta vulgaris* (Chenopodiaceae) in which cutan is the principal polymer (Tegelaar, 1990) and cutin may not be detectable (Holloway and Baker, 1970). The cuticle of *B. vulgaris* has a CP of about 100–200 nm thickness with a pronounced periclinal lamellate structure, composed of 5–15 lamellae (Type 5; Holloway, 1982a), and often lacks significant development of the CL.

Histochemical reactivity of the opaque lamellae in *C. miniata* and *A. americana* suggests that polar molecules or substituted groups are present, at least in younger leaves. Thus lamellar contrast is reported by several authors to be enhanced by treatment with ruthenium red (Wattendorff and Holloway, 1980; Kruger *et al.*, 1996; Figure 2.5d), and with KMnO_4 or $\text{Ba}(\text{MnO}_4)_2$ (Hoch, 1979; Mérida *et al.*, 1981; Wattendorff and Holloway, 1982; Holloway, 1982a; Figures 2.4d, 2.5b) pointing to the presence of carboxyl groups. Further, the dense lamellae of *A. americana* react positively with $\text{H}_2\text{SO}_4\text{-I}_2/\text{KI}$ -silver proteinate (Wattendorff and Holloway, 1980; Figure 2.5c), which is believed specifically to identify epoxy groups in the C₁₈ type cutins by the formation of epoxide iodohydrin derivatives that reduce silver proteinate to metallic silver (Wattendorff and Holloway, 1980, 1982; Holloway *et al.*, 1981).

Schmidt and Schönherr (1982; Figures 2.8d,e) observed that following exhaustive extraction of enzyme-isolated *C. miniata* CM in chloroform/methanol (to remove SCL) and 6 M HCl, 120°C for 24 h (to hydrolyse proteins and polysaccharides), the lamellate structure of the CP of *C. miniata* was distorted but not destroyed by treatment with 20% BF_3 -methanol, at 80°C for 24 h, which cleaves ester bonds in cutin (see also Domínguez and Heredia, 1999). Its appearance also predates the appearance of identifiable ester cutin in the membrane (Riederer and Schönherr, 1988). These findings have several implications. Thus, while the electron-lucent lamellae of the CP may be assembled from extractable SCL, and the electron-dense lamellae from cutin, these constituents are rapidly modified into an insoluble and non-saponifiable hydrocarbon structure. The CP in these species thus fails von Mohl's (1874) definition that it should be completely saponifiable, and the possibility is now raised that either of two polymers, cutin and cutan, or various mixtures of them, may equally be involved in forming eD lamellae that are morphologically indistinguishable.

2.3.2 *The algal cuticle*

Although the thallus of *Chondrus crispus* (Rhodophyta) is covered by a detachable film of lamellar, extracellularly secreted material that looks superficially like a cuticle or pellicle (Craigie *et al.*, 1992), the structure is composed of proteins and polysaccharides with lipids only as minor components (Hanic and Craigie, 1969). The Green algae (Chlorophyta) and, in particular, the Charales with their multicellularity, tissue specialisation and biochemical affinity with land plants (Embryophytes) are regarded as their probable ancestors. Cook and Graham (1998) have suggested that the osmiophilic surface of the pellicle covering *Coleochaete orbicularis* may be analogous to the procuticle of higher plants, as defined here and in Jeffree (1996). The veracity of this interpretation depends ultimately on chemical analysis, and the detection of cutin or cutan polymers and waxes in these layers, which are currently of unknown composition and appear to lack lipids.

2.3.3 *Chemical types in Bryophyte and Pteridophyte cutins*

Monoclea gottschei (liverwort) and *Notothylas orbicularis* (hornwort) have osmiophilic surface layers analogous to a procuticle (Cook and Graham, 1998). The occurrence and functional significance of a hydrophobic cuticle of *Marchantia* and other liverworts in preventing water ingress through air pores into photosynthetic tissues was examined by Schönherr and Ziegler (1975). Holloway's TEM image of the cuticle of *Marchantia* shows a simple uniformly electron-lucent layer comparable in thickness (25–30 nm) with the CP of typical higher plant cuticles (Figures 2.4c,e; Table 2.2). The structures of moss cuticles are analogous with those of vascular plants. Three-week old protonemata of *Sphagnum fimbriatum* bear a superficial $\bar{e}D$ layer comparable with the procuticle of vascular plants (Figure 2.4a; Cook and Graham, 1998) that develops by 10 weeks into cuticle containing lenticular $\bar{e}L$ lamellae analogous with the CP lamellae in vascular plants (Figure 2.4b; Cook and Graham, 1998). The sporophytes of mosses and hornworts have stomata and in *Funaria* cuticle structure closely parallels that of vascular plants (Figure 2.4d; Sack and Paolillo Jr., 1983a,b), as does cutin composition in the few mosses which have been examined. *Sphagnum palustre* contains 10,16-dihydroxy hexadecanoic acid identical to that which forms the major constituent of higher plant cutins (Caldicott and Eglinton, 1976). Although the CM is rarely well developed in moss gametophytes the Polytrichales are an exception, having both cuticles and EW on the edges of the photosynthetic lamellae of the leaves. The linear apertures between adjacent photosynthetic lamellae are functionally equivalent to stomata, and the lamellae to mesophyll. By contrast, the Hepaticae lack dihydroxyacids in their cutin, containing instead a unique class of $(\omega - 1)$ -hydroxymonobasic acids (Caldicott and Eglinton, 1976). *Psilotum nudum* also lacks dihydroxy acids, and contain hexadecane-1,8,16-triol as major constituent (40%) together with 16-hydroxyhexadecanoic acid (Caldicott and Eglinton, 1975). The fern and lycopod cutins are chiefly composed of ω -hydroxymonobasic acids (Hunneman and Eglinton, 1972; Caldicott, 1973),

regarded as primitive by Holloway (1982b). However the Isoetales and some members of the Selaginellales contain dihydroxyacids in substantial quantity (Caldicott, 1973).

2.3.4 *Ontogeny, composition and structure of the CL*

The cuticle was thought by Karsten (1857, 1860) and others to be formed by chemical transformation of the polysaccharides of the PCW, the so-called 'metacrass' theory. By contrast, von Mohl (1847) and de Bary (1871, 1884) saw it as superficial to the epidermal cells, lying entirely outside the CW. Many (van Wissenligh, 1895) thought of it as an oxidised lipid film, analogous to the films formed by drying oils such as linseed and poppy oils in varnish and paint, a concept that was prevalent in the twentieth century also (Lee and Priestley, 1924). It was already clear that the epidermis was the origin of the constituents of the cuticle (Damm, 1902; Lee and Priestley, 1924). Successive generations of scientists (von Ziegenspeck, 1928; Pohl, 1928; Martens, 1934; Weber, 1942) conceded that cuticle and wax originated in the epidermal cells, and searched in vain for pores that were considered necessary to transport cuticle constituents to the surface. Prior to the widespread availability of the electron microscope two further seminal observations were made. First that the CM could be thrown into folds and wrinkles on the surfaces of many leaves and petals (Figures 2.9g,h). Martens (1934) argued that these could only arise from over-secretion of cuticular materials, and not from CW modification, leading to the rapid demise of the metacrass theory. Second, Damm (1902) and later Anderson (1934) demonstrated that a cutinised layer could arise on the internal epidermal CW, and that direct external exposure to atmospheric oxygen was not essential for its formation. Epidermal cell wrinkling, seen widely in petal epidermis of angiosperms, including *Tradescantia*, *Narcissus* (Holloway; Figure 2.9h), *Tropaeolum speciosum* (Jeffree, 1986) and *A. thaliana* (Jenks *et al.*, 1996; Chen *et al.*, 2003; Figure 2.10i), and also in the leaf epidermises of e.g. *Aesculus hippocastanum* (Martin and Juniper, 1970), *Syringa vulgaris* (Holloway, 1971) and *Acer pseudo-platanus* (Holloway, 1971; Wilson, 1984), is often mistaken as a manifestation of wax structure (e.g. *Dieffenbachia maculata*; Sutter, 1985), but it is clear from sections of the epidermis (Figures 2.9g,h) that the cuticle itself is folded into ridges and ripples.

Sargent (1976a,c) interpreted the formation of the CL or 'secondary cuticle' as a second secretory event on a fully expanded epidermis. The CP precedes the CL in all species [*Eryngium rostratum* (Chafe and Wardrop, 1973); *Phormium tenax* (Jarvis and Wardrop, 1974); *A. americana* (Wattendorff and Holloway, 1980); *Utricularia laterifolia*, *Athanasia parviflora*, *C. gronovii* (Heide-Jørgensen, 1991); *C. miniata* (Schmidt and Schönherr, 1982; Riederer and Schönherr, 1988); *P. abies*, (Tenberge, 1989, 1992); *H. helix*(Viougeas *et al.*, 1995)], but the development of the CL begins much earlier than the completion of epidermal expansion. In *P. abies*, an amorphous CP (approximately 100 nm) is present on the protodermal cells in-bud (Phase I; Tenberge, 1992; Figure 2.2a). At this stage, the leaves bear no crystalline EW,

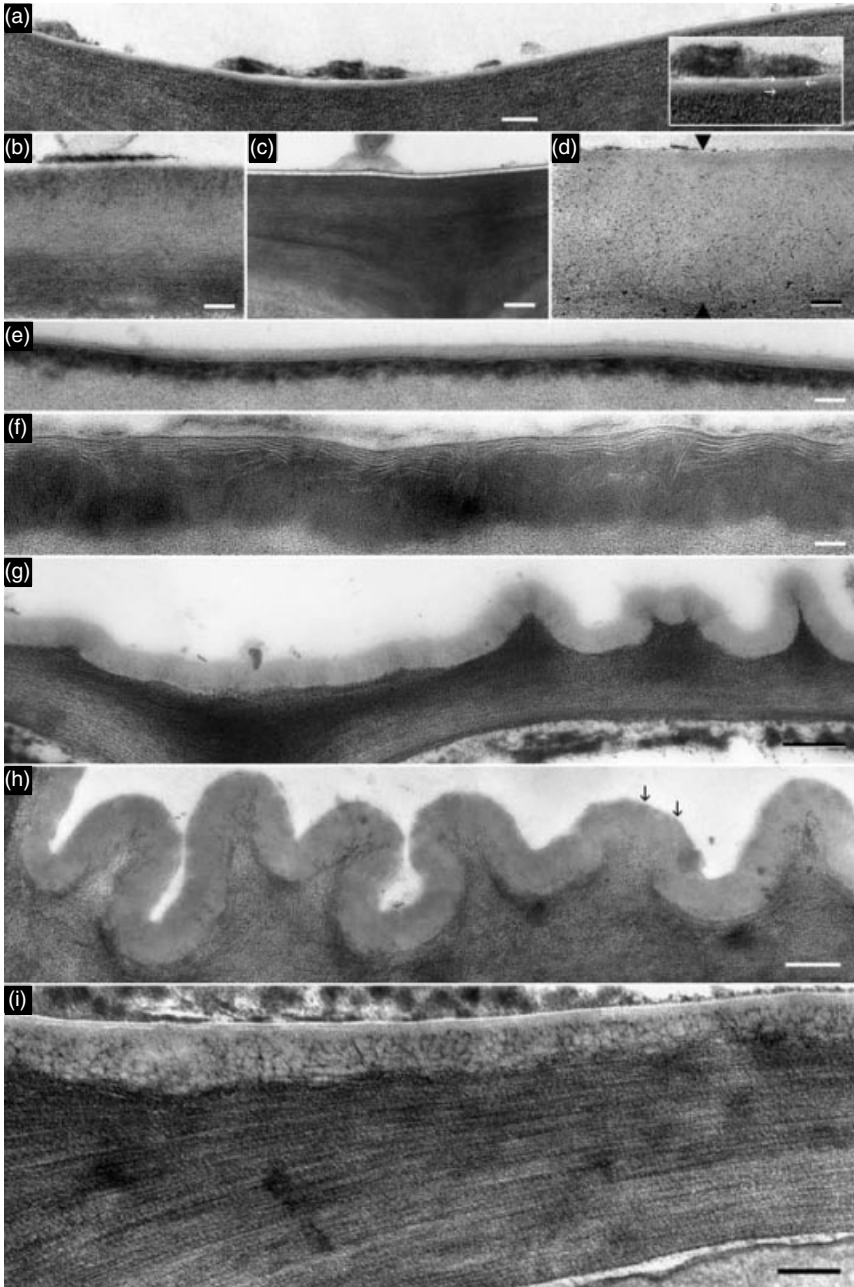


Figure 2.9 (a–d) Cuticle types of various aquatic vascular plants. (a) The thin cuticular membrane (CM) of *Lemna minor* shows at least three layers indicated by arrows in the inset. The outermost electron-lucent layer might have been overlooked if debris had not been deposited on top of it.

which first appears on rapidly expanding leaves prior to the division of the guard cell mother-cells (Jeffree, 1974a; Figure 2.11a,b). Construction of the spruce CL starts in spring, the first accumulations of the CL entrapping polysaccharide fibrils (Figure 2.2b). In *C. miniata*, the CL starts to develop from about 3 cm above the leaf base, where area expansion is the most rapid (Schmidt and Schönherr, 1982), cutin deposition occurring within superficial layers of the PCW, immediately beneath the CP (Riederer and Schönherr, 1988; Figure 2.6d). The boundary of the CL with the CW is marked by globular cutin-rich cystoliths (Mérida *et al.*, 1981) which appear not to migrate through the CW, since they are not observed throughout it, but to arise by accretion of cutin in interfibrillar space in the outer CW, the globules expanding until they coalesce. Consequently, the CL is permeated with electron-dense fibrils of CW polysaccharide. Especially at the base of the CL these appear to be separated into bundles, confined to the interstices between the growing cutinous globules. In *C. miniata*, cell expansion is complete at about 5–6 cm from the leaf base; the amount of ester cutin in the membrane continues to increase, reaching a peak at 10–15 cm (Schmidt and Schönherr, 1982; Riederer and Schönherr, 1988). As in *C. miniata* (Schmidt and Schönherr, 1982; Riederer and Schönherr, 1988), the reticulate CL of spruce appears immediately beneath a clearly demarcated CP, which is amorphous in *P. abies* (Tenberge, 1992; Figures 2.2a–e) and *P. sitchensis* (Figures 2.2f,g; P.J. Holloway; Figure 2.4f, Holloway 1982a), but not necessarily so in all conifers (Table 2.2), and is formed by deposition of cutin within the outer CW.

In the reticulate CL of spruce, the first cutin-embedded fibrils of the CL are carboxylated and alkali-soluble, not cellulose, and early CL development coincides with the disappearance of the pectin lamella as a distinct substratum (Tenberge, 1992; Figure 2.2b). The CL reticulum is PaTAgP-positive, indicating the presence of polysaccharides, and is labelled by cellulase-gold sol, indicating the presence of cellulose or xyloglucan (Tenberge, 1989, 1992; Figures by Tenberge in Jeffree, 1996). The reticulum also stains strongly with KMnO_4 and hydroxylamine-ferric

(Figure 2.9) (b) Similarly the electron-lucent and amorphous CP of *Elodea canadensis* shows little contrast against the background, but is revealed beneath an attached microorganism. (c) The CM of *Potamogeton crispus* is very thin (approximately 50 nm), very electron-lucent and almost amorphous. (d) Aquatic and aerial leaves of *Lobelia dortmanna* have CM of similar thickness. The aquatic CM shown here (between arrowheads) has a faintly lamellate cuticle proper (CP) and reticulate cuticular layer (CL). (e) The CM of Red beet (*Beta vulgaris*) may show scant CL development, as here, but the CP is strongly lamellate. (f) The CP of dandelion (*Taraxacum officinale*) is strongly lamellate, grading into a sparsely lamellate and reticulate CL, but the inner CL layer lacks lamellae. The wrinkled CM of *Brassica napus* leaf (g) and *Narcissus pseudonarcissus* petal (h) are mainly amorphous, with reticulations most conspicuous beneath the wrinkles. However, faint lamellae are visible at arrows in *N. pseudonarcissus* (h). (i) The CL of *Phaseolus vulgaris* leaf CM is strongly reticulate. The fibrillae terminate beneath a sparingly lamellate CP that is terminated by a single continuous outermost electron-lucent lamella, again highlighted by attached superficial debris. (a–c and e–i) Bars = 100 nm; (d,g,h) bars = 200 nm. Figures 2.9a–c and e–i by P.J. Holloway. Figures 2.9d reproduced from Frost-Christensen *et al.* (2003), *Plant, Cell and Environment*, 26, pp. 561–569, with permission from Blackwell Publishing Ltd.

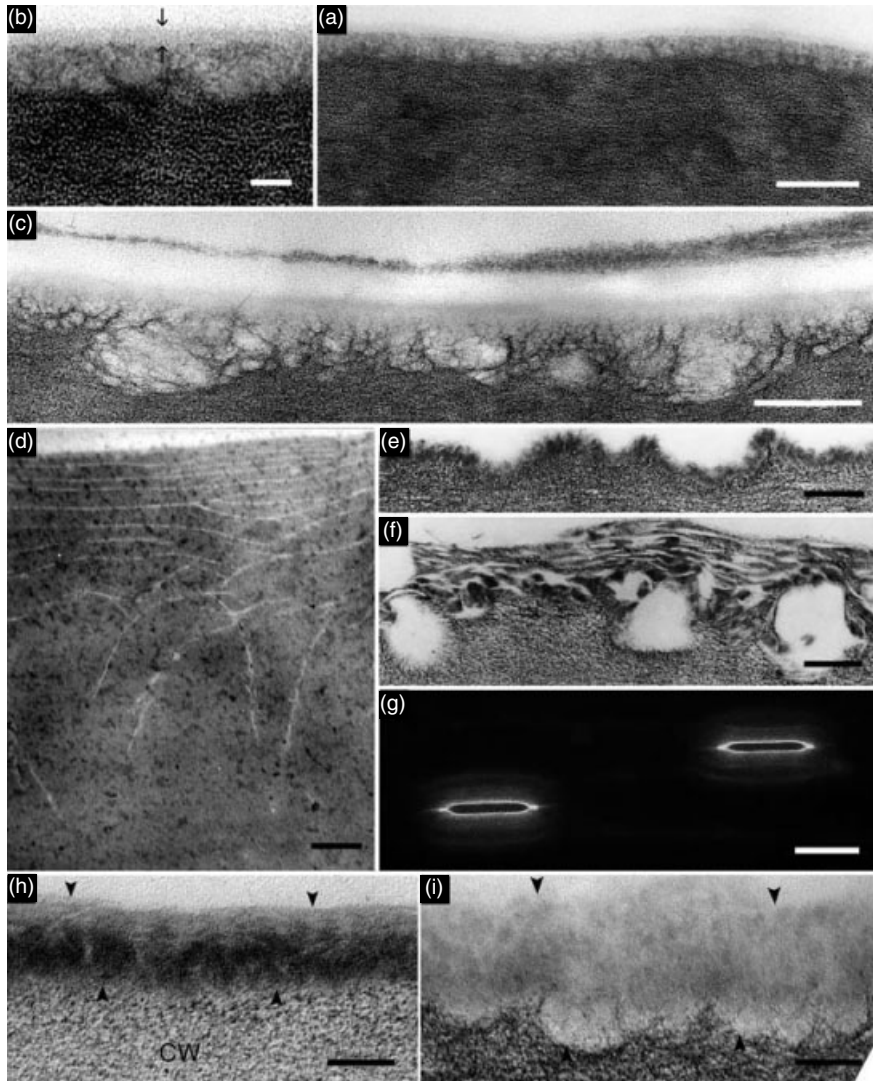


Figure 2.10 (a–c) Cuticles of ferns: (a,b) cuticular membrane (CM) of Hart's tongue fern (*Phyllitis scolopendrium*) fixed in Ga/Os and section stained with lead, shows reticulate cuticular layer (CL) structure beneath a faint amorphous cuticle proper (CP) (b, between arrows). (c) The cuticle of *Dryopteris filix mas* showing strongly reticulate structure of the CL beneath amorphous CP. (d) Fossil cuticle of *Ticoa harissii* (Cycadales) from the Cretaceous of Argentina, showing lamellate structure in the outer regions (*en bloc* staining with OsO₄, section staining with KMnO₄). (e) The amorphous, wrinkled cuticle of the stem epidermis of wild-type *Arabidopsis thaliana* Columbia Col-0/*gl1*. (f) Cutinase-expressing transgenic *A. thaliana* Col-0/*gl1*. The cuticle shows lamellate structure throughout, interrupted by large voids or chambers. (g) Wheat (*Triticum aestivum*) stomatal complex, stained with Nile red and viewed by fluorescence microscopy. The guard cell ledges are selectively stained, and may represent exposed cutin. (h,i) Cuticles of *A. thaliana* ecotype C24 and *wax2* mutant stem.

chloride consistent with the presence of pectin, both reactions being abolished by extraction with ammonium oxalate (Tenberge, 1989, 1992).

Contrary to Sargent's interpretation that the lamellate CP constructs the EW and is consumed in the process (Sargent, 1976a,b), the CP in spruce remains constant in appearance and thickness throughout leaf expansion and CM development and during synthesis of the crystalline epicuticular layer. In *H. helix*, cuticles of young unexpanded leaves consist of a lamellate zone only (CP), sharply demarcated from the underlying PCW (Gilly *et al.*, 1997). The thickness of the lamellate zone remains constant during leaf expansion, representing a third of the thickness of the CM in young leaves and about 11% in fully expanded ones, while the number of lamellae declines from 14 to 9 between these stages. Since the CP is neither thinned nor depleted during expansion of epidermal surface area, it follows that sufficient material is continuously added to the CP during leaf expansion to maintain its thickness and structural integrity, and therefore its assembly, while initiated earlier than the appearance of the CL, continues subsequently.

2.3.5 Layering of the CL

In many cuticles, the CL develops into two broad layers, differentiated by their staining reactions and evidence of differences in composition. This layering arises in part from the process of impregnation of the PCW and SCW layers, but also from a process of centripetal maturation of the deposited cutin. The ICL of the *C. miniata* CM stains strongly with KMnO_4 , while in the ECL staining is less intense (Mérida *et al.*, 1981). This distinction is clear at maturity, and first appears late in expansion phase of leaf growth, about 5–6 cm from the leaf base (Figures 2.6e,f; Figure 2.8). Mérida and Ogura (1987) included the CP in the ECL, but the ICL and ECL are subdivisions of the CL only as defined here, and do not include the CP (Wattendorff and Holloway, 1980; Schmidt and Schönherr, 1982).

Two CL layers are also differentiated in *A. americana* (Wattendorff and Holloway, 1980) and *H. helix* (Holloway *et al.*, 1981; Viougeas *et al.*, 1995). The ECL may be noticeably denser than the ICL when stained with osmium, uranium and lead as in *Nicotiana glauca* (Mérida and Ogura, 1987). The ICL and ECL layers are also distinguishable in *Avena sativa* (O'Brien, 1967), *Eryngium* and *Apium*

(**Figure 2.10**) (h) The stem CM (between arrowheads) of wild-type C24 shows a CP with lamellate structure, and an electron-dense reticulate CL. CW = cell wall. (i) CM of *wax2* stems are thicker, and the CL more electron-lucent than the wild type, but the CW and reticulum stains more strongly. Disorganised lamellae may be present. (a,c,e,f) Bar = 200 nm; (b) bar = 50 nm; (d) bar = 250 nm, (g) bar = 50 μm (h,i) bar = 100 nm. Figures 2.10a–c by P.J. Holloway. Figure 2.10d by Archangelsky *et al.* (1986), *Botanical Journal of the Linnean Society*, **92**, 101–116. Figures 2.10e,f from Sieber *et al.* (2000), *The Plant Cell*, **12**, 721–727 and Nawrath (2002). The biopolymers cutin and suberin, *The Arabidopsis Book*, American Society of Plant Biologists. Figure 2.10g from Collins *et al.* (2001), *Physiological and Molecular Plant Pathology*, **58**, 259–266. Figures 2.10h and i from Chen *et al.* (2003), *The Plant Cell*, **15**, 1170–1185.

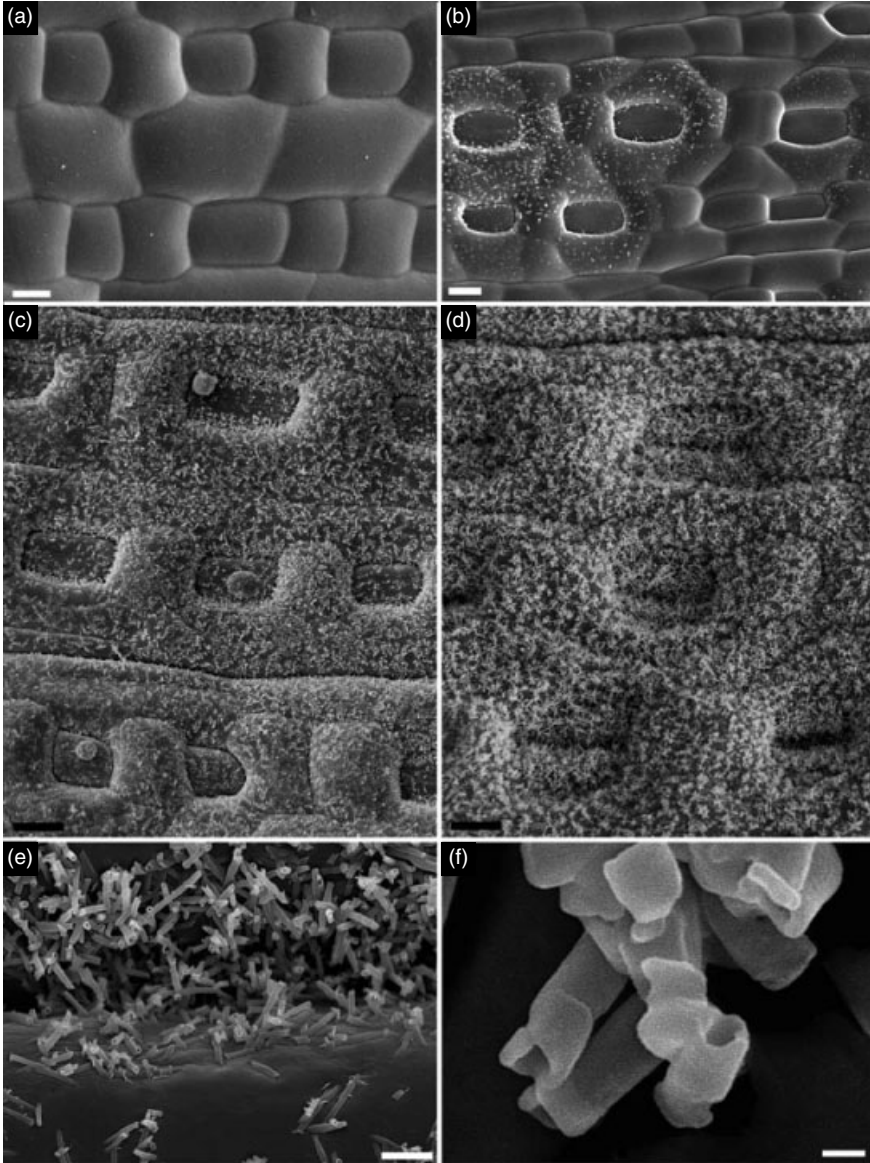


Figure 2.11 Low-temperature scanning electron microscope (SEM) images of stages in the development of the epicuticular waxes of Sitka spruce (*Picea sitchensis*); all stages occur prior to bud-burst and may be observed on a single needle. (a) An almost wax-free cuticle, showing shallow depressions that will become the stomatal antechambers. (b) The earliest stage of wax crystal production occurs just prior to the division of the guard cell mother cells (GCMC), clusters of tubes appearing first on the stomatal accessory cells. (c) At the time of division of the GCMCs, all epidermal cells have a covering of epicuticular wax tubes. (d,e) The first stomatal apertures appear as the antechambers begin to fill with wax tubes, mainly contributed by the accessory cells. (f)

(Chafe and Wardrop, 1973); *P. tenax* (Jarvis and Wardrop, 1974); and *L. elegans* (Sargent, 1976a,b,c), although this was not recognised in these earlier investigations.

Significant chemical differences between the ICL and ECL layers underlie the differences in staining intensity. The outermost 500 nm of the CM in expanding leaves of *C. miniata* withstand saponification in BF₃ (Figures 2.8d,e), leaving a residue of 'non-ester cutin' (Schmidt and Schönherr, 1982) that is probably coterminous with the polymethylenic fraction referred to as cutan by Tegelaar (1990). The quantity of cutan in the CL of the *C. miniata* leaf increases as cell expansion and cutin deposition decline, so that cutan ultimately becomes more abundant than cutin, comprising about 60% of the cuticle (Schmidt and Schönherr, 1982; Riederer and Schönherr, 1988; Tegelaar, 1990). The layer of non-ester cuticle is more than 500 nm thick in mature *C. miniata* leaves (Figure 2.6i, Holloway, 1982a). This significant deposit of cutan does not accumulate in the innermost layers of the ICL, but in the ECL immediately beneath the CP, in which position any cutan precursors synthesised *de novo* would have to pass through the CL. Likewise, in *P. abies*, both the amorphous CP (45 nm) and the outer third of the CL (350 nm) appear to be alkali-insoluble, and can be detached as a unit from the underlying alkali-soluble CL (Tenberge, 1992). Evidence from CP/MS ¹³C NMR spectroscopy and analytical pyrolysis suggests, however, that the non-saponifiable residue of spruce CM may be closely related to lignin rather than to cutan (Kögel-Knabner *et al.*, 1994). Despite having been subjected to acid hydrolysis in 6M HCl at 120°C for 24 h, the saponification-resistant ECL remains permeated with electron-dense reticulum at its base, indicating that in this region the polysaccharide is closed for use as a polar transport pathway even for small polar molecules. Also, the alkali-extracted membrane is almost clear of reticulum in the outer ECL (70–80 nm) of the immature cuticle at the 6.5-cm position and reticulum is absent from the superficial 300–400 nm of the mature ECL, despite the fact that the reticulum extended fully to the base of the CP in the earlier stages of development. It is suggested (Nip *et al.*, 1986; Tegelaar, *et al.*, 1989; Tegelaar, 1990) that the cutan of *A. americana* is covalently bound to the reticulum polysaccharide. It might be supposed that cutan is intruded between the cutinised CL and the CP. However, Schmidt and Schönherr (1982) and Riederer and Schönherr (1988) observed that as 'non-ester cutin' increases, ultimately to become the major polymer of the *C. miniata* CM, the ester cutin declines. Cutanisation of the ECL therefore probably does not arise from *de novo* synthesis and secretion of a new layer, but from a maturation process, involving the progressive modification of the previously deposited cutin and any embedded polysaccharide and waxes, *in situ*. The progressive reduction in

(Figure 2.11) (e) Tubular crystals form in all orientations from parallel to normal to the cuticle surface. (f) A proportion of the tubes show spiral striations or open-spiral structure, indicating that they are fundamentally related to chiral ribbons (see also *Cerithe major*, Figure 2.14c). (a) Bar = 6 μm; (b–d) bars = 10 μm; (e) bar = 1 μm; (f) bar = 100 nm. Figures 2.11a–f by C.E. Jeffree, S. Swift and L. St-John Mosse.

reactivity of all components of the ECL indicates that all types of polar functional groups are in the cutin/polysaccharide framework and are systematically eliminated during this maturation phase.

The apparent absence of polysaccharide reticulum in the ECL of mature *C. miniata* leaves recalls von Mohl's (1847) definition that the CP leaves no cellulose residue upon saponification. In *C. miniata* and *P. abies* however, two significant layers contain no cellulose, but neither is saponifiable, casting doubt on which layer it was that he observed. Although his definition may apply in very early *C. miniata* cuticles, it is rapidly defeated by changes in polymer composition in both CP and CL as the leaf develops and matures. It is relevant here also to recall the findings of Sitte and Rennie (1963) that the CL in *F. elastica* may be cellulose-free, and of Hülsbruch (1966a,b) that there may be both cellulosic and cellulose-free regions in *I. integra*. (Sargent, 1976a,c) interpreted the whole CL, or 'secondary cuticle', as interposed between the CP and the PCW in several species of the Iridaceae. Again the apparent absence of cellulose in the ECL probably influenced her interpretation. In many species (e.g. *B. vulgaris*) the CP is saponifiable early in development, but quickly becomes cutanised and unsaponifiable without losing structural integrity in the process. The CL layer is usually polysaccharide-rich at an early stage, but then becomes unsaponifiable and polysaccharide-depleted later.

The CP, occurring as it does outside the CW, is not contaminated with polysaccharide. In *A. americana*, which also has a cuticle containing cutin and cutan (Tegelaar, 1990), the reticulate region extends to the base of the lamellate CP (Wattendorff and Holloway, 1980; Figure 2.7a). The reticulate region also terminates abruptly beneath the amorphous CP of *P. abies* and *P. sitchensis* (Figures 2.2d,f). In both *A. americana* and *C. miniata*, lamellae in the outer CL show a sharp transition from the periclinal-lamellate layering of the CP to anticlinally or randomly oriented lamellae within the CL. Polysaccharide fibrils ramify between these CL lamellae. The mature cuticles of *C. miniata* and *A. americana* show lamellation throughout a bilayered CL, much of which is also reticulate (Wattendorff and Holloway, 1982; Figure 2.7a; Schmidt and Schönherr, 1982; Figure 2.6h). Since CL lamellae precede and survive cutanisation, they are not a manifestation of the presence of cutan, and can occur in CM matrices of both cutin and cutan polymer types.

Further layering of the CL can be recognised in species with heavy CMs. Tenberge (1992) recognised three sub-layers in the mature *P. abies* CL, differentiated chiefly by the form of the fibrils ramifying through them, and Osborn and Taylor (1990) observed an intermediate CL layer in the adaxial leaf cuticle of *Q. velutina*. The differences in the structure of the layers of the CL, which arise successively during development, result from two sources; first the fact that they are formed by cutin impregnation of successively deeper layers of the CW, first the pectin lamella, then the primary CW and later the secondary CW, and second from the progressive centripetal maturation of the CL by conversion to alkali resistant material. They do not signify the development of a third cuticle zone.

2.3.6 *Cutin cystoliths*

The underside of the isolated CM often shows the rounded, pillow-like profiles of the cutinous globules from which the CL layer was constructed. These globules are exposed when the CM is isolated. In thick SCW of fruits, or in leaves of xerophytic species, or where cutin is deposited between epidermal cells to form spandrels, cutin may accumulate as granules separated from the CM to form cystoliths comparable morphologically and chemically to the innermost layers of the ICL. Although it is possible that cutin cystoliths are packets of cutin precursors *en route* from the cell membrane to the CL (Mahlberg and Kim, 1992), their entanglement in polysaccharide microfibrils indicates that they are outlying sites of accumulation of cutin within the CW.

2.4 Cuticle structural types

Holloway (1982a) summarised the variation in cuticle structure by defining six types, as shown in Table 2.1, that are widely accepted as the standard descriptive tool for analysis of variation in cuticular structures. Many examples taken from the literature and elsewhere are listed in Table 2.2. Holloway's definitions specify the inner and outer regions of the CM as lamellate, amorphous or reticulate, and do not refer explicitly to those regions as the CP and CL as defined here or in Jeffree (1996). However, in considering them further here the explicit connection is made between the outer region and the CP and the inner region and the CL. This makes it possible to resolve certain type conflicts in Holloway's set, and leads to greater consistency in the allocation of cuticles to type.

2.4.1 *Cuticle types 1 and 2*

Of Holloway's cuticle types, four out of six are concerned with the intensity of the images of lamellae and their allocation between inner (CL) and outer (CP) layers. The cuticles of Type 1 have a strongly lamellate CP in which the lamellar orientation is almost invariably parallel-periclinal. The type species include *C. miniata* (Schmidt and Schönherr, 1982) and *A. americana* (e.g. Wattendorff and Holloway, 1980, 1982, 1984) with thick, substantial cuticles (Figures 2.5b–e, 2.6, 2.7, 2.8), but many species with much thinner CMs also fall into Type 1, such as *Utricularia* (Figure 2.3). The cuticle of *Apium graveolens* petiole was shown by Juniper and Cox (1973) to have a conspicuously lamellate CP and reticulate CL (Type 1) and by Hallam and Juniper (1971) to have a faintly-lamellate CP with a reticulate CL, thus Holloway's Type 2. However, in the same species, Chafe and Wardrop (1972) showed no evidence of a CP in *A. graveolens*, making it Type 4. This variation in the image contrast of the lamellae might derive either from intrinsic differences in the capacity of the lamellae to stain at different ages or developmental stages, or to differences in staining and other specimen preparation procedures between individual

workers. Holloway (1982a) placed the adaxial CM of *P. vulgaris* in which only one–three pairs of lamellae are present (Figure 2.9i, P.J. Holloway) in Type 2, but on the same developing guard cell the upper cuticular ridge, which has clear lamellae in the CP and a reticulate CL (Figure 2.1d), could equally be in Type 1. However, the lower (internal) cuticular ridge covering the guard cell bordering the intercellular space (ICS) lacks lamellae entirely, and is therefore Type 3. Since these variations of cuticle type occur in functionally distinct regions of the same cell, it seems clear that the *P. vulgaris* CMs cannot be constructed on fundamentally different models but arise from variations in the expression of different features under the control of the same genome. Such intermediates between cuticle types occur frequently within and between closely related species resulting in frequent boundary conflicts between types. Similarly, the leaf cuticle of *P. major* normally shows no lamellae (Type 3), or only the faintest trace of lamellae on the CM of the general epidermal surfaces (Type 2), but Holloway’s image of the stomatal lip (not shown) contains extensive chaotic lamellation, demonstrating that the capacity to produce lamellae is present, even though it is not uniformly expressed over all of the epidermal surface.

2.4.2 Cuticle type 3

In the section of a mature Sitka spruce cuticle shown in Holloway’s 1982 paper, the CP is not clearly evident, the outer region showing low contrast with fibrillar material close to the surface. He therefore placed Sitka spruce in Type 4. However, in other negatives of leaves at earlier developmental stages (Figure 2.2f) a clearly-demarcated electron-lucent zone 15–25 nm thick is present outside the limit reached by the electron-dense CW-derived microfibrils, which are thus capped by this layer, and do not in fact reach the surface. I interpret this layer as the CP, and therefore allocate Sitka spruce to cuticle Type 3, rather than 4. Later, maturation of the CL layer converts the outer region to a low-density material in which fibrillar contrast is reduced, against which the CP cannot easily be distinguished (Figure 2.2g). Its earlier existence must however be acknowledged, and unless it can be demonstrated that the CP is lost at maturity in *Picea*, for which there is no evidence, it must (1) remain functionally significant as a permeability barrier and (2) continue to cap the ends of the polysaccharide microfibrils. Similarly, the images of *P. abies* cuticles by Tenberge (1992) show a clearly demarcated $\bar{e}L$ layer of dimensions consistent with the CP lying on top of abruptly terminated $\bar{e}D$ microfibrils. In the earliest stages of development, prior to the appearance of the CL layer in spruce, the CP stains more darkly than in maturity (Tenberge, 1992; Figure 2.2a), its electron density later reducing to match the density of the CL matrix (Figure 2.2c). In more mature spruce CMs the region clear of microfibrils increases to a few hundred nanometres thick, presumably by progressive cross-linking, cutanisation and modification of the polymers of cuticle matrix and embedded polysaccharides. The cuticle of Sitka spruce, like that of in *P. abies*, is therefore Type 3 and in all probability is Type 3 also in other spruce species, and the microfibrils do not in fact reach the surface. These amorphous CPs with very low electron density may easily be lost against the

background, especially when photographed with slight over-exposure, giving the impression that the fibrils reach the surface. The lemon (*C. limon*) cuticle (Holloway, 1982a; Type 4) is closely similar to that of Sitka spruce, again showing a clearly demarcated layer capping the CW microfibrils embedded in the ECL and is therefore properly assigned to Type 3, not Type 4.

The leaf cuticle of *A. thaliana* is normally shown to have a Type 3 or 4 CM with a reticulate CL and an indistinct or amorphous CP without visible lamellae. However, there are lamellae in the cuticle of the stem of this species (Jenks *et al.*, 1996; Chen *et al.*, 2003; Figure 2.10i), and in cutinase-expressing *A. thaliana* (Sieber *et al.*, 2000) there is an exaggerated globular lamellate structure consisting of osmiophilic electron-dense globules and electron-lucent lamellae (Figure 2.10e,f). The layer is interrupted by large voids, presumably a consequence of cutinolytic activity, but in all essentials this assemblage strongly resembles the structure of lamellate cuticles and suberised lamellae during their assembly phases, and demonstrates that the same fundamental lamellate structural paradigm is present even when it is not normally visible or expressed in Type 3 cuticles.

Even in submerged aquatics with Type 3 cuticles [*Mentha aquatica*, *Lobelia dortmanna* (Figure 2.9d), *Berula erecta*; Frost-Christensen *et al.*, 2003], the microfibrils do not reach to the outer surface of the CM, but are capped by non-reticulate CP.

2.4.3 Cuticle type 4

By definition, Type 4 cuticles are all-reticulate, and therefore fibrils must reach the outer surface. This may apply to the cuticle of *Funaria* (Sack and Paolillo, 1983a; figure 6d). Since the CP is external to the CW by the definition used here, it is never reticulate. Logically then, fibrillar reticulum can only reach the surface of the CM if the CP is lost, or if the distribution of the CP is discontinuous, either from the start, or as a consequence of its failure to keep pace with area expansion of an organ. The question of whether a CP is present or absent has important functional implications for the CM as permeability barrier for water and polar, hydrophilic molecules (Chapters 8 and 9).

In *Hydrangea macrophylla* (Type 4; Holloway 1982a) the polysaccharide fibrils appear to be close to the surface of the CM. However, in sections stained with HI AgP a continuous non-reactive zone of the dimensions of a typical CP is demarcated at the surface on top of a CL subdivided into an outer ECL and an inner ICL layer. As in *P. abies* and *C. limon*, *H. macrophylla* is therefore more appropriately categorised as Type 3. In the same family (Solanaceae), Mérida and Ogura (1987) and Kruger *et al.* (1996) detected lamellae in the CP of *Nicotiana tabacum* and *Nicotiana glaucum*, contradicting the assignment to Type 4 in Holloway (1982a) based on the data of Akers *et al.* (1978). The CP of *I. integra* was thought by Hülsbruch (1966a,b) to be lost after synthesis of the CL, which might give it the appearance of a Type 4 cuticle. Likewise, Sargent (1976a,c) noted the loss of the lamellate structure of the CP in the mature CM of *Sisyrinchium filifolium*. The CP

in Type 3 cuticles is often of very low electron-density, and in TEM exposures calculated for the dense CL layer or CW the brightness may saturate, thus losing it from the image. Most cuticles putatively of Type 4 are the cuticles of fruits, such as tomato (*Lycopersicon esculentum*), and in some examples the fibrils may come to the surface, perhaps accounting for their high permeability to water compared with cuticles of Type 1 (Schreiber and Riederer, 1996), but this hypothesis, and indeed confirmation of the existence of Type 4 cuticles, both remain to be confirmed.

2.4.4 Cuticle types 5 and 6

Holloway (1982a) included *B. vulgaris* (Figure 2.9e) and *Taraxacum officinale* (Figure 2.9f), among examples of Type 5 CMs. In *B. vulgaris* the tightly ordered periclinally lamellate region of the CM is similar in both size and appearance to the lamellate CP of Type 1 cuticles, such as that of *I. germanica* (Figure 2.5a), and is clearly a lamellate CP with only scant development of a CL layer. The CL in the *B. vulgaris* cuticle consists of a narrow region of disordered lamellae into which CW microfibrils penetrate, but only to a depth of a few tens of nanometres (Figure 2.9e). The *B. vulgaris* genome therefore codes for CL development, but it is not extensively expressed, and *B. vulgaris* therefore has no lesser case to be included in Type 1 than does *A. americana*. Interestingly, the Chenopod *Spinacea oleracea* closely related to *B. vulgaris* has a cuticle of Type 2 or 4, with no CP lamellae or only very faint traces. In *S. oleracea*, the CL layer is much further developed than in *B. vulgaris*, and contains a conspicuous, periodate-Schiff-positive reticulum in which occasional lamellae are also visible (Holloway, unpublished TEM images). The CM of *A. americana* is amplified to enormous thickness compared with that of *B. vulgaris*, and has the trademark lamellate CP of Type 1 cuticles, but the ECL and ICL are also both densely lamellate and reticulate almost to the boundary of the ICL with the PCW. Can the *A. americana* cuticle (Holloway Type 1) be excluded legitimately from Type 5? The predominant structural distinction between the two cuticles is not of structural type but of the extent of the development of the CL layer.

As in *A. americana*, the Type 5 CM of *T. officinale* (Figure 2.9f) has not one but two main zones, the CP and the CL, each with distinctive structures. The outer region is a strongly periclinally lamellate CP, while in each case the inner shows lamellae running in less regular pattern, at a wider variety of spacings and angles. The lamellate region in *T. officinale* is therefore not equivalent with the lamellate CP of Type 1 CMs, but has lamellate CP and CL layers, nor is it identical structurally to the cuticle of *B. vulgaris*. The *B. vulgaris* CM is analogous to the outermost region of the *T. officinale* and *A. americana* cuticles, and may also be compared with an early developmental stage of the cuticle of *Utricularia* (Heide-Jørgensen, 1991; Figure 2.3c,d), while the thicker cuticle of *T. officinale* compares well with later developmental stages in *Utricularia* (Figure 2.3). In much thicker cuticles still, the two outer regions, CP and ECL, often correspond in appearance

with the *T. officinale* cuticle, with lamellate CP and less ordered ECL, while the inner CL (ICL) is extensively reticulate. However, even in the innermost ICL there may be lamellae right down to the boundary with the PCW [*A. americana* (Wattendorff and Holloway, 1984; Figure 2.7)]. As in *T. officinale*, periclinally ordered lamellae are the signature of the CP, and the boundary between the CP and the CL is usually marked by a descent into more disordered and ultimately chaotic orientation of lamellae. Holloway (1982a) included *Eucalyptus cinerea* in Type 5 following Hallam (1964; Hallam figure 4.2 in Martin and Juniper, 1970) who described its cuticle as having anastomosing channels through which waxes might migrate to the surface (Hallam, 1964, 1967). In this CM the superficial three or four lamellae appear to be in tight order, and probably represent the CP, while the remainder show much more variable electron-dense lamella thickness and anastomoses between the electron-lucent lamellae and probably represent the ECL rather than the CP.

Holloway (1982a) shows the *Potamogeton* cuticle (Figure 2.9c) as Type 6, but close inspection of his negatives indicates that it would also be possible to classify it as Type 3, since microfibrils penetrate the flocculent structure at the base, resulting in a weakly-developed reticulate CL. In Holloway's images of *Lemna* (Figure 2.9a) and *Elodea* (Figure 2.9b) and *Potamogeton* (Figure 2.9c) attached epiphytic microorganisms demarcate the upper limit of the very electron-lucent CP layer, which is amorphous in *Elodea*, but contains one or two pairs of faint lamellae in *Lemna minor*. The cuticle of *L. minor* is thus an example of a minimally developed Type 1 cuticle, rather than Type 6.

The caveats that apply to Type 5 CMs also apply to Type 6. Classifications must be made on uniform criteria. Holloway's definitions of the cuticle types refer to 'Inner' and 'Outer' regions without explicitly connecting the outer region and the CP, although the two coincide in Type 1 cuticles for example. This makes it possible to include in Types 5 (all lamellate) and Type 6 (all amorphous) three categories of CMs that are radically different structurally and ontogenetically: cuticles that are all-CP with little or no CL development; cuticles that are all-CL without any evident CP; and cuticles with well-developed CP and CL.

I therefore propose that this connection should be formally made, making it necessary to specify separately the structure of the CP and CL layers.

2.4.5 A seventh cuticle type?

In a variation that falls outside Holloway's six types the adaxial leaf cuticles of *M. pumila* and *P. communis* both show a clear, amorphous CP with a chaotically lamellate ECL penetrated by CW microfibrils. Holloway (1982a) placed these CM in Types 2 (*P. communis* and *M. pumila* leaf adaxial cuticles; Figures 2.1a and c), but the presence of lamellae deep into the CL is an uncomfortable fit with either of these types. Lamellae are apparently absent in the abaxial leaf cuticles of the same species which are therefore Type 3 (see Holloway, 1982a).

2.5 Summary of the cuticle types

The fine structure of cuticles varies predominantly in four parameters: the presence or absence of lamellae, the presence or absence of reticulum, the subdivision or otherwise of the CL layer into inner ICL and outer ECL layers and the presence or absence of any of the four layers (CP, CL, ECL, ICL). Probably, the major differences between cuticle structural types can be ascribed to the relative extent of the development of the CM and of the underlying CW, rather than to fundamental differences in mechanism, or biochemistry. Thus, for example, the simple lamellate CM of *B. vulgaris* (Holloway, 1982a) is structurally analogous to those of *C. miniata* (Figure 2.6b) and *A. americana* at the earliest stages of development which have been documented (Wattendorff and Holloway, 1980; Riederer and Schönherr, 1988). Indeed it is striking that across almost all cuticles for which adequate ultrastructural data are currently available the range of variation in structure displayed between fully developed CMs of increasing thickness, from example, *Potamogeton* to *B. vulgaris*. *P. vulgaris* and *T. officinale* through *I. germanica*, *H. helix*, *C. miniata* and *A. americana*, is paralleled by the changing complexity of the thickest of these cuticles during stages of their development (Jeffree, 1996). There are detailed variations in cuticle size, structure and composition between the cuticles of Bryophytes and vascular plants, between one plant family and another, and between plants adapted to contrasting ecological conditions and lifestyles; but it is also evident that they all contain elements of a common set of ultrastructural features. The cuticles of all land plants are therefore all variants on a common theme, based on the same fundamental paradigm, not on a set of six or seven different paradigms.

2.5.1 Cuticle structure/ecology

What if anything is the functional relevance of cuticle structural types? Contrasting ecological types, such as tropical rainforest species and temperate mesophytes can both have the same structural types. For example, cuticles of *F. elastica* and *P. vulgaris* and *S. oleracea* are all included in Holloway's cuticle Type 2. Structural Type 1 also occurs in *A. americana*, *Phormium*, *Iris*, *Hedera* and *Apium*. Species with lamellate CP *sensu lato* (Types 1, 2, 5) occur in genera as diverse as *Beta*, *Taraxacum*, *Humulus*, *Spartocytisus*, *Agave*, *Clivia*, *Pistacia*, *Pseudotsuga* and *Polytrichum*, while contrasting types occur in related taxa: *Abies spp.* and *Picea spp.* are in structural Type 4 (Holloway, 1982a) but probably best allocated to Type 3 as here, since there is often a clearly demarcated amorphous CP layer (Table 2.1), while cuticles of *Cunninghamia* and *Pseudotsuga* leaves have a lamellate CP, and are thus Type 1.

2.5.2 Cuticle thickness and environment

Cuticle thickness, morphology and composition differ between plants cultivated under glass, in tissue culture and in the field (Martin and Juniper, 1970; van den Ende

and Linskens, 1974). So also do the thickness and morphology of the cuticle differ between aerial and submerged leaves of amphibious plants (Frost-Christensen *et al.*, 2003; Table 2.2). In *Q. velutina*, sun leaf CMs are thicker than shade leaf CMs due to massive amplification of the reticulate ICL (Osborn and Taylor, 1990). In the adaxial cuticles, Osborne and Taylor (1990) reported a thickened amorphous CP. However, at 0.76 μm thick this region is an order of magnitude or thicker than a typical CP as defined here, and is mounted atop a massive reticulate ICL at least 1.4 μm thick. This thick amorphous region is almost certainly not only the CP but includes an intensively cutanised ECL, and it is probable that a study of the ontogeny of this species would demonstrate the existence of an equivalent reticulate layer in expanding leaves that loses its reticulate appearance as the ECL matures. An amorphous CP, as appears to be present on abaxial leaf surfaces, would be hard to distinguish from the amorphous ECL, but its presence would again be revealed by an ontogenetic study. Different CM structures were observed by Osborne and Taylor (1990) on different cells of the abaxial epidermis, normal epidermal cells having an amorphous CP (Type 3), while subsidiary cells have reticulate CM that traverse most of the outer CM without a CP (thus Type 4). However, in my judgement, both of these CMs have a clearly distinguishable amorphous zone outside the termination zone of the CL fibrils, with dimensions within the typical range for a CP (Table 2.1). Both sun and shade outer guard cell CMs are therefore Type 3. The CM continues through the stomatal aperture to line the inner periclinal wall of the guard cells. Moreover an internal CM, which is only sparsely reticulate, lines substomatal chambers. The internal CM of sun leaves is thicker and extends considerably deeper into substomatal chambers than in shade leaves.

2.5.3 *Cuticle structure and phylogeny*

Norris and Bukovac (1968) commented that ‘because of technical difficulties, many early anatomical investigations were limited to plants with thick cuticles, such as *Yucca*, *Dasyllirion*, *Clivia*, *Agave* and *Ficus*. No information is available to establish how well these early findings represent the cuticle of mesophytic economic crop plants’. The cuticles for which we have structural data remain a trivial subset of world species, and crop plant cuticles are relatively poorly sampled. Notably missing from Table 2.2 are records for rice, most grain and forage grasses, sugarcane and indeed the cuticle structures of the vast majority of the world’s top crops, and the dominant species of the world’s forests and other vegetation formations are either unknown or poorly characterised.

Most of the generalisations about cuticle structure discussed so far appear to apply across the spectrum of extant vascular plants including the ferns although records for important basal groups such as the Lycopsidea and Equisetopsida are lacking. Cuticles were an enabling innovation in the attainment of homoiohydry by land plants (Raven, 1977), appearing in the earliest axial pre-vascular and vascular land plants of the Silurian, although the ‘cuticles’ of some other coeval

land plants such as *Nematothallus* did not contain cutin or cutan (Edwards *et al.*, 1996). The gametophytes and sporophytes of many mosses have both stomata and cuticles analogous in fine structure to those of Tracheophytes (see e.g. *Funaria hygrometrica*; Figure 2.4d). The gametophytes of the Polytrichales (*Dawsonia*, *Polytrichum*) also have waxy cuticles at the ends of the photosynthetic lamellae at their leaf bases. The cuticle fine structure of these has not been reported, although EW similar to those of vascular plants are present (Neinhuis and Jetter, 1995). By contrast, the cuticle of the advanced thalloid liverwort *Marchantia* (gametophyte, Marchantiopsida) is very thin, completely without lamellae or fibrillar/reticulate structure, and presumably falls into Type 6. The reaction is negative to staining with uranyl and lead salts and KMnO_4 indicating that it is waxy, impermeable and non-polar. It seems likely that common biosynthetic pathways for the production of these compounds are present in the most primitive groups of extant land plants as well as in representatives of the most advanced, and that waxes from these widely distributed taxa can have closely comparable compositional profiles. The implication of this is that the biosynthetic pathways involved may already have been present in the most basal land plants of the Silurian, and that they have probably been strongly conserved during their subsequent evolutionary radiation.

2.6 The epicuticular wax

The EW often form a visible waxy bloom on plant surfaces. They are readily isolated and examined by light and electron microscopy, and their properties, structure and chemistry are consequently much studied (Chapters 4, 5, 7–9; Baker, 1982; Barthlott and Frölich, 1983; Jeffree, 1986; Barthlott, 1990, 1993; Barthlott *et al.*, 1998). Waxes occur on the surfaces of all land plants. EW of mosses and liverworts may contain identical compounds to those of Gymnosperms and Angiosperms, suggesting that they appeared early in land plant evolution, but although cuticles survive in the fossil record, waxes are fugitive. Plants with waxy bloom and thick cuticles are correlated in the minds of many with xeric habitats of deserts, coasts and dunes, but there is no straightforward relationship, and the Lotus effect, synonymous today with ultra-hydrophobicity caused by wax nano-structures (Barthlott and Neinhuis, 1997; Neinhuis and Barthlott, 1997; Furstner *et al.*, 2000), is named after the sacred lotus *Nelumbo nucifera*, an aquatic species with glaucous leaves that uses waxes to repel water rather than conserve it. The ultrastructure of the EW and that of other species is both complex and varied. On grapes, where the EW layer is developed sufficiently to scatter light, the surface has a matt, bluish, glaucousness, contrasting with the glabrous bright green glossiness of species carrying little EW. The wax structures of sugarcane (*Saccharum officinarum*) are sufficiently large to be seen individually with a powerful hand lens or light microscope, and were accurately described by de Bary (1871, 1884), but on most species wax structure is only visible in the electron microscope.

EW are often described as SCL, which does not distinguish between cuticular waxes embedded in the CM and EW located outside the CM. The terms 'surface lipids' and 'superficial wax' are also common in the literature. EW is defined here solely on the basis of its position on the leaf surface as wax, freely soluble or otherwise, deposited externally to the CP. This definition includes overtly crystalline and amorphous wax structures and any background wax film, structureless or otherwise, whether freely soluble or not. The question of whether EW is visible or otherwise in the scanning electron microscope (SEM; Neinhuis and Barthlott, 1996) is irrelevant. This definition is based on more than an arbitrary choice of boundary. There has long been evidence that wax embedded in the cuticle (intracuticular wax, CW) is chemically distinct from EW (Martin, 1960; Baker, 1977), and recent developments in methods of isolating the EW without contaminating the extract with CW have strengthened this view (Jetter *et al.*, 2000).

The quantity of EW may be as little as $1 \mu\text{g cm}^{-2}$ on leaves of some temperate annuals but the fan palm *Copernicia cerifera*, the source of carnauba wax, and the ouricuri palm *Syagurus coronata*, both of arid north-eastern Brazil, have thick EW crusts weighing several milligrams per square centimetre that can be mechanically harvested by thrashing the dried leaves. Carnauba wax has the highest melting point of any natural wax, and is the most commercially important, used to give a hard, abrasion-resistant shine to products as diverse as cars, cabinets and candy. The Andean wax palms *Ceroxylon spp.* by contrast inhabit the cool, humid cloud forests at altitude in South America. Obligate submerged aquatic and amphibious plants have little or no EW, in which case their surfaces are fully wettable [e.g. *Myriophyllum spp.* (Hallam, 1982); *Potamogeton crispus* (Frost-Christensen, *et al.*, 2003)]. Many mesophytic species of commercial importance including *Lactuca sativa*, *P. vulgaris* (Baker, 1982), *B. vulgaris* (Baker and Hunt, 1981) and *N. tabacum* (Jeffree *et al.*, 1975; Jeffree, 1986) have no visible wax, but carry a measurable load nevertheless. Visible wax loads are absent on many weed species too, making them easy to wet with herbicide sprays (*Rumex obtusifolia*, *Stellaria media* and *Myosotis arvensis*; Baker and Bukovac, 1971). In the majority of species the EW layer is visible by electron microscopy (Juniper and Bradley, 1958; Amelunxen *et al.*, 1967; Jeffree, 1986 and references therein). The wax loads on fruits are invariably heavier than those on the leaves of the same species, which in turn may carry different amounts of wax on their adaxial and abaxial surfaces. The chemical composition of the wax may differ from place to place on a plant, and the fine structure may vary correspondingly. Notable examples are the leaves of peas and wheat, which have different wax chemistry and wax ultrastructure on the adaxial and abaxial surfaces.

2.6.1 Epicuticular wax types

Classification of EW types on the evidence of SEM images was first attempted by Amelunxen *et al.* (1967), who extended de Bary's original scheme (1871, 1884) based on observations using light microscopy (LM). Amelunxen *et al.* (1967)

proposed five main classes:

- (1) 'Wachskörnchen' or grains,
- (2) 'Wachsstäbchen und -fäden' or rods and filaments,
- (3) 'Wachsplättchen und -schuppen' or plates and scales,
- (4) 'Wachsschichten und -krusten' or films and crusts and
- (5) 'Flüssiger und schmieriger Wachsüberzug' or fluid and greasy wax layers.

Each class contained two–five types making sixteen basic types. A further class dealt with massed accumulations of the grains, rods and filaments already included in their classes 1 and 2.

Only waxes with discrete structures large enough to be resolvable in LM were included in de Bary's (1871, 1884) classification. The fact that his classification stood the test of a technology capable of an order of magnitude better resolution is in one sense a remarkable testament to his powers of observation, but it is also attributable to the fact that simple paradigms for EW forms occur repeatedly at a range of size scales. Those gigantic rods he observed on *S. officinarum* are paralleled by ten-fold smaller rodlets on other species. It is now clear that many of the examples of rod and filament waxes (*Rosa canina* fruit, *Tulipa gesneriana* fruit and leaf, *Eucalyptus globulus* leaf) of Amelunxen *et al.* (1967) and Metcalfe and Chalk (1979) are in fact members of a more recently recognised tube-type (Hallam, 1967; Johnson and Jeffree, 1970). In the early SEM images of tube waxes, resolution was scarcely adequate to confirm the existence of a hollow centre in these tubes (see however Johnson and Jeffree, 1970; Jeffree, 1974a; Jeffree *et al.*, 1976). Misclassification frequently arises out of inadequate image resolution (arguably the same is true for many reportedly non-lamellate cuticles), but there are many instances of clear misclassification in the literature even when resolution had been adequate, and classifications based on morphology alone are often unsound. Re-examination of figure 8 of Hallam and Juniper (1971) clearly shows that the 'granules' of wax on the surface of a fruit of *Prunus sp.* in a crisp micrograph of a TEM replica (supplied by D. Skene) are short curls and tubes overlying plate-like structures. The EW of ripe fruit of *Prunus domestica* contains about 48% of asymmetrical secondary alcohols, mostly 10-nonacosanol (Holloway *et al.*, 1976), now known to produce hollow tubular epicuticular crystals (Jeffree, 1974a; Jeffree *et al.*, 1975, 1976; Jetter and Riederer, 1994).

The most commonly encountered crystal morphologies in plant EW remain amorphous films, grains or granules, plates (simple or crenate, polygonal or rounded or spiky, prostrate or erect), filaments, rods, generally terete in cross-section, but other shapes are possible: tubes with a hollow centre; elongated flattened ribbons with various forms of edge decoration; and plates (Table 2.3), and there are good reasons to take a conservative approach in classifying EW types. First, although attempts have been made to describe and classify these structures over many decades, our understanding of the morphological range is still limited by poor resolution of the morphology in many cases, and by a crude vocabulary for description of variations

Table 2.3 The main epicuticular wax morphological types, and their distribution among species

Species	Family or higher taxon	Organ	Reference
Amorphous films, or no visible wax			
<i>Acer mono</i>	Aceraceae	Lab	Barthlott and Neinhuis (1997)
<i>Aesculus hippocastanum</i>	Hippocastanaceae	L	Juniper and Bradley (1958)
<i>Arabidopsis thaliana</i>	Brassicaceae	L	Jenks <i>et al.</i> (2002)
<i>Arabidopsis thaliana</i>	Brassicaceae	Sepal	Figure 2.12a
<i>Beta vulgaris</i>	Chenopodiaceae	LAd	Baker and Hunt (1981)
<i>Blechnum capense</i>	Blechnaceae	L	Hall and Burke (1974)
<i>Bromus interruptus</i>	Poaceae	A	Jeffree, unpublished
<i>Chamerion angustifolium</i>	Onagraceae	P	Figure 2.14f
<i>Chenopodium album</i>	Chenopodiaceae	L	Baker and Bukovac (1971)
<i>Chionochloa rigida</i>	Poaceae	LAd	Hall and Burke (1974)
<i>Chrysanthemum morifolium</i>	Asteraceae	L	Sutter (1985)
<i>Citrus limon</i> cv. 'Adamopolou'	Ranunculaceae	L	Jeffree <i>et al.</i> (1975); Jeffree (1986)
<i>Clematis vitalba</i>	Ranunculaceae	L	Baker (1982)
<i>Coffea arabica</i>	Rubiaceae	LAd	Silva Fernandes (1965a,b)
<i>Dieffenbachia maculata</i>	Araceae	L	Sutter (1985)
<i>Euphorbia cerifera</i>	Euphorbiaceae	L	Juniper and Jeffree (1983)
<i>Fagus sylvatica</i>	Fagaceae	LAd	Barthlott and Neinhuis (1997)
<i>Gaultheria depressa</i>	Ericaceae	LAd	Hall and Burke (1974)
<i>Gerbera jamesonii</i>	Asteraceae	L	Sutter (1985)
<i>Gnetum gnemon</i>	Gnetaceae	L	Barthlott and Neinhuis (1997)
<i>Griselinia littoralis</i>	Cornaceae	LAd	Hall and Burke (1974)
<i>Heliconia densiflora</i>	Heliconiaceae	LAd	Barthlott and Neinhuis (1997)
<i>Hydrangea hortensis</i>	Hydrangeaceae	L	Silva Fernandes (1965a,b)
<i>Hydrocotyle bonariensis</i>	Apiaceae	L	Barthlott <i>et al.</i> (1998)
<i>Juncus inflexus</i>	Cyperaceae	L	Juniper (1960)
<i>Lactuca sativa</i>	Asteraceae	L	Baker (1982)
<i>Lycopersicon esculentum</i>	Solanaceae	F, L	Jeffree <i>et al.</i> (1975); Jeffree (1986)
<i>Magnolia grandiflora</i> L.	Magnoliaceae	LAd	Barthlott and Neinhuis (1997)
<i>Malus hupehensis</i>	Rosaceae	LAd	Baker and Hunt (1981)
<i>Myosotis arvensis</i>	Boraginaceae	L	Baker and Bukovac (1971)
<i>Myriophyllum</i> spp.	Haloragaceae	SL	Hallam (1982)
<i>Nicotiana tabacum</i>	Solanaceae	L	Jeffree <i>et al.</i> (1975); Jeffree (1986)
<i>Nothofagus solandri</i> var. <i>Cliffortioides</i>	Nothofagaceae	LAd	Hall and Burke (1974)
<i>Nothofagus</i> spp.	Nothofagaceae	LAd	Hall and Burke (1974)
<i>Phaseolus vulgaris</i>	Fabaceae	L	Baker (1982)
<i>Potamogeton crispus</i>	Potamogetonaceae	SL	Hallam (1982)
<i>Prunus laurocerasus</i>	Rosaceae	L	Davis (1971)
<i>Prunus laurocerasus</i>	Rosaceae	LAd	Jetter <i>et al.</i> (2000)
<i>Prunus persica</i> cv. 'Red Haven'	Rosaceae	LAd	Jeffree <i>et al.</i> (1975); Jeffree (1986)
<i>Rhododendron ponticum</i>	Ericaceae	Lab	Holloway and Jeffree (2005)
<i>Rumex obtusifolius</i>	Polygonaceae	L	Juniper and Bradley (1958); Baker and Bukovac (1971)

Continued

Table 2.3 Continued

Species	Family or higher taxon	Organ	Reference
<i>Spathiphyllum wallisii</i>	Araceae	LAd	Sutter (1985)
<i>Spinacia oleracea</i>	Chenopodiaceae	L	Baker (1982)
<i>Stellaria media</i>	Caryophyllaceae	L	Baker and Bukovac (1971)
<i>Tamus communis</i>	Dioscoreaceae	L	Baker (1982)
<i>Taraxacum officinale</i>	Asteraceae	L	Baker and Bukovac (1971)
<i>Tilia cordata</i>	Tiliaceae	LAd	Schreiber (1990)
<i>Trifolium repens</i>	Fabaceae	LAB	Holloway (1971); Baker (1982)
<i>Ulex europaeus</i>	Fabaceae	S	Zabkiewicz and Gaskin (1978)
<i>Vicia faba</i>	Fabaceae	LAd	Holloway and Jeffree (2005)
<i>Vitis vinifera</i>	Vitaceae	LAB	Baker (1982)
Granules			
<i>Aegiceras corniculatum</i>	Aegicerataceae	L	Barthlott <i>et al.</i> (1998)
<i>Citrus limon</i> cv. 'Adamopolou'	Rutaceae	F	Jeffree <i>et al.</i> (1975, 1976); Baker (1982)
<i>Citrus paradisi</i> cv. 'Marsh seedless'	Rutaceae	JV	Fahn <i>et al.</i> (1974)
<i>Conocephalum conicum</i>	Bryophyta	G	Schönherr and Ziegler (1975)
<i>Exomotheca bullata</i>	Bryophyta	G	Schönherr and Ziegler (1975)
<i>Hedera helix</i>	Araliaceae	LAd	Martin and Juniper (1970)
<i>Helianthus annuus</i>	Asteraceae	LAd	Hallam and Juniper (1971)
<i>Lunularia cruciata</i>	Bryophyta	G	Schönherr and Ziegler (1975)
<i>Macaranga triloba</i>	Euphorbiaceae	S	Markstädter <i>et al.</i> (2000)
<i>Marchantia paleacea</i>	Bryophyta	G	Schönherr and Ziegler (1975)
<i>Marchantia polymorpha</i>	Bryophyta	G	Schönherr and Ziegler (1975)
<i>Pistacia vera</i>	Anacardiaceae	LAd	Baker (1982)
<i>Plagiochasma rupestre</i>	Bryophyta	G	Schönherr and Ziegler (1975)
<i>Reboulia hemisphaerica</i>	Bryophyta	G	Schönherr and Ziegler (1975)
<i>Targionia hypophylla</i>	Bryophyta	G	Schönherr and Ziegler (1975)
Soft waxes			
<i>Crataegus prunifolia</i>	Rosaceae	F	Metcalf and Chalk (1979)
<i>Malus</i> sp.	Rosaceae	F	Metcalf and Chalk (1979), Martin and Juniper (1970)
Rodlets			
(a) <i>Aristolochia</i> Type: <i>Transversely ridged rodlets</i>			
<i>Actinidia melanandra</i>	Actinidiaceae	L	Barthlott <i>et al.</i> (1998)
<i>Ammophila arenaria</i>	Poaceae	LAd	Figure 2.12b
<i>Aristolochia</i>	Aristolochiaceae	L	Frölich and Barthlott (1988)
<i>Aristolochia gigantea</i>	Aristolochiaceae	L	Meusel <i>et al.</i> (1999)
<i>Centranthus ruber</i>	Valerianaceae	L	Meusel <i>et al.</i> (1999)
<i>Fritillaria meleagris</i>	Liliaceae	L	Frölich and Barthlott (1988)
<i>Fritillaria pallidiflora</i>	Liliaceae	L	Meusel <i>et al.</i> (1999)
<i>Gypsophila acutifolia</i>	Caryophyllaceae	L	Meusel <i>et al.</i> (1999)
<i>Laurus nobilis</i>	Lauraceae	L	Meusel <i>et al.</i> (1999)
<i>Leucjum aestivum</i>	Liliaceae	L	Meusel <i>et al.</i> (1999); Frölich and Barthlott (1988)
<i>Liriodendron</i> sp.	Magnoliaceae	LAB	Neinhuis and Barthlott (1996)
<i>Magnolia</i> sp.	Magnoliaceae	LAB	Neinhuis and Barthlott (1996)

Table 2.3 Continued

Species	Family or higher taxon	Organ	Reference
<i>Monodora sp.</i>	Annonaceae	LAB	Neinhuis and Barthlott (1996)
<i>Nicotiana glauca</i>	Solanaceae	L	Meusel <i>et al.</i> (1999)
<i>Osmunda regalis</i>	Osmundaceae	L	Jetter and Riederer (1999b, 2000)
<i>Paeonia mlokosewitschii</i>	Paeoniaceae	L	Meusel <i>et al.</i> (1999)
<i>Paeonia officinalis</i>	Paeoniaceae	L	Meusel <i>et al.</i> (1999)
<i>Williamodendron quadrilocellatum</i>	Lauraceae	LAB	Neinhuis and Barthlott (1996)
<i>Brassica napus</i> Rigo mutant	Brassicaceae	L	Jeffree <i>et al.</i> (1975, 1976); Baker (1982)
<i>Brassica oleracea</i> mutant gl2	Brassicaceae	L	Jeffree <i>et al.</i> (1975, 1976); Baker (1982)
<i>Galanthus nivalis</i>	Amaryllidaceae	L	Koch <i>et al.</i> (2004)
(b) <i>Strelitzia</i> Type: Longitudinally striated rods without transverse ridges			
<i>Arundinaria sp.</i>	Poaceae	S	Jeffree <i>et al.</i> (1976)
<i>Benincasa hispida</i>	Cucurbitaceae	F	Meusel <i>et al.</i> (1994)
<i>Carex flacca</i>	Cyperaceae	L	Frölich and Barthlott (1988)
<i>Colletia cruciata</i>	Rhamnaceae	L	Barthlott <i>et al.</i> (1998)
<i>Dendrocalamus giganteus</i>	Poaceae	L	Frölich and Barthlott (1988)
<i>Eryngium sp.</i>	Apiaceae	L	Meusel <i>et al.</i> (1994)
<i>Hedychium flavum</i>	Zingiberaceae	L	Frölich and Barthlott (1988)
<i>Heliconia collinsiana</i>	Heliconiaceae	L	Barthlott <i>et al.</i> (1998)
<i>Maranta leuconeura</i>	Marantaceae	LAd	Sutter (1985)
<i>Musa paradisica</i>	Musaceae	L	Jeffree <i>et al.</i> (1975, 1976); Baker (1982)
<i>Phenacospermum guianense</i>	Strelitziaceae	L	Barthlott <i>et al.</i> (1998)
<i>Phragmites australis</i>	Poaceae	L	Jeffree, unpublished
<i>Saccharum officinarum</i>	Poaceae	LS	de Bary (1871); Jeffree <i>et al.</i> (1976)
<i>Sorghum bicolor</i>	Fabaceae	L	Blum (1975)
<i>Strelitzia reginae</i>	Strelitziaceae	LAB	Meusel <i>et al.</i> (1994)
<i>Syagrus coronata</i>	Arecaceae	LAB	Machado and Barros (1995)
<i>Typha angustifolia</i>	Typhaceae	L	Djebrouni (1989)
<i>Typha elephantina</i>	Typhaceae	L	Djebrouni (1989)
<i>Typha latifolia</i>	Typhaceae	L	Djebrouni (1989)
(c) Simple Rod Type			
<i>Butomus umbellatus</i>	Butomaceae	L	Frölich and Barthlott (1988)
<i>Galtonia candicans</i>	Hyacinthaceae	L	Frölich and Barthlott (1988)
<i>Maranta leuconeura</i>	Marantaceae	L	Sutter (1985)
<i>Sceletium compactum</i>	Aizoaceae	L	Barthlott <i>et al.</i> (1998)
(d) Polygonal Rod Type			
<i>Daphne tangutica</i>	Thymelaceae	L	Barthlott <i>et al.</i> (1998)
Filaments			
(a) Simple filament or thread			
<i>Citrus paradisi</i> cv. 'Marsh seedless'	Rutaceae	JV	Fahn <i>et al.</i> (1974)
<i>Cyathodes colensoi</i>	Epacridaceae	LAB	Figure 2.12f

Continued

Table 2.3 Continued

Species	Family or higher taxon	Organ	Reference
<i>Drosera burmanni</i>	Droseraceae	L	Barthlott <i>et al.</i> (1998)
<i>Leptospermum laevigatum</i>	Rosaceae	L	Hallam and Juniper (1971)
<i>Pisum sativum</i>	Fabaceae	LAB	Juniper and Bradley (1958); Juniper (1959); Martin and Juniper (1970); Jeffree <i>et al.</i> (1975, 1976)
<i>Saelania glaucescens</i>	Musci	L	Haas (1982)
<i>Solanum tuberosum</i>	Solanaceae	Tu	Hayward (1974)
<i>Sorghum bicolor</i>	Poaceae	LS	Atkin and Hamilton (1982)
(b) <i>Flattened filament or ribbon</i>			
<i>Macaranga hypoleuca</i>	Euphorbiaceae	S	Markstädter <i>et al.</i> (2000)
<i>Pisum sativum</i>	Leguminosae	LAB, P, St, T	Juniper (1960); Baker and Holloway (1971); Holloway (1971); Jeffree <i>et al.</i> (1975, 1976)
<i>Wooleya farinose</i>	Mesembryanthemaceae	L	Ihlenfeldt and Hartmann (1982)
(c) <i>Chiral filament or ribbon</i>			
<i>Macaranga lamellate</i>	Euphorbiaceae	S	Markstädter <i>et al.</i> (2000)
(d) <i>Filament or ribbon network</i>			
<i>Acer mono</i>	Aceraceae	LAB	Neinhuis and Barthlott (1996)
<i>Acer pseudoplatanus</i>	Aceraceae	LAB	Holloway (1971); Wilson (1984)
<i>Fragaria ananassa</i> cv. 'Cambridge Favourite'	Rosaceae	LAB	Mackerron (1976)
<i>Fragaria ovalis</i>	Rosaceae	LAd	Baker (1982)
<i>Potentilla fruticosa</i>	Rosaceae	L	Rentschler (1971)
<i>Rosa sp.</i>	Rosaceae	LAB	Figure 2.14e
Brassica type, columns and plates			
<i>Arabidopsis thaliana</i>	Brassicaceae	S	Teusink <i>et al.</i> (2002); Jenks <i>et al.</i> (2002)
<i>Brassica napus</i>	Brassicaceae	L	Holloway and Jeffree (2005)
<i>Brassica napus</i>	Brassicaceae	L	Armstrong and Whitecross (1976)
<i>Brassica oleracea</i> var. <i>capitata</i>	Brassicaceae	LAd	Martin and Juniper (1970); Figures 2.5, 4.28
<i>Brassica oleracea</i> var. <i>capitata</i>	Brassicaceae	L	Martin and Juniper (1970); Hallam and Juniper (1971)
<i>Brassica oleracea</i> var. <i>gemmifera</i>	Brassicaceae	L	Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Bupleurum salicifolium</i>	Apiaceae	L	Barthlott <i>et al.</i> (1998)
<i>Centranthus ruber</i>	Valerianaceae	L	Baker (1982)
<i>Clarkia elegans</i>	Onagraceae	L	Hunt <i>et al.</i> (1976); Baker (1982)
<i>Crambe maritima</i>	Brassicaceae	L	Rentschler (1971)

Table 2.3 Continued

Species	Family or higher taxon	Organ	Reference
<i>Erythronium rostratum</i>	Liliaceae	L	Frölich and Barthlott (1988)
<i>Polygala myrtifolia</i>	Polygalaceae	L	Rentschler (1971)
<i>Thellungiella halophila</i>	Brassicaceae	LAd	Jenks <i>et al.</i> (2002)
<i>Thellungiella parvula</i>	Brassicaceae	LAd	Jenks <i>et al.</i> (2002)
Tubes			
(a) <i>Asymmetrical secondary alcohol tubes</i>			
<i>Abies concolor</i>	Pinaceae	L	Hanover and Reicosky (1971)
<i>Agathis australis</i>	Pinaceae	L	Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Aquilegia alpinum</i>	Ranunculaceae	L	Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Aquilegia formosa</i>	Ranunculaceae	L	Rentschler (1971)
<i>Aquilegia vulgaris</i>	Ranunculaceae	L	Rentschler (1971); Holloway and Jeffree (2005)
<i>Atrichum undulatum</i>	Polytrichales	Cap	Neinhuis and Jetter (1995)
<i>Berberis</i>	Berberidaceae	L	Barthlott and Theisen (1995)
<i>Callitris columnaris</i>	Cupressaceae	LAB	Attwill and Clayton-Greene (1984)
<i>Chamaecyparis lawsoniana</i>	Cupressaceae	L	Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Chamaecyparis obtusa</i>	Cupressaceae	L	Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Chelidonium majus</i>	Papaveraceae	L	Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Cotinus coggygria</i>	Anacardiaceae	L	Rentschler (1971)
<i>Dawsonia beccarii</i>	Polytrichaceae	Cap	Neinhuis and Jetter (1995)
<i>Dawsonia beccarii</i>	Polytrichales	Cap	Neinhuis and Jetter (1995)
<i>Exochorda racemosa</i>	Rosaceae	L	Silva Fernandes (1964); Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Ginkgo biloba</i>	Ginkgoaceae	L	Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Oligotrichum hercynicum</i>	Polytrichaceae	Cap	Neinhuis and Jetter (1995)
<i>Papaver somniferum</i>	Papaveraceae	L	Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976); Rentschler (1973)
<i>Papaver somniferum</i>	Papaveraceae	Cap	Barthlott <i>et al.</i> (1996)
<i>Picea abies</i>	Pinaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Picea glauca</i>	Pinaceae	L	Hanover and Reicosky (1971)
<i>Picea pungens</i>	Pinaceae	L	Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Picea pungens</i>	Pinaceae	L	Hanover and Reicosky (1971)
<i>Picea pungens</i> var. <i>glauca</i>	Pinaceae	L	Hanover and Reicosky (1971)
<i>Picea sitchensis</i>	Pinaceae	L	Jeffree (1974b); Jeffree <i>et al.</i> (1975, 1976)
<i>Picea sitchensis</i>	Pinaceae	L	Hanover and Reicosky (1971)

Continued

Table 2.3 Continued

Species	Family or higher taxon	Organ	Reference
<i>Pinus balfouriana</i>	Pinaceae	L	Jeffree, unpublished
<i>Pinus nigra</i>	Pinaceae	L	Hanover and Reicosky (1971)
<i>Pinus nigra</i> var. <i>maritima</i>	Pinaceae	L	Campbell (1972)
<i>Pinus radiata</i>	Pinaceae	L	Rook <i>et al.</i> (1971); Leyton and Juniper (1963)
<i>Pinus strobus</i>	Pinaceae	L	Hanover and Reicosky (1971)
<i>Pinus sylvestris</i>	Pinaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Pinus</i> , 51 species	Pinaceae	L	Yoshie and Sakai (1985)
<i>Pogonatum belangeri</i>	Polytrichales	Cap	Neinhuis and Jetter (1995)
<i>Pogonatum urnigerum</i>	Polytrichaceae	Cap	Neinhuis and Jetter (1995)
<i>Pogonatum urnigerum</i>	Polytrichales	Cap	Neinhuis and Jetter (1995)
<i>Polytrichum commune</i>	Polytrichales	Cap	Neinhuis and Jetter (1995)
<i>Polytrichum formosum</i>	Polytrichales	Cap	Neinhuis and Jetter (1995)
<i>Polytrichum juniperinum</i>	Polytrichaceae	Cap	Neinhuis and Jetter (1995)
<i>Polytrichum juniperinum</i>	Polytrichales	Cap	Neinhuis and Jetter (1995)
<i>Prunus domestica</i>	Rosaceae	L	Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Prunus domestica</i> cv. 'd'Agen'	Rosaceae	F	Bain and McBean (1967, 1969)
<i>Prunus</i> sp.	Rosaceae	F	D. Skene in Hallam and Juniper (1971); Martin and Juniper (1970)
<i>Pseudotsuga menziesii</i>	Pinaceae	L	Campbell (1972); Thair and Lister (1975); Lister and Thair (1981)
<i>Rhus cotinus atropurpurea</i>	Anacardiaceae	L	Silva Fernandes (1964); Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Rosa</i> sp. cv. 'Baccara'	Rosaceae	S	Silva Fernandes (1965b); Holloway (1971); Baker and Holloway (1971); Baker (1982)
<i>Thalictrum flavum</i>	Ranunculaceae	L	Barthlott <i>et al.</i> (1996)
<i>Tropaeolum majus</i>	Geraniaceae	L	Rentschler (1971); Jeffree <i>et al.</i> (1975, 1976); Brunegger <i>et al.</i> (1982)
<i>Tropaeolum speciosum</i>	Geraniaceae	L	C.E. Jeffree
<i>Tulipa gesneriana</i>	Liliaceae	L	Rentschler (1971); Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Tulipa kaufmanniana</i>	Liliaceae	L	Johnson and Jeffree (1970)
Waxes of apple, hawthorn and rose also contain 10-nonacosanol and other asymmetrical secondary alcohols	Rosaceae	L	Wollrab (1969)
(b) Asymmetrical secondary alkandiol tubes			
<i>Nelumbo nucifera</i>	Nelumbonaceae	L	Barthlott <i>et al.</i> (1996); Barthlott and Neinhuis (1997); Neinhuis and Barthlott (1997)
(c) β -diketone tubes			
21 of 27 sp. of <i>Avena</i>	Poaceae	Gl, Lm	Baum and Hadland (1975)
77 species of <i>Eucalyptus</i>	Myrtaceae	L	Hallam (1967); Hallam and Chambers (1970); Hallam and Juniper (1971)

Table 2.3 Continued

Species	Family or higher taxon	Organ	Reference
<i>Andromeda polifolia</i>	Ericaceae	LAb	Jeffree (1986)
<i>Chrysanthemum frutescens</i>	Asteraceae	L	Rentschler (1971)
<i>Dianthus caryophyllus</i>	Caryophyllaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Eragrostis curvula</i>	Poaceae	L	Leigh and Matthews (1963)
<i>Eucalyptus camaldulensis</i>	Myrtaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Eucalyptus glaucescens</i>	Myrtaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Eucalyptus globulus</i>	Myrtaceae	L	Hallam (1967), Hallam and Juniper (1971); Jeffree <i>et al.</i> (1975, 1976)
<i>Eucalyptus gunnii</i>	Myrtaceae	L	Jeffree (1974b)
<i>Eucalyptus nova anglica</i>	Myrtaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Eucalyptus perriniana</i>	Myrtaceae	LAd	Hallam (1967)
<i>Eucalyptus viminalis</i>	Myrtaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Festuca glauca</i>	Poaceae	L	Jeffree <i>et al.</i> (1975, 1976)
Glumes and lemmas of grasses	Poaceae	Gl, Lm	Baum and Hadland (1975)
<i>Hordeum vulgare</i>	Poaceae	F LS	von Wettstein-Knowles (1974); Jeffree <i>et al.</i> (1975, 1976); Baker (1982)
<i>Leymus arenarius</i>	Poaceae	LAd	Meusel <i>et al.</i> (2000)
<i>Poa colensoi</i>	Poaceae	L	Hall <i>et al.</i> (1965)
<i>Triticum aestivum</i>	Poaceae	FLAb	Netting and von Wettstein-Knowles (1973); von Wettstein-Knowles (1974); Jeffree <i>et al.</i> (1975, 1976)
<i>Vaccinium ashei</i>	Ericaceae	L	Freeman <i>et al.</i> (1979)
(d) delta-lactone tubes			
<i>Cerinth minor</i>	Boraginaceae	L	Jetter and Riederer (1999a)
(e) uncharacterized tubes			
<i>Amorphophallus maximus</i>	Araceae	L	Barthlott <i>et al.</i> (1998)
<i>Columellia oblonga</i> Ruiz and Pav	Columelliaceae	L	Barthlott <i>et al.</i> (1998)
<i>Corydalis cava</i>	Fumariaceae	L	Barthlott <i>et al.</i> (1998)
<i>Hosta sieboldiana</i> Hook. Engl	Hostaceae	LAd	Figure 2.14d
Open spirals			
<i>Buxus sempervirens</i>	Buxaceae	L	Barthlott <i>et al.</i> (1998); Meusel <i>et al.</i> (1999)
<i>Cerinth major</i>	Boraginaceae	L	Figure 2.14c
<i>Chrysanthemum segetum</i>	Asteraceae	L	Juniper and Bradley (1958); Martin and Juniper (1970); Barthlott <i>et al.</i> (1998); Meusel <i>et al.</i> (2000)
<i>Lonicera korolkovii</i> Stapf	Caprifoliaceae	L	Barthlott <i>et al.</i> (1998)
<i>Papaver somniferum</i>	Papaveraceae	Cap	Jetter (1993); Jetter and Riederer (1994)
<i>Pogonatum rubenti-viride</i>	Polytrichales	Cap	Neinhuis and Jetter (1995)
<i>Sedum telephium</i>	Crassulaceae	L	Rentschler (1971)
Dendritic plates			
<i>Allium cepa</i> cv. 'Ailsa Craig'	Liliaceae	L	Jeffree <i>et al.</i> (1975, 1976); Jeffree (1986)

Continued

Table 2.3 Continued

Species	Family or higher taxon	Organ	Reference
<i>Allium porrum</i>	Liliaceae	L	Jeffree <i>et al.</i> (1975, 1976); Baker (1982)
<i>Brassica oleracea</i> var. <i>caulo-rapa</i>	Brassicaceae	L	Burkhardt <i>et al.</i> (2001)
<i>Brassica oleracea</i> var. <i>gemmifera</i> , high temp and humidity	Brassicaceae	L	Baker (1974, 1982)
<i>Brassica oleracea</i> var. <i>capitata</i>	Brassicaceae	L	Figure 2.12d
<i>Euphorbia lathyris</i>	Euphorbiaceae	L	Jeffree <i>et al.</i> (1975, 1976); Jeffree (1986)
<i>Euphorbia peplus</i>	Euphorbiaceae	LAB	Holloway (1971)
<i>Lupinus albus</i>	Fabaceae	LAd	Martin and Juniper (1970), Figure 8.3
<i>Lupinus luteus</i>	Fabaceae	L	Rentschler (1971)
Simple plates			
(a) <i>Uncharacterised plates</i>			
<i>Anarthia scabra</i>	Anarthriaceae	L	Frölich and Barthlott (1988)
<i>Aristolochia durior</i>	Aristolochiaceae	L	Rentschler (1971)
<i>Aristolochia elegans</i>	Aristolochiaceae	L	Rentschler (1971)
<i>Barbacenia tubulosa</i>	Velloziaceae	L	Frölich and Barthlott (1988)
<i>Carludovica palmata</i>	Cyclanthaceae	L	Frölich and Barthlott (1988)
<i>Colochasia esculenta</i>	Araceae	L	Barthlott and Neinhuis (1997)
<i>Dawsonia superba</i>	Polytrichales	G L	Troughton and Sampson (1973)
<i>Elegia verticciaris</i>	Restionaceae	L	Frölich and Barthlott (1988)
<i>Equisetum arevense</i>	Equisetaceae	S	Baker and Holloway (1971)
<i>Gossypium hirsutum</i>	Malvaceae	L	Hallam and Juniper (1971)
<i>Habrop et alum dawei</i>	Dioncophyllaceae	L	Barthlott <i>et al.</i> (1998)
<i>Hypericum hircinum</i>	Clusiaceae	L	Rentschler (1971)
<i>Kensitia pillansii</i>	Mesembryanthemaceae	L	Ihlenfeldt and Hartmann (1982)
<i>Kleinia articulata</i>	Asteraceae	LAd	Martin and Juniper (1970); Figure 4.24
<i>Lecythis chartacea</i>	Lecythidaceae	L	Barthlott <i>et al.</i> (1998)
<i>Nicolaia elatior</i>	Zingiberaceae	L	Frölich and Barthlott (1988)
<i>Oxalis hedysaroides</i>	Oxalidaceae	L	Rentschler (1971b)
<i>Paeonia suffruticosa</i>	Paeoniaceae	L	Rentschler (1971b)
<i>Pandanus montanus</i>	Pandanaceae	L	Frölich and Barthlott (1988)
<i>Polygala myrtifolia</i>	Polygalaceae	L	Rentschler (1971b)
<i>Polytrichum juniperinum</i>	Polytrichaceae	G L	Neinhuis and Jetter (1995)
<i>Prosopis</i> spp.	Fabaceae	L	Bleckmann and Hull (1975); Hull <i>et al.</i> (1979)
<i>Rosa</i> cv. 'Baccara'	Rosaceae	L	Silva Fernandes (1965b); Holloway (1971); Baker and Holloway (1971)
<i>Simmondsia chinensis</i>	Buxaceae	L	Gülz and Hangst (1983); Gülz (1986)
<i>Vitis vinifera</i> cv. 'Siebel'	Vitaceae	F	Jeffree <i>et al.</i> (1975)
<i>Vitis vinifera</i> cv. 'Sultana', 'Kishmish', 'Thompson seedless'	Vitaceae	F	Possingham (1972); Baker (1982)

Table 2.3 Continued

Species	Family or higher taxon	Organ	Reference
(b) <i>Primary alcohol plates</i>			
<i>202 species of Eucalyptus</i>	Myrtaceae	L	Hallam (1967); Hallam and Chambers (1970); Hallam and Juniper (1971)
<i>Acacia pycnantha</i>	Fabaceae	LAB	Baker (1982)
<i>Acacia sp.</i>	Fabaceae	LAd	Neinhuis and Barthlott (1997)
<i>Agropyron repens</i>	Poaceae	LAd	Coupland and Caseley (1975)
<i>Avena fatua</i>	Poaceae	L	Whitehouse <i>et al.</i> (1982)
<i>Avena</i> , glumes of 15 species	Poaceae	Gl	Baum and Hadland (1975)
<i>Avena sativa</i>	Poaceae	L	Rentschler (1971b)
<i>Avena sativa</i> cv. 'Black supreme'	Poaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Baptisia australis</i>	Fabaceae	L	Rentschler (1971b)
<i>Bromus interruptus</i>	Poaceae	L	Jeffree, unpublished
<i>Calligonum comosum</i>	Polygonaceae	S	Lyshede (1977b)
<i>Chamerion angustifolium</i>	Onagraceae	L	Rentschler (1971); Figure 2.18b
<i>Chenopodium album</i>	Chenopodiaceae	L	Taylor <i>et al.</i> (1981)
<i>Dactylis glomerata</i>	Poaceae	LAB	Holloway (1971)
<i>Eucalyptus cloeziana</i>	Myrtaceae	L	Martin and Juniper (1970); Figure 4.26
<i>Eucalyptus coccifera forma</i>	Myrtaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Eucalyptus microcarpa</i>	Myrtaceae	L	Attwill and Clayton-Greene (1984)
<i>Eucalyptus miniata</i>	Myrtaceae	L	Hallam and Juniper (1971)
<i>Eucalyptus pauciflora</i>	Myrtaceae	L	Jeffree (1974a,b); Jeffree <i>et al.</i> (1975, 1976)
<i>Eucalyptus platypus</i>	Myrtaceae	28d L	Knight <i>et al.</i> (2004)
<i>Festuca arundinacea</i>	Poaceae	L	Pitcairn <i>et al.</i> (1986); Figure 2.12c
<i>Hordeum sativum</i>	Poaceae	LAd	Martin and Juniper (1970) Figure 2.4; Juniper and Bradley (1958)
<i>Hordeum vulgare</i>	Poaceae	L	Rentschler (1971)
<i>Hordeum vulgare</i> cv. 'Proctor'	Poaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Laburnum sp.</i>	Fabaceae	LAd	Neinhuis and Barthlott (1997)
<i>Oryza sativa</i>	Poaceae	LAd	Mendgen (1996)
<i>Pisum sativum</i>	Fabaceae	LAd	Juniper (1959); Martin and Juniper (1970); Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Pisum sativum</i> cv. 'Meteor', 'Kelvedon Wonder', 'Alaska'	Fabaceae	LAd	Juniper (1959); Martin and Juniper (1970); Jeffree <i>et al.</i> (1975, 1976); Holloway <i>et al.</i> (1976)
<i>Poa nemoralis</i>	Poaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Quercus pubescens</i>	Fagaceae	LAB	Jeffree and van Gardingen (1993)
<i>Quercus robur</i>	Fagaceae	LAB	Schreiber (1990)
<i>Robinia sp.</i>	Fabaceae	LAd	Neinhuis and Barthlott (1997)

Continued

Table 2.3 Continued

Species	Family or higher taxon	Organ	Reference
<i>Secale cereale</i> cv. 'Lovaspatonia'	Poaceae	L	Jeffree <i>et al.</i> (1975, 1976)
<i>Sorghum bicolor</i>	Poaceae	LAd	Atkin and Hamilton (1982)
<i>Trifolium pratense</i>	Fabaceae	L	Juniper and Bradley (1958)
<i>Trifolium repens</i>	Fabaceae	LAd	Hall and Donaldson (1963); Hall (1967a,b); Rentschler (1973)
<i>Trifolium repens</i>	Fabaceae	LAd	Hall and Donaldson (1963)
<i>Trifolium repens</i>	Fabaceae	LAd	Holloway, in Martin and Juniper (1970) Figure 2.6
<i>Triticum aestivum</i>	Poaceae	L	Netting and von Wettstein-Knowles (1973); von Wettstein-Knowles (1974); Jeffree <i>et al.</i> (1975, 1976)
<i>Zea mays</i>	Poaceae	LAd	Martin and Juniper (1970) Figure 4.23
<i>Zea mays</i>	Poaceae	L	Rentschler (1971)
(c) Aldehyde plates			
<i>Fagus sylvatica</i>	Fagaceae	L	Markstädter (1994)
(d) Alkane plates			
<i>Brassica napus</i> mutant <i>nilla</i>	Brassicaceae	L	Holloway and Jeffree (2005)
(e) Symmetrical secondary alcohol plates			
<i>Brassica oleracea</i> mutant <i>g14</i>	Brassicaceae	L	Baker (1974); Jeffree <i>et al.</i> (1975, 1976)
(f) Crenate and dendritic plates			
<i>Allium cepa</i>	Liliaceae	L	Scott <i>et al.</i> (1958)
<i>Allium cepa</i> cv. 'Ailsa Craig'	Liliaceae	L	Jeffree <i>et al.</i> (1975)
<i>Andrachne telephioides</i>	Euphorbiaceae	LAB	Verdus (1973)
<i>Euphorbia characias</i>	Euphorbiaceae	LAd	Verdus (1973)
<i>Euphorbia milii</i>	Euphorbiaceae	L	Rentschler (1971)
<i>Euphorbia peplus</i>	Euphorbiaceae	LAd	Holloway (1971)
<i>Euphorbia serrata</i>	Euphorbiaceae	LAB	Verdus (1973)
<i>Grevillea bipinnatifida</i>	Proteaceae		Barthlott <i>et al.</i> (1998)
<i>Odosicyos</i> sp.	Cucurbitaceae		Barthlott <i>et al.</i> (1998)
<i>Olea europea</i>	Oleaceae	F	Baker (1982)
(g) Convallaria plate types			
<i>Anthericum liliago</i> L.	Asphodelaceae	L	Frölich and Barthlott (1988)
<i>Alstromeria aurantiaca</i>	Alstromeriaceae	L	Barthlott <i>et al.</i> (1998)
<i>Anabasis articulata</i>	Chenopodiaceae	S	Lyschede (1977b)
<i>Asparagus retrofractus</i>	Asparagaceae	Phy	Frölich and Barthlott (1988); Barthlott <i>et al.</i> (1998)
<i>Bupleurum ranunculoides</i>	Apiaceae	L	Figure 2.18c
<i>Burmannia biflora</i>	Burmanniaceae	L	Frölich and Barthlott (1988)
<i>Calibanthus hookeri</i>	Dracaenaceae	L	Frölich and Barthlott (1988)
<i>Camassia cusickii</i>	Hyacinthaceae	L	Frölich and Barthlott (1988); Barthlott <i>et al.</i> (1998)
<i>Chenopodium album</i>	Chenopodiaceae	L	Taylor <i>et al.</i> (1981)
<i>Convallaria majalis</i>	Convallariaceae	L	Frölich and Barthlott (1988); Barthlott <i>et al.</i> (1998)

Table 2.3 Continued

Species	Family or higher taxon	Organ	Reference
<i>Eriospermum triphyllum</i>	Eriospermaceae	L	Frölich and Barthlott (1988)
<i>Hemerocallis citrina</i>	Hemerocallidaceae	L	Frölich and Barthlott (1988)
<i>Herpolorion novae-zelandiae</i>	Doryanthaceae	L	Frölich and Barthlott (1988)
<i>Herreria montevidensis</i>	Herreriaceae	L	Frölich and Barthlott (1988)
<i>Melaleuca hypericifolia</i>	Myrtaceae	LAd	Neinhuis and Barthlott (1997)
<i>Narthecium asiaticum</i>	Melanthiaceae	L	Frölich and Barthlott (1988); Barthlott <i>et al.</i> (1998)
<i>Phormium cookianum</i>	Phormiaceae	L	Frölich and Barthlott (1988); Barthlott <i>et al.</i> (1998)
<i>Prenia sladeniana</i>	Mesembryanthemaceae	L	Ihlenfeldt and Hartmann (1982)
<i>Stypandra glauca</i>	Dianellaceae	L	Frölich and Barthlott (1988)
<i>Uvularia floridiana</i>	Colchicaceae	L	Frölich and Barthlott (1988)
<i>Watsonia bulbifera</i>	Iridaceae	L	Frölich and Barthlott (1988)
<i>Yucca filamentosa</i>	Agavaceae	LAb	Frölich and Barthlott (1988); Neinhuis and Barthlott (1997)
(h) Orientated plates, various types			
<i>Amherstia nobilis</i>	Fabaceae	LAb	Neinhuis and Barthlott (1996)
<i>Erythroxylum coca</i>	Erythroxylaceae	L	Rentschler (1971)
<i>Eucalyptus camaldulensis</i>	Myrtaceae	L	Jeffree (1974a,b); Jeffree <i>et al.</i> (1975, 1976); Figure 2.12e
<i>Eucalyptus pauciflora</i>	Myrtaceae	L	Jeffree (1974a,b); Jeffree <i>et al.</i> (1975, 1976)
<i>Hypericum bucklei</i>	Hypericaceae		Barthlott <i>et al.</i> (1998)
<i>Acacia sp.</i>	Fabaceae	LAd	Neinhuis and Barthlott (1997)
<i>Calliandra haematoma Bert Benth.</i>	Fabaceae		Barthlott <i>et al.</i> (1998)
<i>Laburnum sp.</i>	Fabaceae	LAd	Neinhuis and Barthlott (1997)
<i>Robinia sp.</i>	Fabaceae	LAd	Neinhuis and Barthlott (1997)
<i>Quercus pubescens</i>	Fagaceae	L	Jeffree and van Gardingen (1993)
<i>Quercus robur</i>	Fagaceae	L	Schreiber (1990)
<i>Trifolium repens</i>	Fabaceae	L	Hall and Donaldson, (1963); Hall (1967a); Rentschler (1973)
(i) Miscellaneous plate types			
<i>Benthamia alyxifolia</i>	Loranthaceae		Barthlott <i>et al.</i> (1998)
<i>Nepenthes alata</i>	Nepenthaceae	Pt	Riedel <i>et al.</i> (2003)
<i>Nepenthes rufescens</i>	Nepenthaceae	Pt	Juniper and Burras (1962); Martin and Juniper (1970); Juniper (1986)
<i>Prosopis tamarugo</i>	Fabaceae	LAd	Bleckmann and Hull (1975); Hull <i>et al.</i> (1979)
<i>Tilia magnifica</i>	Tiliaceae	LAb	Neinhuis and Barthlott (1997)
Mixed tubes and plates			
36 species of <i>Eucalyptus</i>	Myrtaceae	L	Hallam (1967); Hallam and Chambers (1970); Hallam and Juniper (1971)

Continued

Table 2.3 Continued

Species	Family or higher taxon	Organ	Reference
<i>Brassica napus</i>	Brassicaceae	L	Rentschler (1973)
<i>Lupinus albus</i>	Fabaceae	LAd	Juniper (1960)
<i>Malephora</i> spp.	Aizoaceae	L	Ihlenfeldt and Hartmann (1982)
<i>Pinus sylvestris</i>	Pinaceae	L	Leyton and Juniper (1963); Martin and Juniper (1970); Figure 4.27
<i>Rhus cotinus atropurpurea</i>	Anacardiaceae	S	Baker (1982)
Farinaceous			
<i>Cheilanthes</i> sp.	Adiantaceae	L	de Bary (1871); Wollenweber (1982)
<i>Notholaena</i> sp.	Cryptogrammeae	L	de Bary (1871); Wollenweber (1982)
<i>Pityrogramma triangularis</i>	Adiantaceae	L	de Bary (1871); Chance and Arnott (1981)
<i>Primula</i> spp.	Primulaceae	LAB	de Bary (1871); Blasdale (1947)
<i>Pteris</i> sp.	Pteridaceae	L	de Bary (1871); Wollenweber (1982)
Substantial wax crusts			
<i>Ceroxylon andicola</i>	Arecaceae	S	Martin and Juniper (1970)
<i>Ceroxylon andicola</i>	Arecaceae	L	Martin and Juniper (1970)
<i>Copernica cerifera</i>	Arecaceae	L	Martin and Juniper (1970)
<i>Copernica cowellii</i>	Arecaceae	L	Barthlott <i>et al.</i> (1998)
<i>Copernica hospita</i>	Arecaceae	L	Martin and Juniper (1970)
<i>Copiapoa cinerea</i>	Cactaceae	L	Barthlott <i>et al.</i> (1998)
<i>Cynanchum sarcostemma</i>	Asclepiadaceae	L	Barthlott <i>et al.</i> (1998)
<i>Eucalyptus platypus</i>	Myrtaceae	L	Knight <i>et al.</i> (2004)
<i>Euphorbia cerifera</i>	Euphorbiaceae	L	Juniper and Jeffrey (1983)
<i>Salix alba agentea</i>	Salicaceae	S	Juniper and Southwood (1986)
<i>Sapium sepiiferum</i>	Euphorbiaceae	SC	Martin and Juniper (1970)
<i>Sapium sepiiferum</i>	Euphorbiaceae	L	Martin and Juniper (1970)
<i>Syagrus coronata</i>	Arecaceae	LAd	Machado and Barros (1995)
<i>Terminalia cf seyrigii</i>	Combretaceae	L	Barthlott <i>et al.</i> (1998)

Abbreviations used: A = anther, Cap = capsule, F = fruit, FLAb = flag leaf abaxial surface, FLS = flag leaf sheath, G = gametophyte, Gl = glume, JV = juice vesicles, L = leaf, LAB = abaxial leaf surface, LAd = adaxial leaf surface, Lm = lemma, LS = leaf sheath, P = pod, Phy = phylloclade, Pt = pitcher, S = stem, SC = seed coat, St = stipule, T = trichome, Tu = tuber.

in the shape and size of objects in general. The range of types recognised by de Bary (1871, 1884) and Amelunxen *et al.* (1967) very nearly consumes the available words in the English language for describing such structures, reflecting the fact that we discriminate between comparatively few basic shapes. Second, our understanding of the relationships between wax morphology and the underlying chemical and genetic basis for specifying it is still incomplete, although substantial progress has been made in the past 35 years. Superficial classification based on morphological characters alone without understanding of the underlying chemistry will lead to errors in interpretation of taxonomy and phylogeny. A common trap, for example, is to assume that supposedly amorphous waxes are fundamentally different from

obviously crystalline ones. It is therefore strongly recommended that morphology-based classifications should be used informally and with circumspection.

2.6.2 Chemical and structural classification of EW

Predominantly, EW are derivatives of *n*-acyl alkanes with chain lengths in the range C16–C35 (Chapter 4). The hydrocarbon chains may have substituted groups in terminal (fatty acids, primary alcohols, aldehydes) or mid-chain positions (β -diketones, secondary alcohols). With some exceptions (estolides of gymnosperm waxes; von Rudloff, 1959), and polymeric aldehydes in the waxes of *S. officinarum* (Lamberton and Redcliffe, 1959; Haas *et al.*, 2001) and *Nepenthes* (Riedel *et al.*, 2003) the constituents of plant EW are freely soluble in non-polar solvents such as hexane, benzene, chloroform and diethyl ether, but insoluble in water. The more polar constituents such as fatty acids are sparingly soluble in the polar solvents ethanol and ethyl acetate. The detailed description of the range and distribution of the compounds that have been detected in EW is outside the scope of this chapter, but the main constituent compound classes are summarised in Table 2.4. A detailed treatment of wax chemistry is given in Chapter 4.

EW often fall into classes by virtue of having a single predominating constituent compound class, and it is commonly noted that such waxes have a characteristic structural type. The aliphatic constituents of the EW of most grass and *Eucalyptus* species fall into one of two types: those in which the primary alcohols hexacosanol or octacosanol predominate in the wax and those in which β -diketones hentriacontan-14,16-dione or tritriacontan-12,14-dione predominate (Tables 2.3 and 2.4). Sometimes as in wheat (*Triticum aestivum*) the waxes rich in primary alcohols (1-octacosanol) and β -diketones (tritriacontan-16,18-dione) may both be present, but are usually segregated into distinct regions of the flag leaf and other organs such as glumes and lemmas (Tulloch, 1973; Baum and Hadland, 1975; Baum *et al.*, 1989). In these groups, the primary alcohol-rich waxes almost always have a simple plate morphology (Figures 2.12c,e and 2.13c) while the β -diketone-rich waxes are tubular, plates usually being absent (Figures 2.13a,b and 2.14d). Plate-type waxes dominated by primary alcohols are widespread in the Fabaceae (*Acacia*, *Trifolium*, *Pisum* Figure 2.15a) and Myrtaceae (*Eucalyptus*, Figure 2.12e), and also appear frequently in the Poaceae (*Triticum*, *Hordeum*; Figure 2.13c, *Poa*, *Zea*, *Elytrigia*, etc.) and other monocotyledonous groups (Table 2.3). However, plates are not only correlated with primary alcohols. In grapes (*V. vinifera*) plate morphology is accompanied by the presence of oleanolic acid as a major constituent (Baker, 1982). Ketones are dominant compounds in the wax of *Allium spp.* and *Euphorbia*, which again have plate-like crystals, but usually of a more complex type than in primary alcohol species, with edges which are lobed or serrated or spiked (Jeffree *et al.*, 1975; Baker, 1982, Figure 2.16a). The morphology is not always reliably distinguishable from the more common primary alcohol plate type. There is also some evidence that the dendritic structures (Figure 2.12d) sometimes observed in *Brassica* waxes are attributable to ketones (Baker, 1982).

Table 2.4 Principal classes of compounds in plant epicuticular waxes, with examples of representative constituents, plant species, organs and associated crystal morphologies

Compound class	C-No.	Range	Common example	Representative species	Organ	Associated wax type
Alkanes	Odd	17–35	Nonacosane	<i>Brassica</i> spp.	Leaf adaxial	Columns, plates
Alkyl esters	Even	36–72	Henriacotane	<i>Pisum sativum</i>	Leaf abaxial	Ribbons
	Odd	23–33	Octadecyl hexacosanoate	<i>Copernicia cerifera</i>	Leaf	Crust
Ketones	Even	22–32	Octacosyl octacosanoate	<i>Musa paradisica</i>	Leaf	Large rods
	Odd	21–33	Nonacosan-15-one	<i>Brassica</i> spp.	Leaf	Columns, plates
Aldehydes	Even	22–32	Hexacosanal	<i>Allium porrum</i>	Leaf	Spiked plates
	Odd	21–33	Octacosanal	<i>Fagus sylvatica</i>	Leaf	Granular
Secondary Alcohols	Even	22–32	<i>Asymmetrical</i>	<i>Citrus limon</i>	Fruit	Granular
	Odd	21–33	Nonacosan-10-ol	<i>Aquilegia vulgaris</i>	Leaf	Tubes
Secondary alcohols	Even	21–33	<i>Symmetrical</i>	<i>Tropaeolum majus</i>	Leaf	Tubes
	Odd	21–33	Nonacosan-15-ol	<i>Dawsonia superba</i>	Sporophyte	Tubes
Secondary alkandriols	Odd	21–33	Nonacosan-4,10-diol	<i>Brassica</i> spp.	Leaf	Columns, plates
	Odd	29–33	Henriacontan-14,16-dione	<i>Clarkia elegans</i>	Leaf	Columns, plates
β -Diketones	Odd	21–33	Henriacontan-16,18-dione	<i>Arabidopsis thaliana</i>	Stem	Columns, plates
	Even	22–32	hexacosanol	<i>Nelumbo nucifera</i>	Leaf	Tubes
Primary alcohols	Even	22–32	Octacosanol	<i>Eucalyptus globulus</i>	Leaf	Tubes
	Odd	29–33	Henriacontan-9-ol-4,16-dione	<i>Triticum aestivum</i>	Flag leaf sheath	Tubes
Hydroxy- β -diketones	Even	20–32	Hexacosanoic acid	<i>Buxus sempervirens</i>	Leaf	Open spirals
	Even	20–32	Octacosanoic acid	<i>Pisum sativum</i>	Leaf adaxial	Plates
Fatty acids	Even	20–32	Hexacosanoic acid	<i>Triticum aestivum</i>	Leaf	Plates
	Even	20–32	Octacosanoic acid	<i>Trifolium repens</i>	Leaf	Plates
Polymeric aldehydes	Even	22–32	Estolides	<i>Triticum aestivum</i>	Leaf	Tubes
	Even	22–32	Ursolic acid	Esterified in <i>Copernicia cerifera</i>	Leaf	Crust
δ -Lactones	Even	22–32	1,5-hexacosanolide	Esterified in <i>Musa paradisica</i>	Leaf	Large rods
	Even	22–32	Oleanolic acid	Esterified in <i>Musa paradisica</i>	Leaf	Large rods
Triterpene acids	Even	22–32	Ursolic acid	<i>Saccharum officinarum</i>	Leaf sheath, stem	Large rods
	Even	22–32	Oleanolic acid	<i>Gymnosperms</i>	Leaf	?Amorphous
Triterpene acids	Even	22–32	Ursolic acid	<i>Cerithia minor</i>	Leaf	Tubes
	Even	22–32	Oleanolic acid	<i>Malus, Prunus</i>	Fruit	Plates, greasy film
				<i>Vitis vinifera</i>	Fruit	Plates

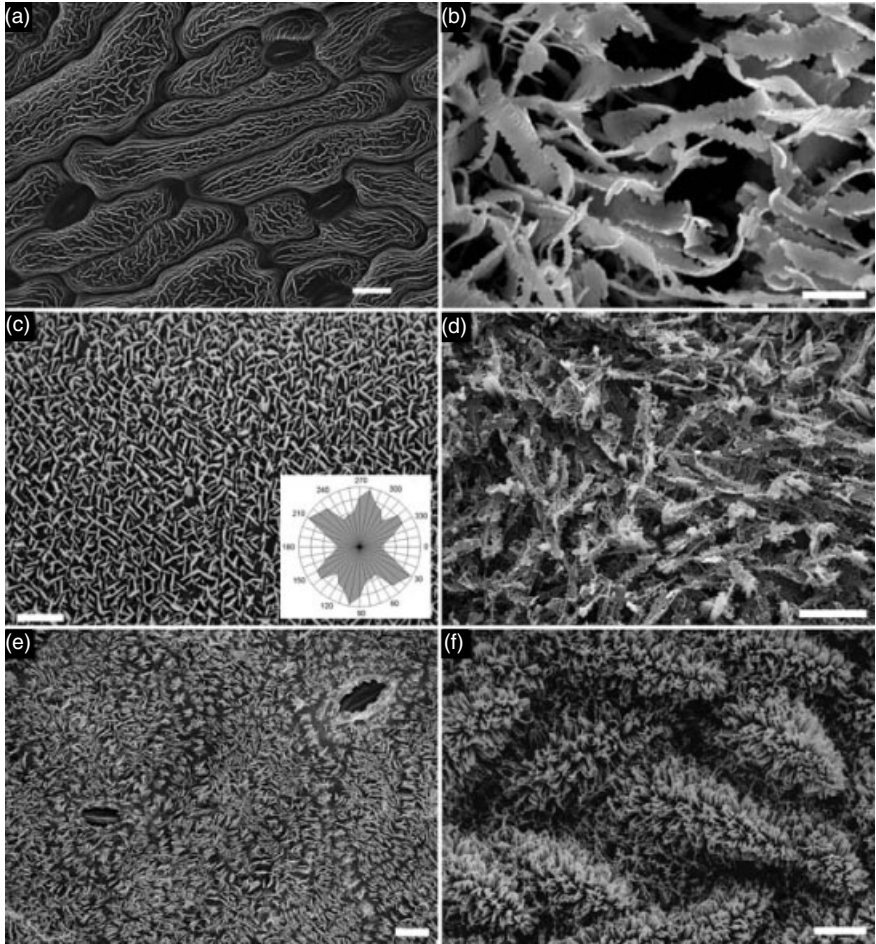


Figure 2.12 Cuticular and epicuticular structure in various species. (a) Patterns of cuticular ridges such as these on a sepal epidermis of *Arabidopsis thaliana* are often mistaken for epicuticular wax, but are caused by cuticular wrinkling. No epicuticular wax structures are visible by SEM on this surface (cf. Figures 2.9g,h). (b) *Aristolochia*-type transversely ridged ribbons on the leaf adaxial surface of mar-ram grass (*Ammophila arenaria*). (c) Simple plates of the primary alcohol type are frequently orientated in preferred orientations. On a leaf of *Festuca arundinacea*, the crystals prefer three orientations roughly 120° apart (see inset). (d) Dendritic structures growing from the tops of wax columns on a cabbage leaf may be related to the production of ketones. (e) *Convallaria*-type patterning of simple primary alcohol plates on a leaf of *Eucalyptus camaldulensis*. Rows of plates orientated transverse to the rows may traverse many different cells. (f) Looped filaments on the abaxial leaf surface trichomes of *Cyathodes colensoi*. (a,f) Bars = $10\ \mu\text{m}$; (c,e) bars = $2\ \mu\text{m}$; (b) bar = $1\ \mu\text{m}$; (d) bar = $5\ \mu\text{m}$. Figures 2.12a,b,d,f by C.E. Jeffree, Figure 2.12c from Pitcairn *et al.* (1986), *Plant, Cell and Environment*, **9**, 191–196, Figure 2.12e from Jeffree *et al.* (1976).

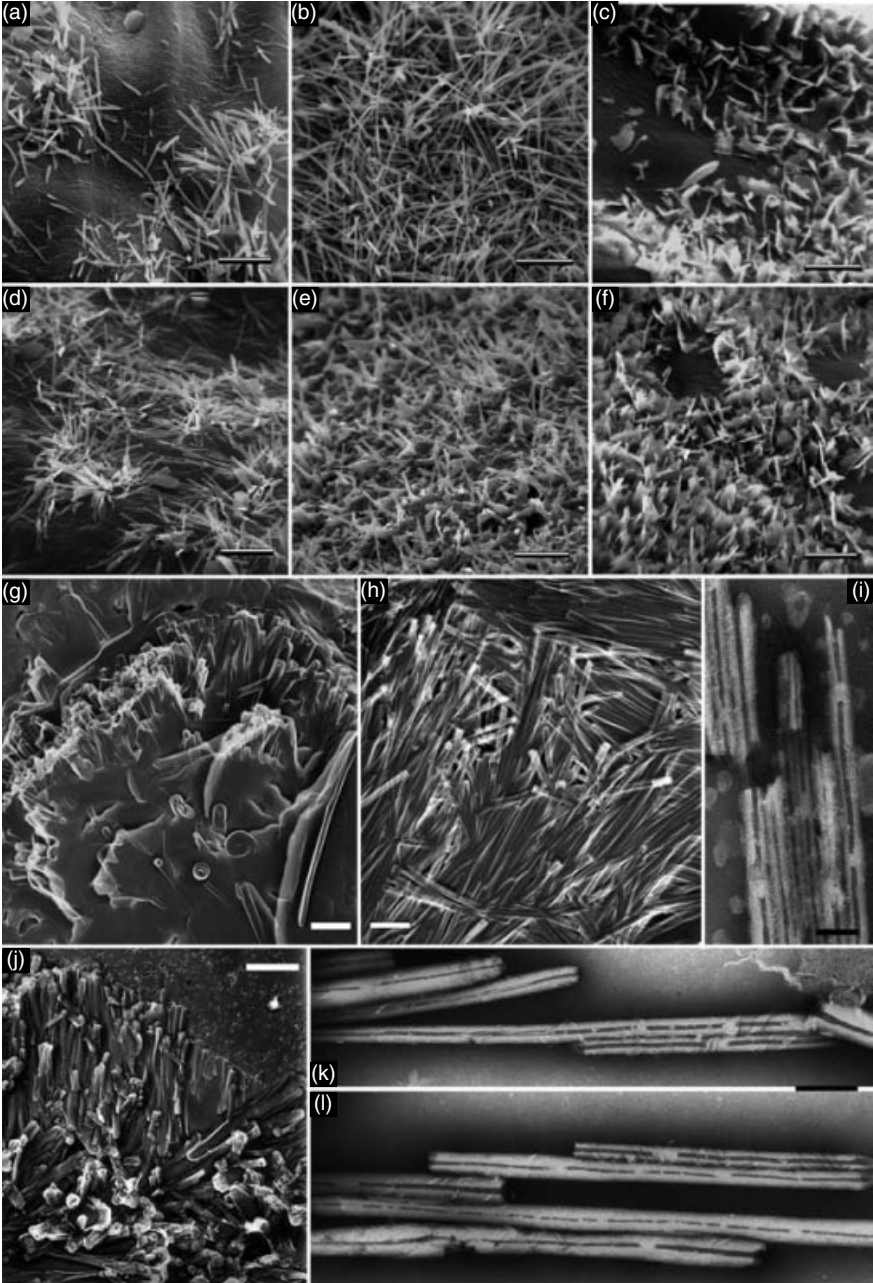


Figure 2.13 Structure and recrystallisation of epicuticular waxes, mainly of barley (*Hordeum vulgare*). (a) β -Diketone tube waxes on a barley flag leaf sheath and (b) on a barley lemma. (c) Primary alcohol plate wax on a barley leaf adaxial surface. (d) Recrystallised tube from barley leaf sheath wax.

2.6.2.1 Granules

The term granules [the 'Körnchen' of de Bary (1871, 1884) and Amelunxen *et al.* (1967)] is used as a bin for a range of indeterminate shapes that can be minute featureless particles (but usually with some kind of shape: round, polygonal, slightly flattened, elongated), or consist of compact aggregates of small rods, tubes or platelets or other sub-polygons. It is possible that granules are truly amorphous in some cases, but in others, such as those of *Aegiceras corniculatum* shown by Barthlott *et al.* (1998) they are clearly aggregates of small crystals. Lack of crystallinity may not therefore be assumed, and there may be little common ground between the granular wax types either morphologically or chemically.

2.6.2.2 Filaments

Massed, interwoven filaments 60–80 nm thick with lengths 3–10 μm occur in the tuber lenticels of potatoes (*Solanum tuberosum*; Hayward, 1974). The composition has not been reported, but filaments are often associated with triterpenes. Thread-like crystals of wax on the gametophyte of the moss *Saelania glaucescens* were found by Haas (1982) to contain the diterpenoid hydroxy kaurane as 94.6% of the wax. Wax crystals with fine thread and ribbon structures are also associated with the stems of several *Macaranga* species that are adapted to act as specific anti-climb barriers protecting their partner symbiotic ants from competition and predation (Federle *et al.*, 1997). In all of the glaucous *Macaranga* species, triterpenoids were present in concentrations between 52 and 88%, containing epitaraxerol and taraxerone as major constituents, with smaller proportions of taraxerol, β -amyrin, and friedelin (Markstädter *et al.*, 2000). However, in glossy species triterpenoids are absent, or present in much smaller quantities. A distinctive kind of structure loosely referred to here as a filament for want of a name occurs in *Fragaria*, *Rosa* (Figure 2.14e), and *Potentilla* and some other Rosaceae, and similar structures occur on the abaxial leaf surfaces of *Acer spp.* These structures often appear to have a triangular cross-section.

2.6.2.3 Plates

Plate-type waxes vary considerably in shape, chemical composition and spatial pattern. The primary alcohol plate types represented by *Eucalyptus*, grasses and

(**Figure 2.13**) (e) Tubes recrystallised from the β -diketone fraction of barley leaf-sheath wax. (f) Plates recrystallised from barley leaf adaxial surface wax. (g,h) Replicas of recrystallised β -diketones (g) and hydroxy- β -diketones (h) from barley leaf-sheath wax, both showing tubular structure. (i) Negative-stained tubes recrystallised from barley leaf sheath whole wax. (j) Pt/C replica of tubes recrystallised from a solution of *Ginkgo biloba* wax in hexane. (k,l) Negative-stained tubes recrystallised from *G. biloba* wax showing evidence of spiral substructure. (a,b,d,e,j) Bars = 4 μm ; (c,f,g) bars = 2 μm ; (h) bar = 1 μm ; (k,l) bar = 300 nm. Figures 2.13a,c,d,f from Jeffree *et al.* (1975, 1976). Figures 2.13g,j from Jeffree *et al.* (1976). Other images by C.E. Jeffree, E.A. Baker and P.J. Holloway.

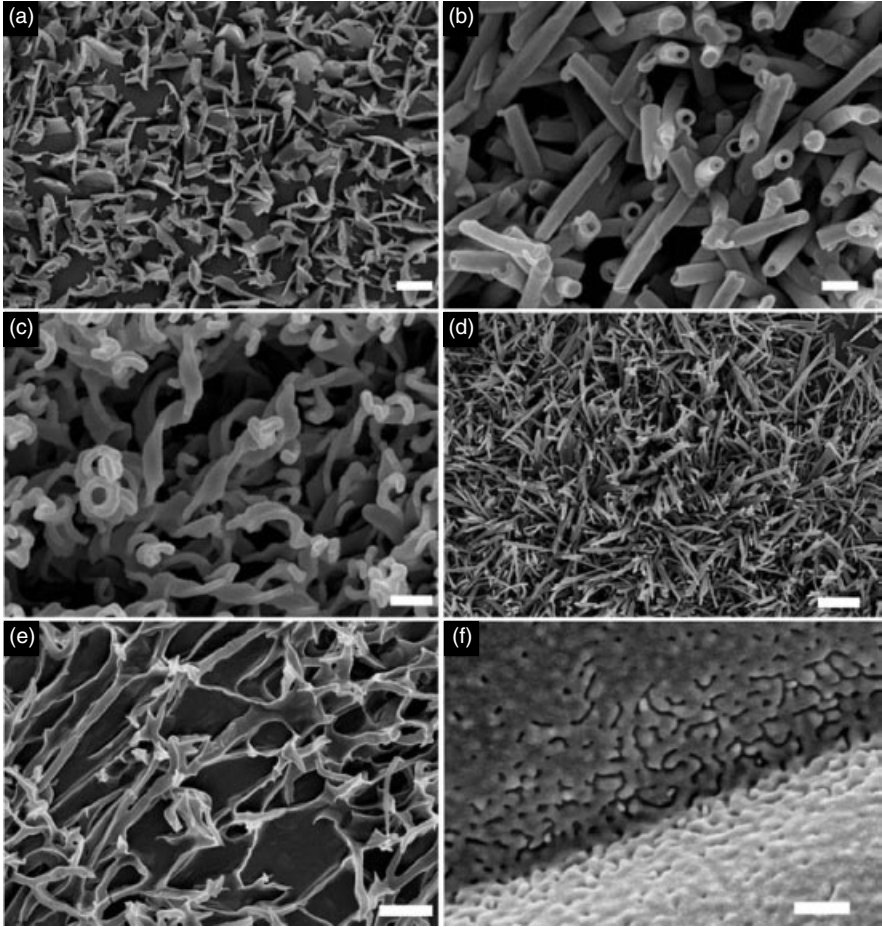


Figure 2.14 Field emission low-temperature scanning electron micrographs of epicuticular wax structures of various species. (a) *Quercus pubescens* leaf abaxial surface, showing simple plate-type waxes. (b) *Mahonia aquifolium* young leaf adaxial surface, showing hollow tubes of the 10-nonacosanol type. (c) Spiral ribbons on a leaf of *Cerinthe major purpurascens* are intermediate between tubes and plates. (d) A dense layer of β -diketone tubes on a leaf of *Hosta sieboldiana*. (e) A filament network on a leaf abaxial surface of *Rosa sp.* The filaments appear to have a triangular cross-section. (f) A structured wax film on the surface of a trichome on the seed-pod of rosebay willowherb (*Chamerion angustifolium*). See also Figure 2.18b. (a,e) Bars = 1 μm ; (b,c) bars = 200 nm; (d) bar = 3 μm ; (f) bar = 500 nm. Figures 2.14a–f by C.E. Jeffree.

Fabaceae are probably the commonest morphological group. Other plate morphologies based on or modified by alkane, aldehyde, ester, ketone, symmetrical secondary alcohol and fatty acid constituents are comparatively poorly defined and there is still only weak discrimination between plate morphological variants or their chemical basis. This situation must be corrected before plate wax characters can be used safely

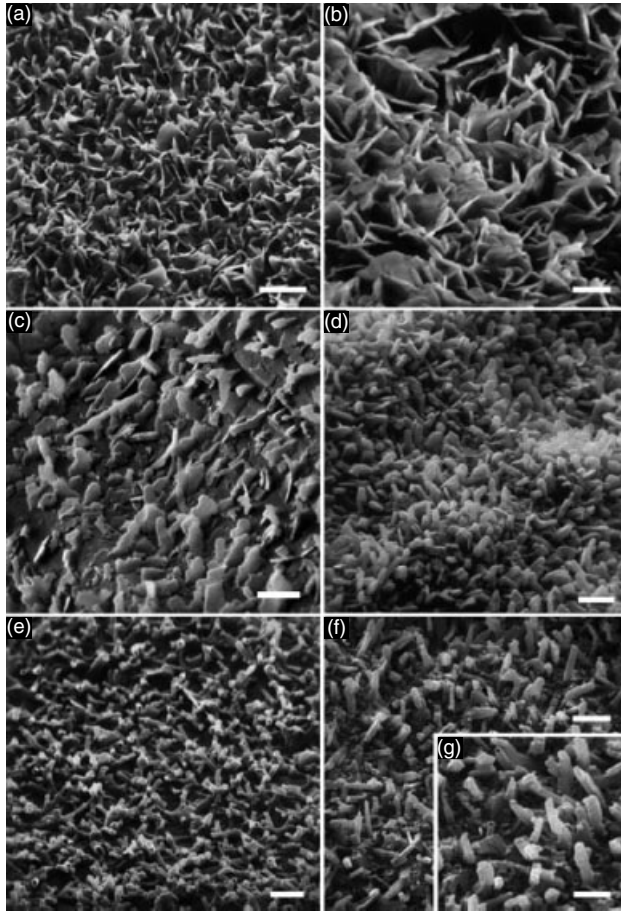


Figure 2.15 Recrystallisation of epicuticular waxes. (a) The adaxial leaf surface of *Pisum sativum* cv. ‘Meteor’ has a primary alcohol-rich plate-type wax that recrystallises in a plate form, as does (b) the primary alcohol fraction from the wax. (c) By contrast, the abaxial leaf surface, petioles, tendrils (shown here) and stipules bear elongated lobed ribbons. (d) The aldehyde fraction from the pea leaf abaxial surface approximates the morphology of the tendril wax. (e) Wax tubes on the leaf of *Exochorda racemosa*. The wax contains about 30% of asymmetrical secondary alcohols of which 93% is 10-nonacosanol. (f) *Exochorda* wax recrystallised in a ribbon morphology analogous to that of the pea tendril. However, short tubes are abundant at the bases of the ribbons (inset g). (a,c,d) Bars = 2 μm ; (b,e,f) bars = 1 μm . (g) bar = 0.5 μm . Images by C.E. Jeffree, E.A. Baker and P.J. Holloway. Figures 2.15e, from Jeffree *et al.* (1975).

in taxonomy and phylogeny. I have struggled to restrain myself from including a special category for plate waxes shaped like ‘moose antlers’ (*Prosopis*; Hull *et al.*, 1979); yet, there is frankly a lack of appropriate terminology to describe variation in plate wax forms. Most of the primary alcohol ‘simple plate type’ described here, and epitomised by the EW of the Eucalypts and Poaceae have distinctly lobed or crenate

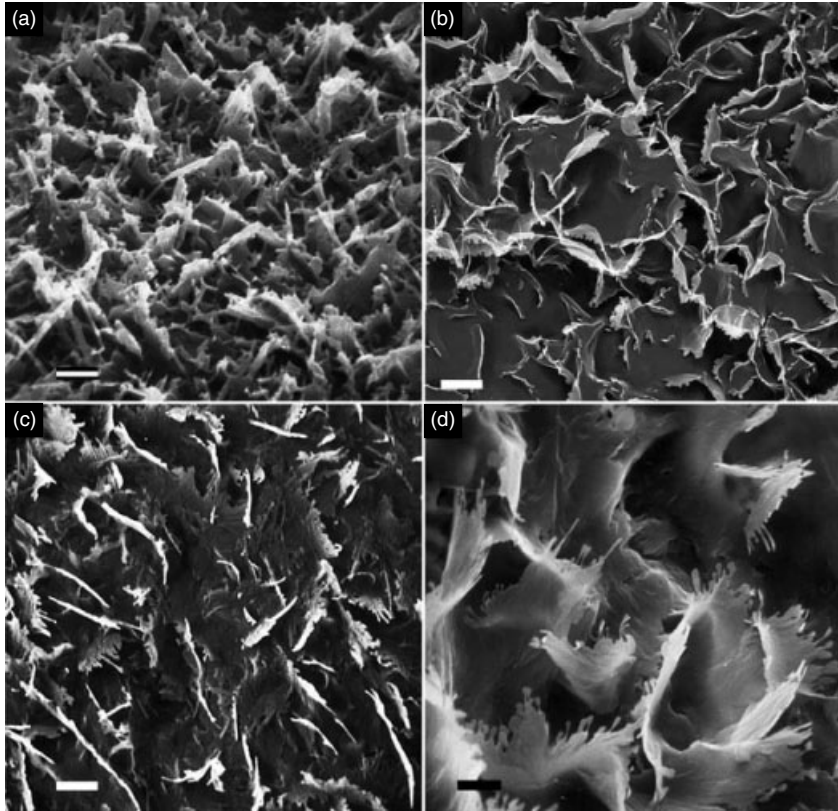


Figure 2.16 Structure and crystallisation of ketone waxes. (a) Spiked plates and filaments on the leaf surface of leek (*Allium porrum*). (b) Replica of the recrystallised ketone fraction from leek wax, showing plates with lobed margins. (c) Recrystallised leek wax, showing plates with spiked and lobed margins. In some instances, regularly grouped dendritic extensions are visible, analogous to the dendritic crystals of *Brassica* waxes (see Figure 2.12d). (d) The ketone fraction from Brussels sprout wax crystallises in much the same form as the ketone fraction from *A. porrum* (b) despite having different constituents. (a) Bar = 1 μm ; (b,c) bars = 2 μm ; (d) bar = 4 μm . Images by C.E. Jeffree, E.A. Baker and P.J. Holloway.

edges when viewed at sufficient resolution. But adequate resolution even to confirm their categorisation as a plate type is often not available from routine SEM images. From this perspective, the work of describing several tens of thousands of wax morphologies using high resolution SEM (Frölich and Barthlott, 1988; Barthlott *et al.*, 1998) has been a major contribution to our understanding. Other wax types, such as the ketone rich plates of *Allium porrum* are chemically and structurally distinct from, for example, the primary alcohol rich plates of *Trifolium repens*, yet could be categorised with them in the absence of knowledge of their chemistry. There is a strong case, therefore, for reworking the wax morphological categories to take account of the correlation between wax chemistry and morphology.

2.6.2.4 Tube-type waxes

Tubes in general. Structures once taken to be rodlets in many species (Amelunxen *et al.*, 1967) are now known to be tubes (Hallam, 1967, 1970b; Johnson and Jeffree, 1970; Jeffree *et al.*, 1975, 1976). Plant surfaces bearing tube waxes are usually highly glaucous, strongly scattering of light enriched in short (blue) wavelengths. They are comparatively fragile (Barber, 1955), contrasting with the more mechanically robust plate type. Tube-type waxes form ultrahydrophobic surfaces, as in lotus *N. nucifera* (Barthlott *et al.*, 1996; Barthlott and Neinhuis, 1997). Terminological confusion still remains at the boundaries between rods and tubes, and also between rods and filaments. Examples include the classification of the coiled crystals of *Buxus* as coiled rods by Barthlott *et al.* (1998) where on chemical considerations they are likely to be related closely to the well-recognised β -diketone tubes. Plant waxes do not always fall neatly quantified into a tube bin or a plate bin. Intermediate forms occur, such as the mixtures of tubes and plates on the leaves of *Pinus sylvestris* (Leyton and Juniper, 1963) and *Eucalyptus umbrawarrensensis* (Hallam and Juniper, 1971) and on the flag leaves of wheat. The term 'tube' is meaningless in these examples without further qualification of their chemical basis. Chemical analyses show that the commonest tube waxes fall into two distinct groups – those containing substantial amounts of asymmetrical secondary alcohols (predominantly 10-nonacosanol and its homologues; Table 2.5) and those containing substantial quantities of β -diketones, such as hentriacontan-14,16-dione in the Poaceae and tritriacontan-16,18-dione in *Eucalyptus spp.* (Horn and Lamberton, 1962; Horn *et al.*, 1964; Hallam, 1967; Hallam and Chambers, 1970; Tulloch and Hoffman, 1971, 1974; von Wettstein-Knowles, 1972; Jeffree *et al.*, 1975, 1976; Holloway *et al.*, 1976; Baker, 1982; Tables 2.3 and 2.4). These two classes of compounds are never recorded as major constituents in the same species or even in the same family. Three further compound classes are associated with simple tubes, hydroxy- β -diketones, secondary alkanediols and delta lactones (see later).

Secondary alcohol tubes. Tubes are the dominant wax crystal type in the Gymnosperm genera *Ginkgo*, *Chamaecyparis*, *Taxus*, *Picea*, *Agathis*, *Pseudotsuga*, *Pinus*, *Abies* and others, in which the asymmetrical secondary alcohol 10-nonacosanol is always an important constituent (Jeffree *et al.*, 1975, 1976) although it can constitute less than a third of the EW load (Holloway *et al.*, 1976). Asymmetrical secondary alcohols, of which 10-nonacosanol is the most important constituent, have also been recorded in a wide range of Angiosperms with tube waxes, mostly dicotyledons (Table 2.5), including *Aquilegia alpinum*, *Chelidonium majus* and *Papaver somniferum*, *Rhus cotinus*, *Exochorda racemosa* and *P. domestica*, *Tropaeolum majus* (Holloway *et al.*, 1976). In *Rosa* cv. 'Baccarra', the variable distribution of tubes is positively correlated with the amounts of 10-nonacosanol present in the EW (Baker, 1982). Tubular crystals were also discovered on *Tulipa kaufmanniana* their hollow centres confirmed by negative staining, as were the tubes of *P. sitchensis* (Johnson and Jeffree, 1970; Jeffree, 1974a; Figure 2.17f). The composition as well

Table 2.5 Asymmetrical secondary alkanols and alkandriols in tube waxes. The abundance of the major constituent as percentage of the secondary alkanol fraction is indicated in bold

Species	Secondary alkanol fraction — Wax (%)		C27 secondary alkanols		C29 secondary alkanols		C31 secondary alkanols				
			Isomer	Fraction (%)	Wax (%)	Isomer	Fraction (%)	Wax (%)	Isomer	Fraction (%)	Wax (%)
From Holloway <i>et al.</i> (1976)											
<i>Brassica oleracea</i> var. <i>gemmifera</i>	12.5		All	0.5	0.06	13-ol	0.9	0.11	All	0.9	0.11
	12.5					14-ol	36.3	4.54			
	12.5					15-ol	60.1	7.51			
<i>Clarkia elegans</i>	3.0		All	tr	tr	13-ol	1.0	0.03	All	nd	nd
	3.0					14-ol	16.0	0.48			
	3.0					15-ol	83.0	2.49			
<i>Pisum sativum</i>	5.5		All	1.0	0.06	13-ol	0.1	0.01	14-ol	2.4	0.13
	5.5					14-ol	0.2	0.01	15-ol	37.1	2.04
	5.5					15-ol	3.1	0.17	16-ol	51.8	2.85
<i>Aquilegia alpinum</i>	57.0		All	0.7	0.40	10-ol	98.3	56.03	All	tr	tr
<i>Papaver somniferum</i>	65.7		10-ol	0.6	0.39	10-ol	99.4	65.31	All	tr	tr
<i>Chelidonium majus</i>	66.0		10-ol	2.7	1.78	10-ol	95.3	62.90	All	tr	tr
<i>Prunus domestica</i>	48.3		7-ol	0.1	0.05	9-ol	2.9	1.40	All	0.6	0.29
	48.3		8-ol	0.8	0.39	10-ol	92.7	44.77			
	48.3		9-ol	0.5	0.24	11-ol	1.0	0.48			
	48.3		10-ol	0.3	0.14						
<i>Exochorda racemosa</i>	30.1		7-ol	0.1	0.03	9-ol	2.3	0.69	9-ol	tr	
	30.1		8-ol	0.4	0.12	10-ol	93.0	27.99	10-ol	1.0	0.30
	30.1		9-ol	0.4	0.12	11-ol	1.0	0.30	11-ol	0.9	0.27
	30.1		10-ol	0.2	0.06						

<i>Rhus cotinus</i>	38.3	All	0.4	0.15	9-ol	2.4	0.92	All	tr	tr
<i>atropurpurea</i>	38.3				10-ol	93.2	35.70			
	38.3				11-ol	2.8	1.07			
<i>Tropaeolum majus</i>	47.5	8-ol	0.2	0.10	9-ol	3.0	1.43	All	tr	tr
	47.5	9-ol	0.5	0.24	10-ol	92.5	43.94			
	47.5	10-ol	0.3	0.14	11-ol	3.0	1.43			
<i>Tulipa gesneriana</i>	37.8	All	2.7	1.02	9-ol	2.9	1.10	All	tr	tr
	37.8				10-ol	92.4	34.93			
	37.8				11-ol	1.0	0.38			
<i>Picea sitchensis</i>	19.7	All	1.0	0.20	10-ol	98.0	19.31	All	1.0	0.20
<i>Picea pungens</i>	10.0	All	0.6	0.06	10-ol	98.8	9.88	All	0.6	0.06
<i>Chamaecyparis lawsoniana</i>	28.6	All	—	—	10-ol	100.0	28.60	All	—	—
<i>Agathis australis</i>	27.8	All	0.8	0.22	10-ol	98.0	27.24	All	1.2	0.33
<i>Ginkgo biloba</i>	48.9	10-ol	1.1	0.54	10-ol	98.9	48.36	All	—	—
From Neinhuis and Jetter (1995)										
<i>Pogonatum urnigerum</i>	16.0				C29 10-ol	91.0	14.56			
<i>Pogonatum belangeri</i>	61.0				C29 10-ol	97.0	59.17			
From Barthlott <i>et al.</i> (1996)										
		Secondary alkanol								
		fraction – wax (%)								
<i>Nelumbo nucifera</i>	28.0									
										Secondary alkandiol
										fraction – wax (%)
<i>Thalictrum flavum</i>	57.0									57.0
										12.0
<i>Papaver somniferum</i>	61.0									11.0

nd = not determined; tr = trace; nd = not detectable.

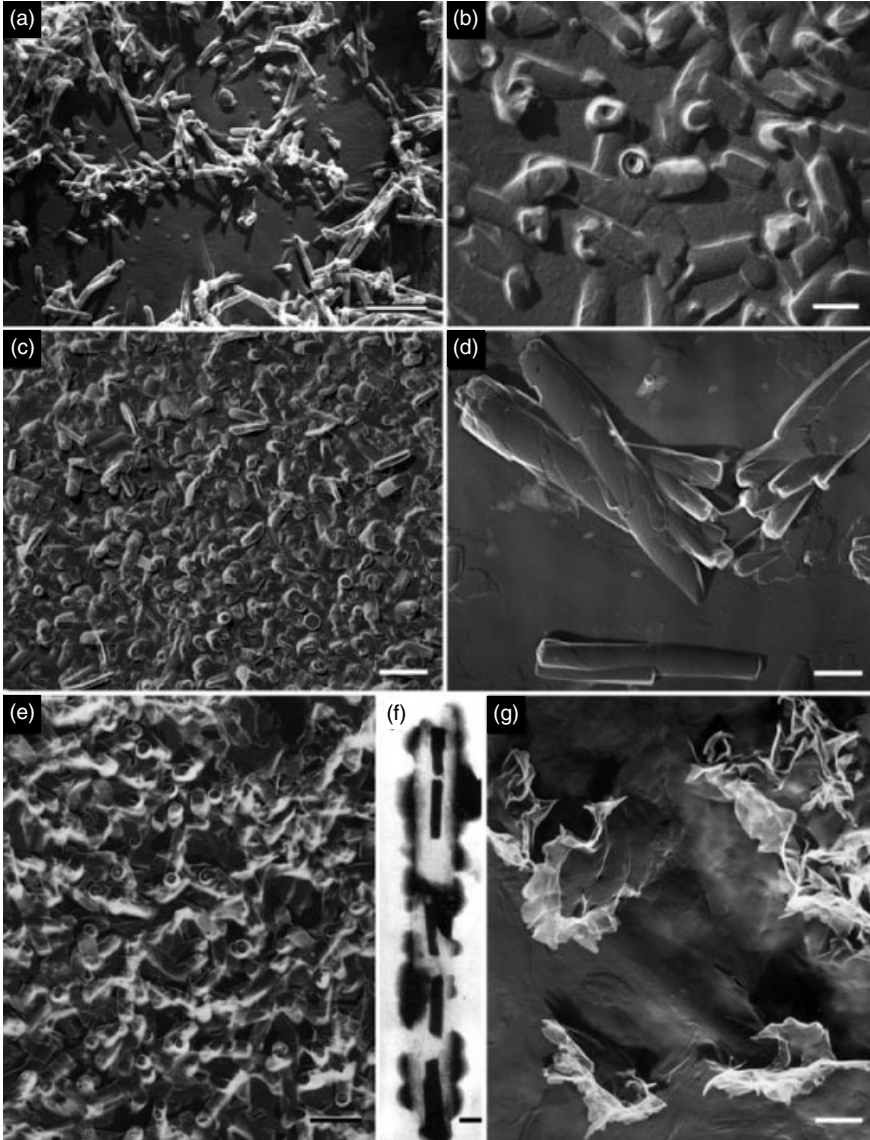


Figure 2.17 Pt/C replicas of the structure and crystallisation of Sitka spruce wax tubes. (a) Wax tubes on a current-season leaf. (b) A tube seen end-on, clearly demonstrating its hollow structure. (c) Epicuticular wax recrystallised from solution in benzene, showing tubular structure. (d) Tubular structure crystallised by slow-cooling of molten Sitka spruce wax. The tubes bear diagonal (spiral) striations at about 30° to the long axis. (e) The secondary alcohol fraction from Sitka spruce wax recrystallised in tube form. (f) A negatively stained Sitka spruce wax tube shows segments of stain in its lumen. (g) The remainder of the wax, depleted in secondary alcohols, forms large plates instead of tubes. (a) Bar = $1\ \mu\text{m}$; (b) bar = $200\ \text{nm}$; (c,e,f) bars = $500\ \text{nm}$; (d) bar = $400\ \text{nm}$; (g) bar = $2\ \mu\text{m}$. Figures 2.17a–e and g from Jeffree (1974a). Figure 2.17f from Johnson and Jeffree (1970), *Planta*, **95**, 179–182.

as the structure of the wax of *T. kaufmanniana* bears comparison with that of *Ginkgo biloba* or *T. majus* (Table 2.5).

β-Diketone tubes. Hallam (1964, 1967) and Hallam and Chambers (1970) found that tubular wax structures and β -diketones in 113 *Eucalyptus* species were strongly correlated, as were plate waxes and primary alcohols in a further 238 eucalypts. In 36 of these species plates and tubes occurred together, and the wax contained both primary alcohols and β -diketones. Further β -diketone-rich waxes with tubular structures occur in the Poaceae (barley leaf sheath, wheat leaf abaxial surface; Netting and von Wettstein-Knowles, 1973; Jeffree *et al.*, 1975, 1976) and *Dianthus caryophyllus*; Table 2.3; Figures 2.13a,b and 2.14e), and in the tubular waxes of Ericaceae (*Rhododendron spp.*; Evans *et al.*, 1975; *Vaccinum ashei*, Freeman *et al.*, 1979; *Andromeda polifolia*, Jeffree, 1986). In the Poaceae, as in the eucalypts, the leaf waxes fall into two contrasting types, sometimes to be found on different parts of the same plant. Secondary alcohol and β -diketone tubes are distinguishable from their morphology as well as their chemistry (see earlier). The secondary alcohol tubes are usually shorter and wider (0.3–1.1 μm long by 0.1–0.2 μm wide) than the β -diketone type (typically 2–3 μm long by 0.2–0.3 μm wide; Jeffree *et al.*, 1975).

Alkandiol tubes. More recently, it has become clear that secondary alkanols and secondary alkandiolols often co-occur in tube wax species, forming about 12% of the wax of *Thalictrum flavum* and 11% of *P. somniferum* (Tables 2.3 and 2.4; Barthlott *et al.*, 1996). Although they occur widely in secondary alcohol-rich waxes, usually as subsidiary compounds, it is rare for a secondary alkandiol to dominate. Currently the only known exception is in the tube wax of *N. nucifera* which contains 57% of secondary alkandiolols [of which nonacosan-4,10-diol (38%), nonacosane-5,10-diol (38%) and nonacosan-10,13-diol (22%)] accompanied by 28% of secondary alkanols, with 10-nonacosanol being the major constituent (Jetter and Riederer, 1995; Barthlott *et al.*, 1996). Similarly, β -diketones and hydroxy- β -diketones occur together in the tube waxes of many Poaceae, although not in dicots. However, there are no known examples of waxes containing both β -diketones and asymmetrical secondary alcohols.

Delta-lactone tubes. Another completely new category of tube-wax forming compounds is the δ -lactones (1,5-alkanolides) reported in *Cerintho minor* by Jetter and Riederer (1999a), the first example of this type that has been found.

Tubes, curls, spirals and branches. Several of the images of tube waxes presented by Barthlott *et al.* (1998) show transitional forms between coils and tubes. Thus, in *Lonicera korolkovii* (their figure 20) the tubes appear to be constructed as tight coils, resulting in a characteristic twist at the distal end, and close spiral ridges down the side of the tube. Their images of *Buxus sempervirens* show small coiled rods (or are they coiled tubes? – more resolution is required to determine this) and much larger, clearly hollow tubes together with intermediate forms that appear to be curved plates.

It is noteworthy that the wax of *Cerithe major purpurascens* shows an open helical structure (Figure 2.14c), indicating a possible relationship between coiled ribbons and closed tubes. Curling of plates and coiling of tubes are also evident in some β -diketone waxes, first demonstrated in *Chrysanthemum segetum* by Juniper and Bradley (1958) in their paper on the then newly developed carbon replica technique. Recently, a similar morphology has been reported in *B. sempervirens* (Meusel *et al.*, 2000). Both forms are associated with C31 β -diketones substituted in unusually asymmetrical 8,10 (*Buxus*) or 10,12 positions (*Chrysanthemum*) compared with the more nearly symmetrical 14,16 positions of the β -diketones in *H. vulgare* (Figures 2.13a,b), which produces tubes but no coiling.

Brassica-type tubes. A third and less frequent category of tubes identified by Baker (1974, 1982) on leaves of *Brassica spp.* and stems of *A. thaliana* also appears in *Clarkia elegans* and *Centranthus ruber* together with comparable, though not identical, chemical compositions (Hunt *et al.*, 1976). The tubes of *Brassica* wax are much larger than those described earlier, up to $8 \times 1 \mu\text{m}$, and have transverse striations visible in negative stained specimens (see Jeffree *et al.*, Holloway, 1976) or in replicas (Martin and Juniper, 1970; Hallam and Juniper, 1971; Figures 2.18a,b). The *Brassica* wax type is characterised by mixtures of symmetrical secondary alcohols (*n*-nonacosan-15-ol), ketones (*n*-nonacosan-15-one) and alkanes (Netting *et al.*, 1972; Holloway *et al.*, 1977). Although short *Brassica* tubes appear to have hollow ends when seen in the SEM (e.g. Neinhuis *et al.*, 1992; their figure 5) negative staining of the wax columns of *Brassica* does not reveal them to be hollow (Figure 2.18b), and it is therefore likely that they become filled as they grow. They are therefore not comparable with true tubes of the types formed by asymmetrical alkanols, alkanediols and β -diketones. Gomez-Campo *et al.* (1999) used the term 'column', and that seems a more appropriate term, distinguishing these characteristic structures from simpler terete rods.

Because of the current importance of *A. thaliana* as a model plant it may be worthwhile to clarify some terminological issues concerning the EW morphologies of this species. Gomez-Campo *et al.* (1999) referred to three layers of crystals in *Brassica* waxes, a continuous sheet (i.e. background film), flat crystals and upright columns. Because the density of crystallites on *Brassica* leaves is very high, replicas of the intact surface crystal become too complex to interpret easily, and clarity of the images of individual crystallites is improved where areas of the wax have been damaged by abrasion, as in figure 4.28 in Martin and Juniper (1970) and figure 12 of Hallam and Juniper (1971). These images indicated two predominant crystal morphologies: lobed or crenate plates, often in low profile on the surface, and longitudinally fluted and transversely-ridged rods or tubes (Figure 2.18a). Recent experiments using a TEM with a goniometer tilting stage reveal that crystals that appear to be 'plates' at one angle resolve as fluted columns when tilted to another. The morphologies are therefore two ends of the spectrum of possibilities for column assembly using the same stacked planar modules. The crenate plates often contain holes, and there may be indications of a hole in images of tubes, but negative staining

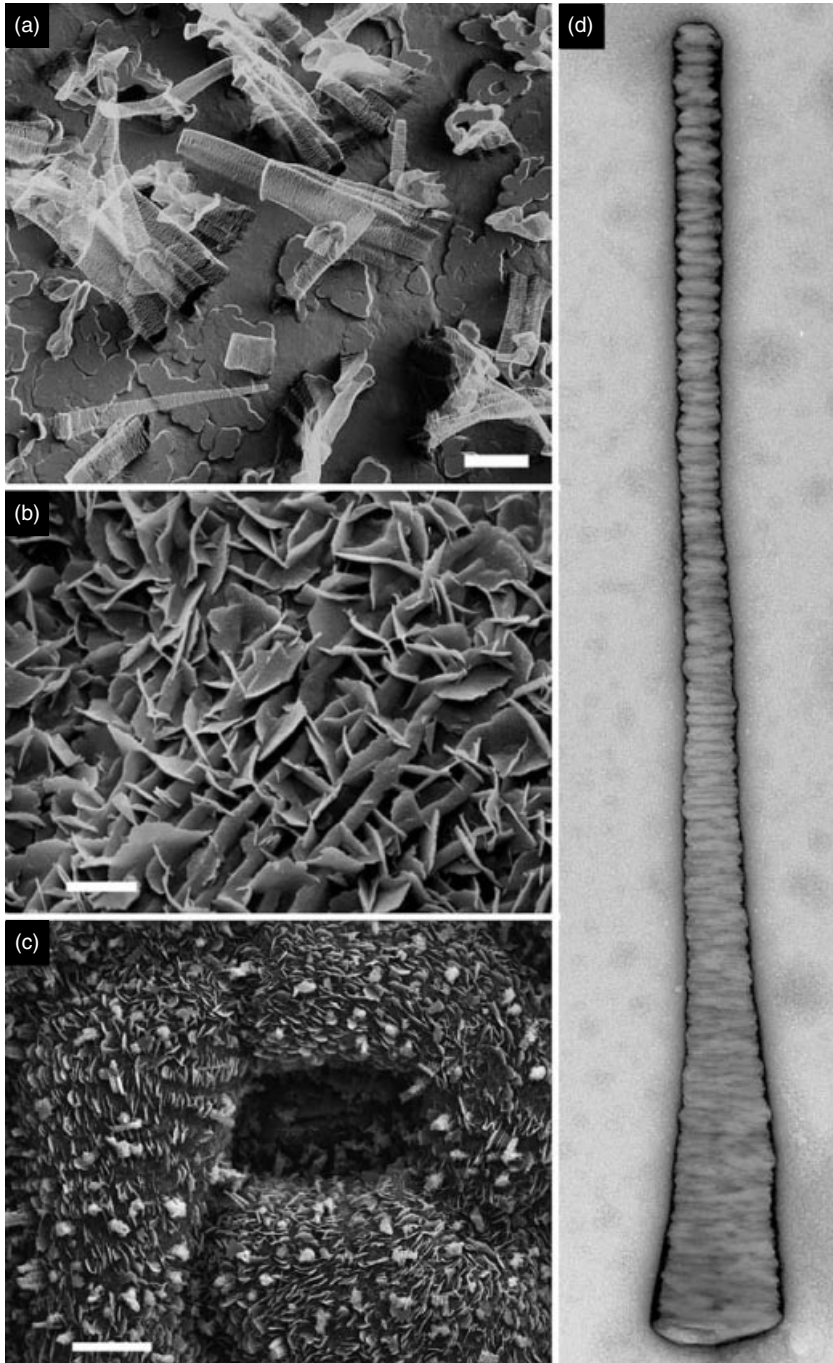


Figure 2.18 (a–d) Wax structures in *Brassica* spp.

does not reveal a hollow core (Figure 2.18b). Successive plates diminish in size from base to apex of the rod, so that the rod usually tapers distally. However, in the *gl2* mutant the direction of tapering is reversed (Figures 2.19e,f).

2.6.2.5 Rodlet-type waxes

Baker (1982) pointed out the correlation between aldehydes in waxes of lemon *C. limon* fruits in which the wax is approximately 43% aldehydes, and short rods. Rods are again observed in the aldehyde-rich wax of sugarcane (*S. officinarum*), but in this species the aldehydes are not fully soluble, and are probably polymeric. Aldehydes, chiefly triacontanal, occur again as 43% in the specialised plate waxes of the *Nepenthes* pitcher (Martin and Juniper, 1970; Riedel *et al.*, 2003).

The Strelitzia rodlet type. Large wax rodlets of a readily recognisable type occur on the leaves of many Poaceae – *S. officinarum* (de Bary, 1871, 1884; Jeffree *et al.*, 1975, 1976; Haas *et al.*, 2001), *Arundinaria* spp. (Jeffree *et al.*, 1976) and *Typha* spp. (Djebrouni, 1989) – and in *Musa paradisica* and *Strelitzia reginae* and *Heliconia* (Meusel *et al.*, 1994; Barthlott *et al.*, 1998). These form co-aligned crystals (Kreger, 1949) aggregated into massive terete rodlets, or in *Strelitzia* and *Heliconia* cylindrical chimneys accurately surrounding each stomatal complex (Meusel *et al.*, 1994; Barthlott *et al.*, 1998). The massive wax films of carnauba palm (*C. cerifera* and *C. cowellii*) are huge accumulations of these co-aligned crystals. This type of wax structure appears to occur in the Monocotyledons almost exclusively in the Commelinidae and Arecacidae and to be absent from the Liliaceae and has been named the *Strelitzia* type (Barthlott and Frölich, 1983; Frölich and Barthlott, 1988; Meusel *et al.*, 1994; Table 2.3). A similar wax type occurs in some Dicotyledons, including *Wooleya farinosa* and *Malephora* sp. (Ihlenfeldt and Hartmann, 1982) *Eryngium* (Frölich and Barthlott, 1988) and fruits of the wax gourd *Benincasa hispida* (de Bary, 1871, 1884; Barthlott, 1990; Meusel *et al.*, 1994), but the list is not exhaustive. The waxes of the Strelitziaceae and Musaceae contain substantial quantities of alkyl esters, or of polymeric aldehydes (11% in *Strelitzia*;

(**Figure 2.18**) (a) Gold–palladium replica of the leaf surface of cabbage (*Brassica oleracea* var. *capitata*) showing stacks of flat-lobed plates, and tapering columns. The former appear to develop into the latter. The replica was made by detaching the sputter coating from a low-temperature SEM specimen using frozen ethanol. (b) Large simple wax plates formed transverse to cuticular ridges on a leaf of rosebay willowherb (*Chamerion angustifolium*). Some plates span two or more ridges. (c) Simple plates form a regular pattern of rows radiating from a stomatal aperture on the abaxial surface of a leaf of *Bupleurum ranunculoides*. (d) A negative-stained wax column from Brussels sprout (*B. oleracea* var. *gemmaifera*) shows superficial transverse ridges, but no evidence of a hollow centre and is therefore not tubular. The pattern is analogous to the ‘*Convallaria*’-type waxes widespread in the monocotyledons (Barthlott *et al.*, 1998), but the plates are also interspersed with transversely ridged rodlets of the *Aristolochia* type (see Meusel *et al.*, 1999). (a) Bar = 1 μm ; (b) bar = 2 μm ; (c) bar = 5 μm ; (d) the wax rod is 4.2 μm long. Figures 2.18a–c by C.E. Jeffree, Figure 1.12d from Jeffree *et al.* (1996).

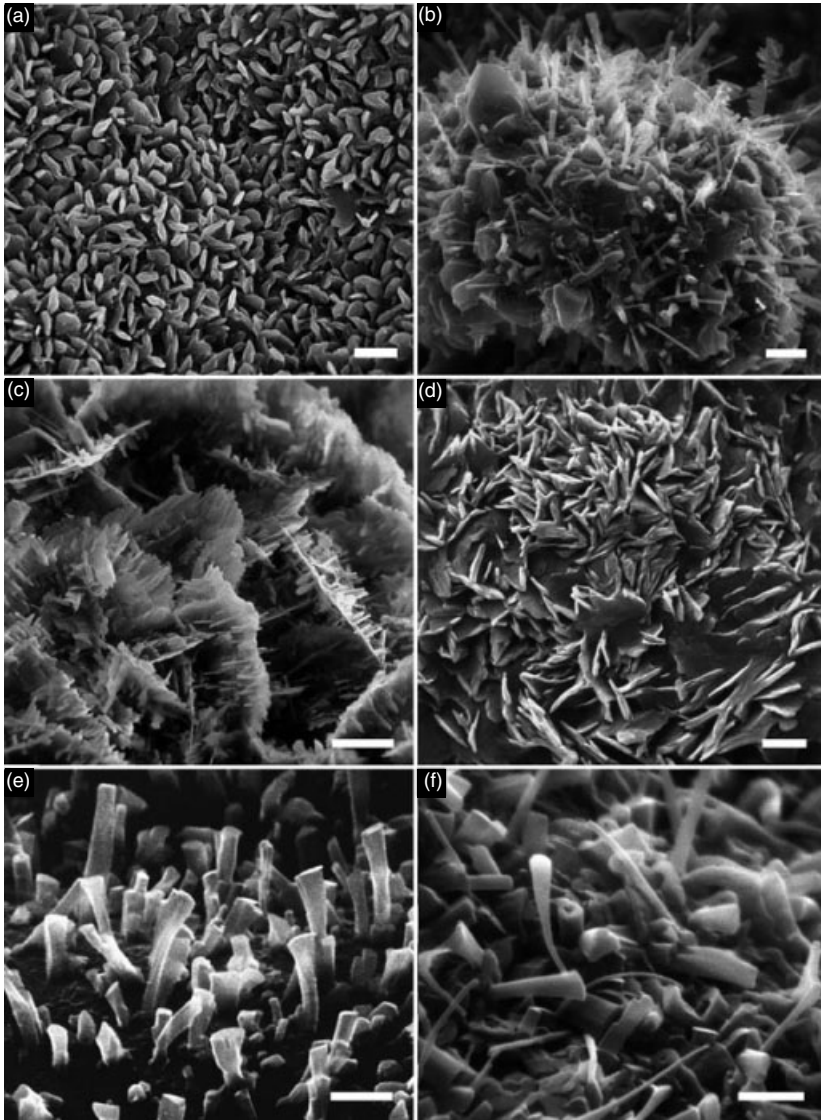


Figure 2.19 Structure and recrystallisation of *Brassica oleracea* waxes. (a) Recrystallised Brussels sprout wax, showing plates unlike those found on leaves. (b) The same wax delivered to a porous disc via a short chromatographic column, showing several crystal motifs characteristic of the epicuticular waxes of *B. oleracea* leaves. (c) Slow-crystallised *B. oleracea* wax, showing dendritic structures analogous to those shown on a cabbage leaf in Figure 2.12d. (d) Plate-like structures formed by recrystallisation of the secondary alcohol fraction from Brussels sprout wax. (e) Tapered columns on the leaf surface of *B. oleracea gl2* mutant have an inverted taper compared with the normal wax, which is reproduced when the wax is recrystallised (f) together with other typical *B. oleracea* motifs, such as columns with a false hollow end. (a,b,d) bar 2 μm , (c,e,f) bar = 1 μm . Figures 2.19a, and c–e by C.E. Jeffree, E.A. Baker and P.J. Holloway. Figures 2.19b,f from Jeffree *et al.* (1975, 1976).

Meusel *et al.*, 1994), but the related wax morphology in *B. hispida* is associated instead with triterpene acetates (Meusel *et al.*, 1994).

The Aristolochia rodlet type. Another characteristic type of wax rodlet found in basal Angiosperm genera of the Magnoliidae such as *Aristolochia*, *Liriodendron* and *Magnolia* is referred to by Barthlott *et al.* (1998) as the *Aristolochia* type (Table 2.3). These substantial rodlets usually have pronounced transverse ridges, and often appear to be assemblages of transversely stacked platelets. The morphology of this group shows considerable overlap with the morphology of *Brassica* type waxes, some of the crystal motifs being shared between them. For example, it would be hard on morphological grounds alone to distinguish between the waxes of *Brassica oleracea* var. *gemmifera* and *Fritillaria pallidiflora*, and the waxes of *N. glauca* and *Leucojum aestivum* show the same type of construction of fluted columns from irregularly curled plates that occur in seakale (*Crambe maritima*). Analysis of these waxes reveals little common ground between them in terms of composition (Meusel *et al.*, 1999). One group, comprising *Aristolochia*, *Paeonia* and *Centranthus*, to which could also be added the fern *Osmunda regalis* (Jetter and Riederer, 2000) has significant amounts of ketones, but others including *Galanthus*, *N. glauca*, *L. aestivum* and *F. pallidiflora* lack them completely, and instead have alkanes as about 70% or more of the wax together with alkyl esters and primary alcohols. In *Paeonia* the major ketone is the symmetrical palmitone (hentriacontan-16-one) and in *Centranthus* nonacosan-14-one, both with important quantities of triterpenoids. The major constituents in *Laurus* are primary alcohols (52%) and fatty acids (Meusel *et al.*, 1999). It therefore appears that the waxes of these species have convergent wax crystal morphologies based on a variety of different chemical solutions. It is clear that in these species, and probably also in the Brassicaceae, a simplistic view of relationships between single major constituents and wax ultrastructure is not appropriate.

Farina. On some ferns (*Pityrogramma* spp.; Wollenweber, 1982), and in many species of the Primulaceae the 'wax' or 'farina' found on the leaves is composed almost entirely of crystalline lipophilic flavonoids. In most of these species the farina forms dense assemblages of narrow rodlets.

2.6.3 *The background EW film*

Jeffree *et al.* (1975, 1976) postulated that the background layer underlying the EW crystallites was a continuous amorphous wax coating covering the CP. The use of the word 'amorphous' to indicate the absence of crystalline projections detectable by microscopy is inadvisable. In some instances (e.g. on the leaves of *B. vulgaris*, *Clematis vitalba* and *P. vulgaris*), there is little visible evidence of epicuticular ultrastructure at the modest resolutions achievable with the SEM of the 1970s. Evidence of planar structures in an otherwise featureless surface layer or in the spaces between crystallites was often visible in TEM replicas of *B. oleracea* var. *capitata*

and *Hyacinthus orientalis* wax (Juniper and Bradley, 1958) and on the cuticle surface of cotton leaf (*G. hirsutum*; Hallam and Juniper, 1971), but confirmation of the existence of a discrete superficial wax layer by SEM is not usually possible. Jeffree (1996) showed a low-temperature SEM image of the surface of a *Quercus pubescens* leaf from which the wax was stripped at -196°C by using pentane as a cryo-adhesive. The image demonstrated that the EW crystallites arise from a continuous covering layer of wax about $0.2\text{--}0.3\ \mu\text{m}$ thick. Likewise, confirmation that the apparently smooth surface of cuticles of *Prunus laurocerasus* nevertheless carries an EW film that is chemically distinct from the embedded cuticular wax has come from elegant experiments by Jetter *et al.* (2000), Ensikat *et al.* (2000) and Jetter and Schäffer (2001) in which the superficial wax layer was removed mechanically using ambient- or cryo-temperature adhesives. A striking observation is that despite the fact that EW must be transported through the cuticle, the soluble wax compositions of the intracuticular and epicuticular domains each appear to be uncontaminated with the other's constituents. The data of Jetter *et al.* (2000) show that the mechanically harvested epicuticular layer of *P. laurocerasus* wax contains exclusively aliphatic compounds, while the triterpenoids ursolic and oleanolic acid represent almost two-thirds of the intracuticular wax. How this chemical segregation is achieved remains unknown. The EW appears to be extended as a film over all parts of the general epidermal surface to form the visible surface exposed between the EW crystallites. No pores or other structures that might correlate with points of origin of the crystallites are visible in the underlying cuticle. Whether the projecting crystals originate directly at the surface of the CP or are rooted somewhere within the EW film is still a matter of conjecture. In *Phragmites australis* leaves cryo-stripped with ethanol, what appear to be the stumps of wax rodlets remain attached. By contrast, Koch *et al.* (2004) found that the longitudinally ridged rodlets of *Galanthus* leaf surfaces were stripped completely with epoxy resin leaving a featureless smooth surface. Neinhuis and Barthlott (1997) stated that the EW penetrate the cuticle to build up films, crusts or distinct surface structures. The word 'penetrate' was probably used here in the sense of 'enter while being transported across'. It is not suggested that epicuticular crystals are rooted deep in the CM, and images of the CM structure beneath EW crystals in various species (Figures 2.4e,f) lend no support to this, implying that the crystal structures originate entirely externally to the CP layer.

Although the background wax film appears amorphous in the SEM, there are good reasons to doubt this, and the consensus of opinion is that the film may be crystalline, even if it does not appear to be so in EM images. Images of the structure of wax films regenerating on plant surfaces have recently been made using Atomic Force Microscopy (AFM) in three species – *Euphorbia lathyris*, *Galanthus nivalis* and *Ipheion uniflorum* (Koch *et al.*, 2004; Figures 2.20a–d). In this groundbreaking study, the first ever direct observation of a time-course of wax regeneration in a living leaf, it is clear that progressively the surface is covered completely with monomolecular and then bimolecular layers of crystalline wax. The images (Figure 2.20a,b) show rodlets growing from the cuticle independently,

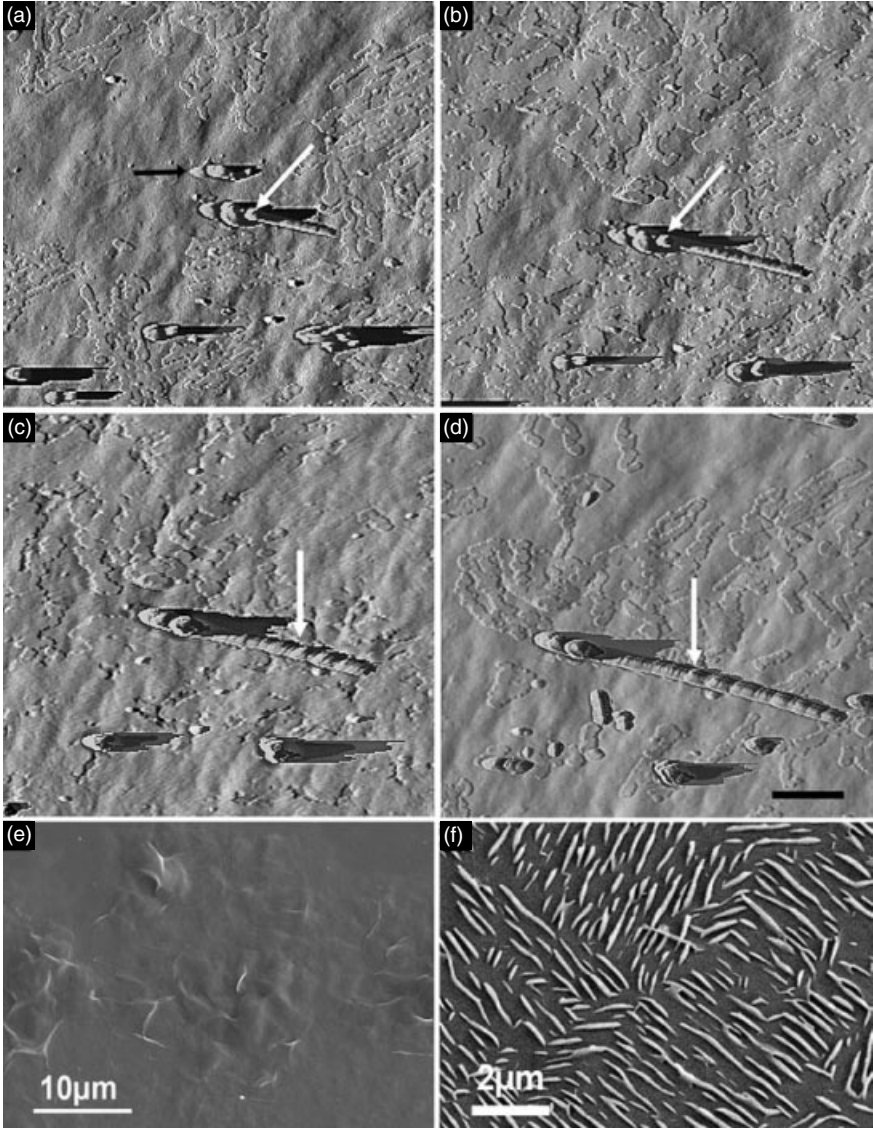


Figure 2.20 Atomic force microscopy (AFM) images of epicuticular wax regenerating on a living *Galanthus nivalis* leaf adaxial surface, following removal of the earlier-secreted wax with epoxy resin glue. The images show changes in a $6 \times 6 \mu\text{m}$ area during an 80-min time frame. Flat, lobed plates extend over the whole surface to form first a monolayer (light grey) (a,b) and then bilayered structures (dark grey) (c,d). Independently, rod-like crystals arise directly from the cleaned cuticle proper (CP) surface, extending by growth at their distal ends, not from the base. Diagonal arrows (a,b) and vertical arrows (c,d) mark reference points that remain in fixed positions during rod extension growth. The black arrow in (a) marks a crystal that in (b) has been removed by the AFM tip. (a–d) bars = $1 \mu\text{m}$; reproduced with permission from Koch *et al.* (2004), *Journal of Experimental Botany*, **55**, 711–718.

beginning from bare areas of cuticle prior to the completion of the coverage of the background film.

Only rarely is the CP exposed at the surface. Fungi, particularly the rusts, respond to cutin monomers by increasing the germination rate or frequency. *Magnaporthe grisea* (rice blast) differentiation is induced by the addition of cutin monomers (Gilbert *et al.*, 1996; de Zwaan *et al.*, 1999) and the fungus produces cutinase during infection (Sweigard *et al.*, 1992). These monomers may be released directly from the cutin polymer, by cutinase secreted by the spores and hyphae, or there may be a sufficient amount of the molecules free in the covering wax film. The responses of germ tubes to stomata indicate that they can be sensed by hyphae, and there is some evidence that cutin is exposed at the stomatal lip of broad bean and cowpea leaves since it stains locally with Nile red (Collins *et al.*, 2001; Figure 2.10g).

2.7 Cuticular pores and permeability of the CM

2.7.1 Permeability of water and solutes

The reticulum of polysaccharide microfibrils ramifying through the CL offers a potential pathway for diffusion (but not mass flow) of water and solutes through the CL (for a functional discussion see Chapters 8 and 9). Although the reticulum demonstrably traverses the entire CL in *A. americana* (Wattendorff and Holloway, 1980) and also in *P. abies* (Tenberge, 1989, 1992) it stops at the base of the ECL in mature *C. miniata* cuticles, which have notably low permeability to water (Schmidt *et al.*, 1981; Schmidt and Schönherr, 1982). The ultrastructure of the pathway for diffusion of water and other molecules across a lamellate CP is probably at intermolecular scale. It has never been observed, and may never be directly by currently available technologies. Periclinal penetration of permanganate is two–four times faster in the electron-dense than in the electron-lucent lamellae of the CP. In the CL, penetration by the fibrillar pathway is some ten times or faster than via the cuticular polymer matrix (Wattendorff and Holloway, 1984). The lamellate cuticle covering the guard cells of *F. hygrometrica* sporophytes (Sack and Paolillo, 1983a,b) is penetrated apparently to the surface by polysaccharide fibrils (Figure 2.4d) which may evaporate water from their tips, but in almost all cuticles putatively of Type 4, the ends of the microfibrils are capped with a thin amorphous CP. Since our understanding of the ontogeny of the CM is that the construction of the procuticle and

(**Figure 2.20**) (e,f) SEM images of wheat leaf wax recrystallised from chloroform on glass forming an amorphous film (e) and on freshly cleaved highly ordered pyrolytic graphite (HOPG; PLANO GmbH, Wetzlar, Germany) (f), producing highly ordered wax crystals; reproduced with permission from Kerstin Koch (see also Koch, K., Barthlott, W., Koch, S., Hommes, A., Wandelt, K., Mamdouh, H., De-Feyer, S. and Broekmann, P. Structural analysis of wheat wax (*Triticum aestivum*, c.v. 'Naturastar' L.): from the molecular level to three dimensional crystals, *Planta*, in press). This figure is produced in colour in the colour plate section, which follows page 249.

CP outside the CW always precedes the process of CW impregnation that produces the CL layer, the only ways that the fibrillar reticulum can become uncapped, and the fibrils exposed at the surface, are by the systematic disassembly or sloughing of the CP, or by the failure of the CP to keep pace with massive area expansion of an organ. There is currently no convincing evidence for any of these in ordinary leaves, but it is possible that in fruit cuticles the CP is discontinuous or lacking.

In cuticles of higher plants, evidence for discrete pores, capable of functioning as pathways for fluid mass flow, or wax delivery is practically confined to secretory cells, including the receptive stigmatic papillae. In glandular hairs of many species, secreted materials accumulate between the PCW and the CM which becomes detached from the CW. Release of secretions may require rupture of these cuticles. A comparatively thick specialised Type 1 stigma cuticle of *Crocus chrysanthus* has a chambered structure in which the CL layer is eroded to the base of the CP to form ovoid chambers containing a protein–polysaccharide secretion. At maturity, the entire CM is underlain with this secretion, separating it from the PCW and permitting the growth of pollen tubes within the layer of secreted material (Heslop-Harrison and Heslop-Harrison, 1982). These processes presumably involve enzyme-mediated local reduction of the CM. In other species, cuticles covering glandular trichomes [e.g. *Drosera* (Chafe and Wardrop, 1973) stigmatic papillae] or absorptive cells [the pitcher epidermis of *Sarracenia* (Joel and Heide-Jørgensen, 1985)] and trap gland cells of *Utricularia monanthos* (Fineran and Lee, 1975) may be discontinuous or of highly variable thickness, or contain pores or slits through which secretion and absorption may occur. The pre-secretory cuticle covering the stigma of rye, *Secale cereale*, is thrown into ridges at the apex of which thinning occurs until mechanical rupture of the cuticle occurs at maturity, releasing a mucilaginous polysaccharide secretion (Heslop-Harrison and Heslop-Harrison, 1982). In *Abutilon venosum*, nectar droplets pass through a cuticle 0.4 μm thick perforated with pores 0.15–0.25 μm across at the tip of each nectary hair (Findlay and Mercer, 1971; Table 2.2). These pores seemed to have valve-like action, periodically releasing nectar accumulated between the cuticle and the CW. Gaps in the CM are reported for other gland types. In many cases the evidence points to mechanical rupture at weak points in the CM. Thus, the thin, discontinuous cuticles of *Drosophyllum lusitanica* (Joel and Juniper, 1982; Joel *et al.*, 1983) rupture at predetermined points of weakness under the tension forces caused by area expansion of the underlying PCW, which reduces in thickness by a factor of more than 2. No gaps or pores were found in the cuticles of glandular trichomes of *Phyllyrea latifolia* (Gravano *et al.*, 1998). The authors therefore thought that the secretory products diffused through the cuticle. In the glandular hairs of *Galphimia brasiliensis* the Type 3 cuticle comes very close to being Type 4, the CP zone free of visible microfibrils being less than 20 nm thick on top of approximately 0.7 μm of reticulate CL.

2.7.2 Wax secretion, cuticular pores and microchannels

The ontogeny of the EW layer (see also Chapters 4 and 5) has been debated for almost 150 years. de Bary (1871, 1884) proposed that wax was exuded to the surface via

pores in the cuticle, and in the same year, Weisner (1871) proposed that the wax was transported to the surface in solution in a volatile solvent. Before the advent of SEM, pores were reported in the cuticles of *S. officinarum* (Wijnberg, 1909) and *M. paradisica* (Mueller *et al.*, 1954), both species with very large rodlets observed by LM by de Bary (1871, 1884). Mueller *et al.* (1954) proposed that the wax was extruded under pressure through these pores as a soft paste (see also Schieferstein and Loomis, 1959). Hall and Donaldson (1962, 1963) and Hall (1967a,b) claimed the existence of pores in direct and secondary TEM replicas of leaves of *T. repens*, *Trifolium pratense*, *B. oleracea* and *Poa colensoi*. Their images showed a network of putative pores on *Trifolium* leaves in locations which might correspond to the positions of plate wax crystals. Fisher and Bayer (1972) also claimed the existence of pores of 2.5 nm diameter traversing the entire CM in sections of *P. major* CM. More recent images of the CM of the same and many other species by Holloway (1982a and unpublished) lend no support to this. Von Wettstein-Knowles (1974) proposed that wax tubes on barley leaves and leaf-sheaths could be extruded from pores, with the wax precursors polymerising in contact with the air. It should be noted that with the exception of the estolides of gymnosperm waxes (von Rudloff, 1959), and the polymerisation of aldehydes in the waxes of *S. officinarum* (Lamberton and Redcliffe, 1959; Haas *et al.*, 2001) and *Nepenthes* (Riedel *et al.*, 2003), polymeric constituents are almost unknown in EW of Angiosperms, and the wax tubes of barley are now known to consist of freely soluble β -diketones and hydroxy- β -diketones. The forms of wax rods on leaf sheaths of *S. officinarum* and nodes of *Arundinaria*, the leaves of *Strelitzia* and the stomatal chimneys of *Heliconia* with their longitudinal striae (Frölich and Barthlott, 1988; Meusel *et al.*, 1994) are suggestive of extruded structures, and Schieferstein and Loomis (1959) thought of them as bundles of extruded wax threads. But it is known from X-ray crystallographic data and electron diffraction studies (Kreger, 1949; Reynhardt and Riederer, 1991, 1994; Reynhardt, 1997; Meusel *et al.*, 2000) that these wax forms are bundles not of extruded threads but of longitudinally aligned crystals. Nevertheless, the issue of wax extrusion through cuticular pores has again been raised in *S. coronata*, the ouricuri palm of Brazil, which bears *Strelitzia*-type waxes assembled into massive epidermal wax crusts, but the true identity of the observed structures by Machado and Barros (1995) cannot be confirmed because of poor image resolution. The morphologies of most other EW types listed in Table 2.3 do not lend themselves to an extrusion-based origin. Tubular crystals would require a complex pore with a central spigot, while crystals with crenate edges, or the transversely ridged structures seen on *Bupleurum* (Figure 2.18d), *Actinidia* (Barthlott *et al.*, 1998) and *Brassica* species (Figures 2.18a,b) would require pores with fluctuating dimensions.

Miller (1982, 1985, 1986) reported the 'anticlinally-oriented transcuticular canals' in leaves of more than 50 species, but his observations made with the light microscope failed to confirm the identity of such features. Electron microscopy has also consistently failed to reveal transcuticular pores in the sense of open transcuticular channels via which wax, or other materials, might pass freely (Roelofsen, 1952; Mueller *et al.*, 1954; Juniper, 1959, 1960; Schieferstein and Loomis, 1959; Crisp, 1965; Jarvis and Wardrop, 1974; Jeffree, 1974a).

Hallam (1964, 1967) suggested that wax might migrate to the surface through the anastomosing channels between the lenticular cuticular (CP) lamellae of *Eucalyptus cinerea*, and that the ultimate pattern of wax arrangement may be partly a result of the exit pathway through the cuticle. But he had already obtained and reported in the same works evidence of their crystalline properties that seemed more persuasive. In a light TEM and SEM study of the adaxial surface of primary needles of ten-week-old *Pinus radiata* (Franich *et al.*, 1977; Wells and Franich, 1977), rows of wax tufts across cuticular ridges consisting of aggregates of wax tubes were observed, and were interpreted as covering clusters of pores from which they were exuded. Well before bud-break, Sitka spruce needles already have a CP and a CL, and EW are crystallising as tubes on top of the CM, which must therefore be permeable to 10-nonacosanol and other constituents of the EW (Figure 2.11). No structure corresponding to a pathway for the wax is visible by SEM or TEM, however (Jeffree, 1974a). The developing surfaces in-bud are completely dry, and there is no sign of the presence of a solvent. The mechanism by which the EW is delivered to the plant surface is currently unknown. AFM images of regenerating wax layers on leaves of various species show extension of mono- and bimolecular films of wax occurring without any visible evidence of a source (Koch *et al.*, 2004).

In the petiole of *Vaccinium reticulatum*, and in sections of leaf cuticles of *P. major* and *A. crenata* (Fisher and Bayer, 1972), the CL is penetrated by a 'channel-like reticulum which emanates from the primary CW', consisting of polysaccharide microfibrillar bundles embedded in cutin (Singh and Hemmes, 1978). Pectic radiating strands in the reticulate CL of *Spartocytisus filipes* were referred to as 'microchannels' by Lyshede (1978) and in *H. suaveolens* by Heide-Jørgensen (1978a,b). These structures, which correspond with massively enlarged forms of the tree-like reticulum in the spruce cuticle (Tenberge, 1989, 1992; and Figure 2.2), are light in TEM negatives, consisting of material more electron-dense than the matrix. They are thus inconsistent with the concept of open pores or channels. The reticulum of polysaccharide fibrils might be thought to represent a possible wax pathway, but their polar reaction, and the evidence of Wattendorff and Holloway's (1984) experiments with KMnO_4 suggesting they are permeable to water and solutes are incompatible with the view that they are pathways for wax mass transport.

2.7.3 Relative sizes of wax crystals and cuticle

Let us also be clear about the relative dimensions of wax crystals, the cuticle and the underlying CW. Images of the EW and cuticle structure usually feature one without the other, making it hard to appreciate the relative scale of wax crystals compared with the thickness and lamellar dimensions of the CP. However, the ghosts of wax crystals sometimes appear in TEM images of the cross-sections of the CM, as in the work of Hallam (1964, 1967, 1970), Hallam and Chambers (1970; tube and plate-type *Eucalyptus* waxes), Jarvis and Wardrop (1974; *P. tenax*), Holloway (1982a; Figure 2.4f, *P. sitchensis*) and Wattendorff (1974; Figure 2.4e; *A. americana*). In a

micrograph of *Eucalyptus papuana* epidermal cells by Hallam in Martin and Juniper (1970, his figure 1.1), the β -diketone wax tubes are as long or longer than the entire thickness of the CW, and the diameter of the tubes approximates the full thickness of the CM. In the daffodil petal cuticle (P.J. Holloway, Figure 2.9g) the cuticular ridges are approximately 0.6–0.9 μm wide. Similarly sized cuticular wrinkles on the leaf of willowherb (*Chamerion angustifolium*; Figure 2.18c) bear wax crystals that span two or more wrinkles. If pores were responsible for wax extrusion in these cuticles they would have to span the wrinkles. Two important conclusions can be drawn from these images: first the lengths (height) of the wax crystallites is typically greater than the thickness of the CP, and in some instances thicker than the entire CM, and second no trace of any pores of dimensions corresponding with the crystallites is ever visible. Pores large enough to act as paths for wax extrusion could not hide undetected. They would be conspicuous, their diameter up to an order of magnitude larger than the thickness of the entire CP, and moreover accumulations of yet-to-be extruded material might be expected to appear beneath them. In the CP of *A. americana* shown in Figure 2.4e, the CP lamellae are in orderly periclinal alignment from side to side of the image. They are not interrupted anywhere, they do not deviate relative to the positions of the wax crystallites or take any account of them whatever, and in the spruce image (Figure 2.4f), the CW microfibrils come close to the surface of the CM, but are much more than an order of magnitude smaller than the diameter of the tubes and their distribution shows no relationship with those of the tubes (see also Jeffree, 1974a). If wax moves through the structure of the CP, as it must, it does so without altering the ordered structure of the CP.

2.8 Crystallisation studies on EW

Although de Bary (1871, 1884) proposed exudation of wax to the cuticle surface, he used the word 'Krystalloid' to describe the wax structures on *S. officinarum* and other plant surfaces and must have regarded their structure as fundamentally crystalline. In an analysis of the optical anisotropy of the waxes from *C. cerifera*, *P. sylvestris* and *P. australis*, Weber (1942) proposed a layered structure in the superficial wax film with vertical orientation of the carbon chains. For terete rodlet waxes he proposed a radial packing arrangement in which various molecular species such as long-chain acids, alcohols and esters might collaborate. Kreger (1949) confirmed by X-ray diffraction studies that EW rods of the type seen in *S. officinarum*, *Arundinaria* and *Strelitzia* are bundles of long crystals, and that their structure must be related to their chemical composition. Prior to the emergence of Bradley and Juniper's groundbreaking replica technique (Juniper and Bradley, 1958), and the later advent of SEM little further progress was possible, but even then the matter remained unsettled for many years.

Weisner (1871) suggested that wax precursors pass through the cuticle in a solvent carrier which evaporates, permitting the wax to crystallise at the surface,

and by simple analogy with related compounds in petroleum products there is no doubt that the constituents of EW are capable of self-assembly into microcrystals, and that the EW structures on plant surfaces are crystalline, as indicated by Kreger (1949). The crystalline behaviour of long-chain hydrocarbons is of technological and economic importance, but although petroleum products include hydrocarbons similar to those in SCL, the results have not been widely assimilated by plant scientists. Many wax alkanes, acids and alcohols exhibit polymorphic behaviour, and slow evaporation of wax solutions in benzene or xylene can result in more than one type of crystal morphology (Amelinckx, 1955). Birdwell and Jessen (1966) reported that 'pure *n*-paraffins exhibit a tabular or platy crystal habit, the most common forms of which are thin hexagonal and rhombic plates'. Homologous series of alkanes generally crystallised compatibly, but EW in which alkanes are dominant constituents show multiple contrasting crystal morphologies, suggesting that the relative proportions of different homologues, or relationships between alkanes and other constituents may be influential.

That the structure of EW crystallites is predominantly a process of self-assembly, with the crystal morphology determined by dominant chemical constituents, is now well established both from correlative and genetic observations (Hallam, 1967; von Wettstein-Knowles, 1974), and by experimental analysis of crystal formation in many species (Jeffree, 1974a,b, 1986; Jeffree *et al.*, 1975, 1976; Holloway *et al.*, 1976; Lister and Thair, 1981; Jetter and Riederer, 1994, 1995; Jetter *et al.*, 1996). Whitecross (1963) was the first worker to examine Weisner's hypothesis (1871) of wax crystallisation from solution experimentally. He found that crystalline structures could be obtained from solutions of *Brassica* wax in organic solvents such as acetone. Later, Hallam (1967) and Hallam and Chambers (1970) who had observed a general correlation between chemical composition and morphology in *Eucalyptus* waxes, demonstrated that the primary alcohol-rich plate wax of *Eucalyptus ovata* and the β -diketone rich tube wax of *E. globulus* would recrystallise from solution in acetone with crystal forms corresponding to the *in vivo* morphology, and that the rate of solvent evaporation influenced the crystal structure obtained.

Jeffree (1974a,b) developed a method for crystallising waxes and their constituent fractions by feeding solutions of them to a porous evaporating surface using a wick, enabling the crystallisation of waxes from a wide range of species and morphological types, together with isolated and purified constituents of the waxes (Jeffree *et al.*, 1975, 1976; Baker, 1982). These experiments confirmed that the plate-type waxes of eucalypts (Jeffree, 1974a,b), grasses and peas, and the primary alcohol fractions isolated from them, could recrystallise *in vitro* as plates, while the remainder of the wax failed to reproduce the characteristic plate morphology. (Figures 2.13c,f, 2.15a,b). Jeffree (1974a) used this method to recrystallise the tube wax of Sitka spruce in its original tubular form from solution in chloroform (Figure 2.17c). The chromatographically isolated asymmetrical secondary alcohols from Sitka spruce, comprising 19.7% of the wax, of which 98% is 10-nonacosanol (Holloway *et al.*, 1976), also recrystallised in tube form (Figure 2.17e; Jeffree, 1974a, 1986; Jeffree *et al.*, 1975, 1976), while the remaining 80.3% of the wax lost the capacity to

recrystallise in tube form (Figure 2.17g). Tube waxes rich in the asymmetrical secondary alcohol 10-nonacosanol isolated from leaves of *Chamaecyparis lawsoniana*, *G. biloba* (Figure 2.13k,l), *Picea pungens*, *C. majus*, *Aquilegia vulgaris*, *T. majus* all recrystallise in tube forms as do the isolated asymmetrical secondary alcohol fractions from these waxes. The secondary alcohol-depleted residues did not form tube crystals. Tube-wax of *E. racemosa* (Figure 2.15e), containing approximately 30% asymmetrical secondary alcohol (Holloway *et al.*, 1976), recrystallised in a *Strelitzia* or *Brassica*-like form, with tubes present but plate-like structures predominating (Figures 2.15f,g), while *Agathis australis* wax (approximately 28% asymmetrical secondary alcohol) recrystallised as stubby plates without visible tubes (Jeffree *et al.*, 1975). The secondary alcohol fractions isolated from these waxes by preparative scale thin-layer chromatography nevertheless always formed tubular crystals. Important recent data has been added by Jetter and Riederer (1995), showing that a range of alkanediols, present in the waxes of many secondary alcohol tube-forming species, also have tube-forming capability. The crystal sizes formed by x,10-nonacosan-diols, where x is 3, 5, 7, 9 and 13, were found by Jetter and Riederer to be uniform and closely comparable with the diameters of the tubes formed in the waxes of their origin, suggesting that they may be capable of collaborating in wax tube formation with 10-nonacosanol, as suggested earlier for *R. cotinus* by Hunt and Baker (1979).

Similarly, both the tubular β -diketone-rich whole waxes of *E. globulus*, *Eucalyptus glaucescens*, *D. caryophyllus*, *T. aestivum*, *Festuca glauca* and *H. vulgare*, and the β -diketone and hydroxy- β -diketone fractions isolated from them crystallise in tube forms. The hollow form of the recrystallised secondary alcohol and β -diketone tube crystals was confirmed by negative staining (Jeffree *et al.*, 1976; Figure 2.13i,j) as it had been earlier for the natural leaf waxes of Sitka spruce and *T. kaufmanniana* (Johnson and Jeffree, 1970; Figure 2.17f).

Not only is the morphology reproduced in recrystallisation experiments, but also the sizes of the crystals are generally closely similar to the corresponding crystals found on plant surfaces (Hallam, 1967, 1970b; Jeffree *et al.*, 1975; Jetter and Riederer, 1994, 1995).

2.8.1 *The tube wax crystal*

Hallam (1967) viewed the wax crystals of *Eucalyptus cephalocarpa* leaves as tubular, reporting a complete cover of compound branching tubes produced in a 24-h period after the crystals had been removed by rubbing. 'Two hours after rubbing it was apparent that the tubes were originating from the cuticle as narrow plates, these appearing to roll over and fuse their edges to form a tube'. Tubular crystals in *Pseudotsuga menziesii* wax probably develop by tight coiling of a narrow filament (Lister and Thair, 1981). Open coils or spirals can sometimes be observed (von Wettstein-Knowles, 1974). Spectacular examples, in which both tubular and helical morphologies occur side-by-side are shown by Jetter (1993) and Jetter and Riederer (1994) from the capsule of *P. somniferum*, by Neinhuis and Jetter (1995)

on the gametophyte of *Pogonatum rubenti-viride* and by Barthlott *et al.* (1998) in *L. korolkovii*. Discussing the phenomenon of plates rolling to fuse along their free edges into hollow structures, Hallam (1967, 1970a) advocated use of the term 'tube' as more appropriate than 'rod' or 'fibril'. This sequence of events is hard to observe in a dynamic sense, but it is clear today that Hallam was fundamentally correct. There is a spectrum of morphologies between straight ribbon, curled ribbon and tube in all types of tube-forming species, both secondary alcohol and β -diketone types and also in a new category of tube-forming waxes dominated by δ -lactones in *C. minor* (Jetter and Riederer, 1999a; see also *C. major* Figure 2.14c). Furthermore, in some species the tubes themselves may become curled or super-coiled as in *B. sempervirens* and in *C. segetum* (Meusel *et al.*, 1999).

Micrographs at adequate resolution show a spiral line on the surfaces of nonacosanol tubes at about 30° to the long axis (Jeffree, 1974a; Figure 2.17d) probably representing the edge seam. A notch is also common at the top of the tube, representing the terminus of the coiling ribbon. Current data suggests that the amount of asymmetry in the crystal-forming compounds is responsible for the tightness of the spiral structure. Branching of the tubes also occurs in many species. In crystals e.g. of quartz, this branching is known as 'twinning' and is observed more commonly in β -diketone-tube waxes (e.g. in several species of *Eucalyptus*; Hallam, 1967) than in the secondary alcohol types.

In the experiments carried out by Jeffree, Baker and Holloway (1975, 1976) the isolated alkane fractions and pure alkanes generally formed crystals of a plate type, as also observed by Chambers, Ritchie and Booth (1976), even when their origin was from a wax that appeared amorphous, as in the wax of *N. tabacum* leaves and lemon fruit (*C. limon*). Jeffree *et al.* (1975) found that aldehydes tended to crystallise as elongated plates or ribbons, and suggested that their presence in the alkane-dominated ribbon wax of the abaxial leaf surfaces of peas (*P. sativum*) might be influential in shifting the crystal morphology away from the isodiametric plate type. This kind of domination of the wax morphology of some conifer waxes was already known in *P. pungens*, e.g. where 10-nonacosanol is less than 10% of the extractable wax (Holloway *et al.*, 1976) but nevertheless determines that the dominant wax morphology is tubular. However, an alternative and more probable interpretation of the situation in *P. sativum* is suggested by the findings of Chambers *et al.* (1976), who showed that mixtures of hydrocarbon, C32 and C20 alkanes with C20 primary alcohol and C12 ketone crystallised as elongated ribbons, distinct from the crystal morphologies of the pure compounds indicating that new crystal morphologies can arise by co-crystallisation of compatible hydrocarbon constituents.

The ketone fractions from *A. porrum* wax (Figure 2.16a), which is 36% palmitone, hentriacontan-16-one (Baker, 1982), recrystallise in a crenate plate form with lobed and spiked edges that approximates the wax type found on the plant surface, lending support to the hypothesis that the wax crystals of *A. porrum* are predominantly composed of palmitone. Barthlott *et al.* (1998) referred to the wax of *Brassica*, *Clarkia* and *Centranthus* as 'polymorphic', implying that the wax

contains crystals of several different morphological types, which can be expressed in different proportions when Brussels sprout plants, for example, are grown under contrasting environmental conditions (Whitecross and Armstrong, 1972; Baker, 1972, 1974, 1982). *Brassica* wax polymorphism may arise from the presence in the wax of several compound classes, each capable of forming distinctive crystal morphologies, but the evidence also suggests the possibility that new crystal forms are specified by co-crystallisation of specific mixtures of these compounds. The wax contains dominant alkanes (nonacosane 46%) and C29 secondary alcohols, in *B. oleracea* var. *gemmifera* the symmetrical isomer, 15-nonacosanol being approximately 7.5%, together with about 4.5% of asymmetrical nonacosan-14-ol (Holloway *et al.*, 1976). Both of these compound classes crystallise in plate-like forms *in vitro* that are not expressed on *Brassica* leaves (e.g. Figure 2.19d). Ketones, of which 95% is nonacosan-15-one in *Brassica* (Baker, 1982), account for about 30% of the wax of *B. napus* and *B. oleracea* (Shepherd *et al.*, 1995) and the isolated ketone fraction recrystallises to form characteristically spiky-edged plates or dendrites (Figure 2.16d). Furthermore, the leek ketones often form dendritic structures (Figure 2.16c, top left) which recall the dendritic crystals of cabbage leaves (Figure 2.12d) and suggest (Jeffree *et al.*, 1975, Jeffree *et al.*, 1976) that ketones may be involved in the dendritic waxes of *Brassica* plants grown at high radiant energy and temperature, conditions which promote an increase in ketone production.

Certain waxes fail to recrystallise in their *in vivo* morphology. The most problematic cases are of waxes that have apparently amorphous structure *in vivo*, but readily form crystallites *in vitro*, and complex waxes of the *Brassica* type that recrystallise correctly only under certain circumstances. The whole wax of *B. oleracea* var. *gemmifera* recrystallises to produce highly variable (polymorphic) results, even within a single experiment. The resultant morphologies appear to respond to variation in local and global conditions during recrystallisation which are not even crudely understood, such as the carrier solvent, rate of solvent feed, concentration of the wax, porosity of the evaporation surface, rate of evaporation from the surface (cf. Figures 2.19a,c). In our experiments, the most successful reproductions of *in vivo* morphology from recrystallised *Brassica* waxes occurred when wax solutions in hexane, chloroform or benzene were delivered to a porous surface via a chromatographic column (silica gel G) that partially separated the constituents in order of polarity, the least polar (alkanes) being delivered first (Jeffree *et al.*, 1975, 1976). Under these circumstances various characteristic elements of the *Brassica* wax morphology were reproduced successfully (Figure 2.19b). The most significant observation is that to date it has not been possible to reproduce the characteristic fluted columns or the lobed plates by recrystallisation of any of the purified individual compounds or compound classes isolated from *Brassica* waxes. Similarly, recrystallisation experiments with the *Aristolochia*-type waxes showed that they could all be recrystallised *in vitro* from the whole wax with morphologies similar to or approximating the epicuticular morphology of the plant surface in some recrystallisation regime. However, in no instance was it possible to identify a wax

constituent capable of re-creating any element of the *Aristolochia*-type morphology (Meusel *et al.*, 1999).

2.8.2 *The single-compound hypothesis*

Barthlott *et al.* (1998) stated that 'Dominant wax components are normally considered to be responsible for the formation of wax crystalloids'. Meusel *et al.* (1994) stated that 'the dominant constituent(s) may be responsible for a particular ultrastructure of the crystalloids'. Neinhuis and Jetter (1995) reported that 10-nonacosanol is the dominating component in cuticular waxes of gymnosperms and moss sporophytes. Dominant in what sense? The asymmetrical secondary alkanols and alkanediols form tubes on their own, and are decisive in determining the functional morphology of tube waxes, and in some cases (*Aquilegia* with approximately 56% 10-nonacosanol and *Nelumbo* with approximately 57% secondary alkandriols, Table 2.5) dominant constituents are responsible for the observed morphology. However, the 10-nonacosanol waxes of gymnosperms rarely contain more than 20–30% of nonacosanol; so the compound class cannot be said to be quantitatively dominant even in those conifers with emphatic tube wax morphology. Why does the remaining 70–80% not have a say in determining crystal morphology? Individually, there is no reason why several other compound classes present in nonacosanol tube waxes should not form distinctive crystals, but together, in approximately the proportions found on the leaf surface, these compounds appear mutually to suppress the appearance of crystal morphs. This sort of trick applied to metals is the metallurgist's Holy Grail. Waxes of conifers contain 16-hydroxyhexadecanoic acid (juniperic acid) and 12-hydroxydodecanoic acid (sabinic acid) interpolymerised into sparingly soluble estolides or etholides. These can be a significant proportion of the soluble material isolated from the leaves of conifers (von Rudloff, 1959) but they are not known to be involved in wax crystal formation.

In general, across a wide variety of inorganic and organic materials, crystallisation can only take place by segregation of different components of mixtures, co-crystallisation occurring only between compounds with closely similar molecular dimensions and characteristics. In water containing KCl and NaCl in solution, rapid freezing will cause all three components to crystallise separately. Jeffree (1974a) and Jeffree *et al.* (1976) argued that in common with other crystalline substances, EW will usually crystallise in pure form from mixtures, and that the EW crystals on spruce leaves are likely to be composed of a single compound or closely similar compounds and isomers. Holloway *et al.* (1976) showed that the secondary alcohol fractions of tube wax species did not usually contain a single compound but homologous series of secondary alcohol compounds and many also contain asymmetrical diols. Likewise, β -diketone waxes also contain homologous series of related compounds, and most waxes of grasses also contain hydroxy- β -diketones that also have the capacity to crystallise as tubes as a purified fraction (Jeffree *et al.*, 1976; Figure 2.13h). EW mechanically detached from *E. globulus* leaves by simulated rain contained exclusively C33 β -diketone, which is only 55% of the

solvent-extracted wax from the same leaves (Baker and Hunt, 1986). This may be taken as evidence that the tube waxes of *E. globulus* are essentially pure crystals of the tritricontan-14,16-dione or are significantly enriched in it. Equally, the C31 alkane hentriacontane predominated in the material detached from pea leaf abaxial surfaces, suggesting that it is the predominant constituent of the mechanically vulnerable wax ribbons on this species. It is also relevant in this respect that the loss of rodlet (tube) structure was reported to coincide with depletion of the wax in β -diketones when leaves of *V. ashei* are weathered (Freeman *et al.*, 1979).

By contrast with these examples, the *Aristolochia*-type and *Brassica*-type waxes represent a suite of types which are capable of self-assembly into crystals, but in which the morphology appears to be determined not by a single dominant constituent or constituent class, but by co-crystallisation of special combinations of compatible compounds (Meusel *et al.*, 1999). Relative proportions of alkanes, secondary alcohols and ketones in rain-detached waxes of *Brassica* (Baker and Hunt, 1986) matched closely the proportions of the three principal compound classes in the solvent-extracted leaf wax. This may be taken as evidence that the three principal compound classes comprising 92% of *B. oleracea* wax are mutually responsible for the observed crystalline structure. It seems probable that the same situation applies in the *Aristolochia*-type species, but in these species it is also likely that the mix of constituents in the crystals differs between species, despite their analogous morphologies. The *Brassica* and *Aristolochia* waxes are therefore the first EW classes to be identified in which the crystallite morphologies are not directly produced by individual compound classes, as in the basal tube and plate types, but by a convergent series of different chemical combinations in different plant groups (Meusel *et al.*, 1999).

2.8.3 How do wax crystals grow?

An important issue which is not resolved by these crystallisation experiments is the mechanism by which wax is delivered to the inner surface of the cuticle (see Chapter 5), and its route through the CM. This is part of a much wider series of unanswered questions about the mechanism by which relatively large hydrocarbons with high melting points can move from place to place – both through the developing CM, and then into their final positions in projecting EW crystallites.

There is no evidence to support a solvent-carrier based mechanism as proposed by Weisner (1871), but the possibility cannot either be conclusively eliminated on current evidence. Jeffree *et al.* (1975) considered the idea that mobility of wax molecules through the CM was facilitated by a solvent, such as isoprene. However, isoprene turned out to be a poor solvent for the constituents of secondary-alcohol-type waxes, and attempts to recrystallise them using isoprene failed. Other considerations militate against a solvent-assisted model, not least the facts that many developing plants secrete very little of any likely candidate volatiles, and that wax crystal development is in many species, such as Sitka spruce, already well advanced inside multiple enclosing layers of bud scales from which evaporation of volatiles would be severely

retarded. EW of *B. oleracea* varieties *gemmifera* and *capitata* also develop while the surfaces are enclosed in layers of older leaves that would strongly inhibit solvent evaporation. As Hallam (1982) pointed out, only very non-polar solvents, such as benzene, hexane and chloroform dissolve EW of most species. These solvents, e.g. chloroform, are also cytotoxic, and present an unsatisfactory model for a carrier system in essentially aqueous plant tissues. Hallam (1982) proposed that wax might be transported through the CW enclosed in an envelope of lipo- or glyco-proteins, which would have hydrophilic external surfaces compatible with the aqueous environment of the CW. Evidence in favour of this hypothesis is accumulating rapidly. Acyl transfer proteins occur in the EW of broccoli (*B. oleracea*; Pyee *et al.*, 1994; Pyee and Kolattukudy, 1995), and the *cer5* gene in *A. thaliana* encodes a protein that localises to the plasmalemma exclusively in epidermal cells. Disruption of *cer5* results in the accumulation of sheets of lipid in the epidermal cytoplasm, accompanied by reduction in the wax loads on the surface. *cer5* thus appears to be an ABC (adenosine phosphate binding cassette) transporter responsible for the export of wax from the cell to the plant cuticle (Pighin *et al.*, 2004; Chapter 5; see also Kunst and Samuels, 2003).

Are the molecules added to growing tubes at their apices or their bases? Von Wettstein-Knowles (1974) envisaged newly exuded wax accreting to the base of the wax structures, pushing the older polymeric material away from the plant surface. However, for the first time, Koch *et al.* (2004) have been able to image the development of individual wax crystals by AFM on the living surface of a *Galanthus nivalis* leaf. Consecutive images of an elongating wax rod (Figures 2.20a–d) show clearly that the extension is occurring at its distal end, not from its base. This finding appears to put the extrusion hypothesis finally to rest, but simultaneously raises again the question of how long-chain hydrocarbon molecules can migrate up the inner or outer surfaces of tubes or plates, to accrete at their distal ends. What form is the wax in – is it mobilised by a solvent, and what is the energy source for such transport?

2.9 Crystal orientation and spatial patterning

In a great many plant species both the crystal orientation and the position of the wax crystals relative to surface features such as stomata, may be patterned in various ways. Spatial patterning in wax crystals implies non-randomness, which in turn carries the implication that both the position and orientation of crystals are under some form of control.

Tufted, clustered tube wax crystallites are common on the leaves of conifers e.g. Sitka spruce (Jeffree, 1974a; Jeffree *et al.*, 1976) and on developing leaves of *T. majus* (Rentschler, 1971). These aggregates of crystals give the appearance of arising from a common point of origin. Consequently it is tempting to search for pores beneath. However, the origin of the clustering is ambiguous. Young leaves are expanding, and this area expansion may separate an even distribution of crystals

into islands, the bare spaces between rapidly filling with new wax (Jeffree, 1974a). Clustered or tufted tubes are also common in β -diketone type tube waxes, e.g. of eucalypts (*E. globulus*, Hallam, 1967; *Eucalyptus camaldulensis*, Jeffree *et al.*, 1976).

Clustering of plates is commonly observed with clusters of at least three types, those which aggregate into clusters side-by-side (*Hypericum bucklei*), those which aggregate into radial clusters (*Erythroxylum coca*, Rentschler, 1971; *Calliandra haematoma*) and those which aggregate into mutually adherent groups that have the appearance of granules (*A. corniculatum*, Barthlott *et al.*, 1998).

In *Prenia sladeniana* platelets are orientated transverse to the stomatal aperture, and are co-aligned at various angles in domains about the size of the epidermal cells (Ihlenfeldt and Hartmann, 1982). The most dramatic such patterning occurs in *Convallaria majalis*, where evenly spaced plates may show parallel alignment over large areas, typically at 90° to the stomatal apertures (Barthlott and Frölich, 1983; Barthlott *et al.*, 1998). Frölich and Barthlott (1988) indicate that the *Convallaria* type wax is 'more or less confined to the Monocotyledons', but Dicotyledonous examples are also widespread. Marked alignment of plate type crystals is reported in *Chenopodium album* leaves (Taylor *et al.*, 1981) and is present in the Apiaceae (*Bupleurum ranunculoides*; Figure 2.18d). Analogous patterning occurs in plate-forming eucalypts such as *E. camaldulensis* in which stripes of transversely aligned plates can traverse multiple epidermal cells (Jeffree *et al.*, 1976; Figure 2.12e). Carr *et al.* (1985) described several types of orientated arrays in *Eucalyptus*, some of which are closely analogous to the *Convallaria* type while others form lines, swirls and rosettes of platelets. In many other primary alcohol plate-type species, such as *P. sativum*, *T. repens* and *Quercus spp.*, and the Poaceae generally (e.g. *Poa nemoralis*, Jeffree *et al.*, 1976; and *Festuca arundinacea*, Figure 2.12c and inset) the spatial distribution is highly regular over large areas and the crystals tend to occur in preferred orientations approximately 120° apart. Dramatic wax crystal patterning occurs in the rodlet-type waxes of *Saccharum*, *Musa*, *Strelitzia* and *Heliconia* where aggregates of co-aligned crystals form massive terete rodlets, the cylindrical chimneys accurately surrounding each stomatal complex in *Heliconia* being a notable example (de Bary, 1871; Barthlott *et al.*, 1998).

In most of these examples the basis of the wax patterning is obscure. Carr *et al.* (1985) raised the possibility of the involvement of ectodesmata, but their punctate distribution is at odds with formation of large-scale ordered crystal arrays. Kreger (1949) recognised that the direct determination of orientation by the cell membrane must be rejected because of the complexity of the pathway between it and the epicuticle. The orientation of new upright crystals could be influenced by crystal orientation of their neighbours, or by a large-scale crystal orientation in a basal wax film. Kreger (1949) discussed the possible nucleation of radial crystal groups by the first-secreted alcohol molecules, which might be expected to orientate head-to-head on the cuticle surface at 120° apart. This model would indicate random orientation of the nuclei themselves, perhaps spawning small-scale ordered domains. However,

the fact that in *Convallaria* plate orientation does not occur at random with respect to large-scale surface features such as stomata, around which the crystals orientate like field lines round a magnet (Barthlott *et al.*, 1998), indicates that random seeding of the pattern must be rejected. Wax crystals are always transverse to guard cells, not in alternative orientations. It is worth considering the possibility that wax crystals may epitax onto ordered or semi-ordered cutin polymer in the substrate CP. Chambers *et al.* (1976) were aware of the possibility of epitaxial growth of crystals on an orientated substrate, but appeared to dismiss the possibility on the grounds that the cuticle is a polymer. However, a striking finding in polymer technology is that transverse epitaxial crystal growth occurs on aligned polyethylene chains (Mackley, 1978). The resulting 'shish-kebab' crystals bear tantalising similarity to the linear tracks of primary alcohol crystals on *Eucalyptus* leaves, which may arise from an analogous process. Since the epidermis is still growing, the cuticle is still undergoing area expansion while wax secretion is taking place. The CM polymer may well be under mechanical stretching during growth, causing molecular alignment of the polymer chains of the CP, on which epitaxial growth of wax crystals can occur. If this hypothesis is true, then the distribution and orientation of the plate-type wax crystals on leaves maps out the distribution and orientation of the underlying cutin polymer chains, and therefore also the stress pattern on the cutin polymers during leaf area expansion. Experimental evidence for the ordered growth of crystals of wheat wax and its major constituent 1-octacosanol on ordered substrates (highly ordered pyrolytic graphite) has now been obtained by Koch *et al.* who found that wheat wax was reluctant to form crystals on amorphous surfaces such as glass (Figure 2.20e), but crystallised readily on crystalline substrates, the orientation of the wax crystals following the crystal orientation (Figure 2.20f). Crystallisation of wheat wax on isolated *C. majalis* cuticles produced the typical wax platelets of wheat, arranged in the complex patterns characteristic of *C. majalis* (Koch *et al.*, in press). These observations strongly indicate that EW crystal orientation is under epitaxial control by an ordered CM structure.

2.10 Degradation of EW

Because of the strong linkage between chemistry and EW crystal morphology it must be assumed that any environmental impacts on wax chemistry will alter the wettability and reflectivity (Chapter 6) of leaves. There has been widespread concern that pollutant impacts accelerate degradation of the EW of conifers, and that this may be responsible for forest decline in Europe and elsewhere. Aldehydes (alkanals) comprising about 35% of the EW of newly expanded leaves of beech (*Fagus sylvatica*) are susceptible to atmospheric photo-oxidation and decline in amount, alkanolic acids of the same chain length correspondingly increasing (Gülz *et al.*, 1992; Markstädter, 1994). With this exception, most common constituents of EW are very resistant to chemical change *in vivo* during the normal lifetime of plant organs.

The leaves of evergreen conifers are long-lived, normally surviving for four–six years in Norway and Sitka spruce, and they must presumably be photosynthetically competent throughout this period. The ancestors of the angiosperms generally had long-lived leaves, and the Lotus effect (Barthlott and Neinhuis, 1997; Neinhuis and Barthlott, 1997) may have been more significant for them than it is today in most mesophytic annuals.

In their discussion of the crystal morphology of secondary alkanols and alkanediols, Jetter and Riederer (1995) suggest that the tubular crystals of 10-nonacosanol should spontaneously, but slowly, transform to planar aggregates. If the structure of the tubes is intrinsically metastable, degrading continuously from leaf maturity onwards, this would have important implications for stomatal gas exchange in conifers where leaves persist for 4–6 years, and must presumably earn their keep.

There is a large body of observational data in the public domain that documents the progressive weathering and degradation of the EW covering of conifers. Wells and Franich (1977) reported that the density of tubiform wax tufts on *P. radiata* leaves was greatest near the stomatal pores where the morphology of the wax changed from tubiform to amorphous as the primary needle matured. The tubular structure of conifer leaves is observed gradually to be degraded by a variety of impacts, mechanical from wind, abrasion and ballistic impacts of rain and detritus, chemical from deposited loads of pollutants, and biotic from the activities of epiphytic microorganisms and insects. Degradation of wax morphology has been reported in *Pinus strobus* and *P. banksiana*, resulting in the fusion of tubes into plate-like sheets (Riding and Percy, 1985). The images, however, show wax structures consistent with mechanical abrasion as described by Pitcairn *et al.* (1986) and van Gardingen *et al.* (1991). Crossley and Fowler (1986) reported a systematic increase in wax tube diameters on Scots pine leaves from approximately 130 nm at time 0 to 220 nm at 30 months leaf age. They also noted that undamaged tubes remained beneath a superficial layer of damaged tubes, suggesting that the change was a consequence of external effects. EW crystals are detached from leaves by repeated droplet-impacts of rain (Baker and Hunt, 1986). The fragile, long, narrow crystal rods, tubes and dendrites of species such as *E. globulus* and *Brassica spp.* (the ‘non-structural’ waxes of Barber, 1955) are more susceptible to such damage than the comparatively robust primary alcohol-type plates of grasses (*Z. mays*, *H. vulgare*).

As waxes degrade, and wettability increases, weathered epicuticular surfaces of conifers may be colonised by fungal hyphae (Chapters 11 and 12) and green algae (principally *Chlorococcus*) that can begin to be visible to the naked eye from the second year onwards reducing photosynthesis of the whole tree by between 4 and 9% (Peveling *et al.*, 1992). The layers of cells can be sufficiently dense to obscure the abaxial surface of three- and four-year-old Sitka spruce needles almost completely (Figure 2.21f). This epiphytic biofilm forms a surrogate abaxial leaf surface that intercepts radiation, and has properties such as wettability, water absorption capacity and albedo that are different from those of the original leaf surface. However, the tubular wax structures in the stomatal antechamber that function as an antitranspirant mechanism (Jeffrey *et al.*, 1971; Figures 2.21a,d,e) remain intact throughout the

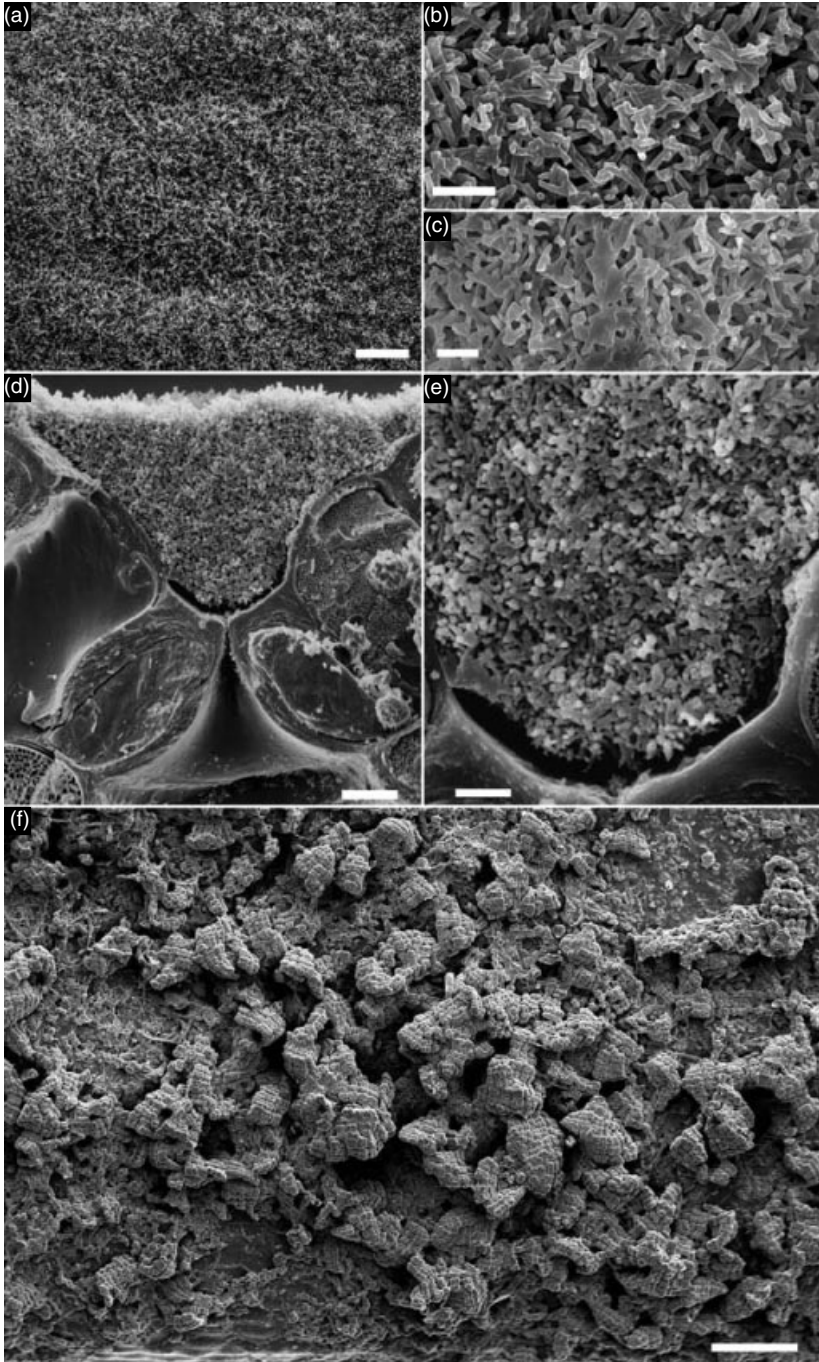


Figure 2.21 Low-temperature scanning electron microscope (SEM) images of the stomatal antechamber wax of Sitka spruce (*Picea sitchensis*).

lifetime of Sitka spruce leaves (Figures 2.21b,c,e). Therefore the wax tubes do not spontaneously degrade or convert to other forms during physiologically relevant periods of time.

Secondary alcohols in plant waxes may be expected to show long-term chemical stability in the natural environment, although Jetter *et al.* (1996) demonstrated that 10-nonacosanol can be oxidised by strong oxidants such as nitrogen dioxide (NO₂) to yield the corresponding ketone, 10-nonacosanone and ultimately alkanic acids that may not have tubular crystal morphologies. Although much higher NO₂ concentrations are required than are experienced in the natural environment, the possibility exists that a chemical transformation of this major structure-determining component of the wax may indeed provide the long-sought explanation for the observed degradation of the tube-crystals on the long-lived leaves of conifers growing in polluted environments. The required acceleration of the reaction at physiologically relevant NO₂ concentrations may turn out to be accounted for by interactions with other factors in the environment, such as water or particulate matter.

2.11 Summary of cuticle ontogeny

Summarising the process of development of plant cuticles, it is evident that an amorphous procuticle (Figures 2.3a, 2.22a), positioned outside the CW of the earliest epidermal surface, becomes flocculent in appearance (Figures 2.3b, 2.22b) and differentiates into the CP (Figures 2.3c, 2.6b and 2.22c). The CP is most often lamellate, but may appear amorphous (Figures 2.2a–g; Figures 2.22c–g, left). The early CP may be subtended by a pectinaceous layer or pectin lamella (Figures 2.6a,b) bonding it to the PCW. Quite rapidly, before leaves are fully expanded, the cutin of the CP may be converted to the non-saponifiable polymer cutan without much, if any, detectable change in ultrastructure. In most species, cutinised layers of the CW begin to develop by impregnation of successive CW layers with cutin polymer. During the impregnation phase, globules of electron-dense cutin precursors enclosed in an electron-lucent lamella accumulate at the base of the CP and appear to fuse laterally first at the surface of the pectic lamella and PCW, and later within successive layers of SCW (Figures 2.6c,d, 2.22e). During the phase of CW cutinisation and at

(**Figure 2.21**) (a) The completed epicuticular wax of a fully expanded leaf, showing the fully developed antitranspirant wax plug in pristine condition. (b,c) Images of the plug structure in a four-year-old leaf, showing (b) tubular structure retained in the centre of the plug. (c) A view of the base of the plug in a four-year-old leaf as seen looking up through the stomatal aperture. The vertical mid-line coincides with the stomatal aperture. (d,e) A vertical cryo-fracture through the wax plug and stomatal complex showing (d) the plug is attached to the accessory cell walls, but not to the guard cells, allowing them to move (e) uniform structure of the intermeshed wax tubes. (f) A dense biofilm of epiphytic algae and fungal hyphae forming a surrogate abaxial (upper in spruce) leaf surface. (a) Bar = 10 μm; (b,c) bar = 1 μm; (d) bar = 5 μm; (e) bar = 3 μm; (f) bar = 100 μm. Figures 2.21a–f by C.E. Jeffree, Figure 2.21d from van Gardingen *et al.* (1991), *Plant, Cell and Environment*, **14**, 185–193.

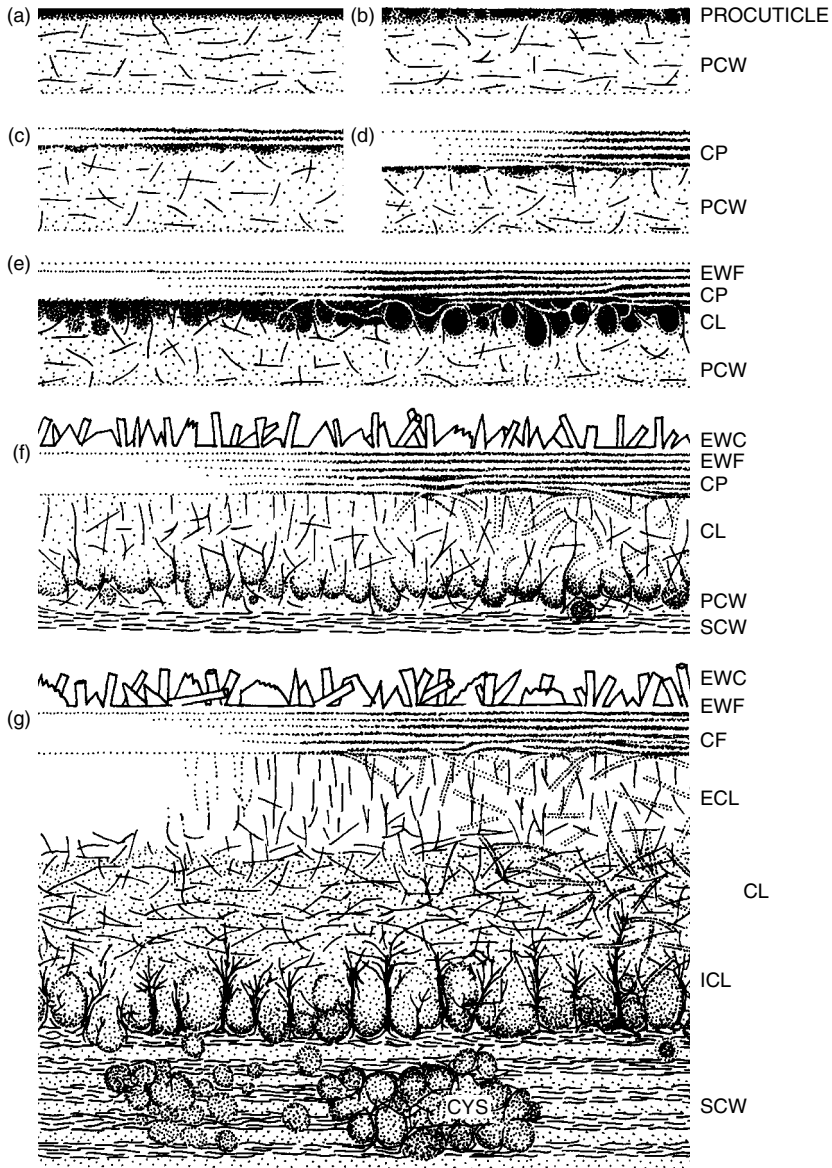


Figure 2.22 Summary diagrams of the development of the plant cuticle. (a) A uniformly electron-dense procuticle is present on the surface of the primary cell wall (PCW). (b) In the procuticle–cuticle-proper (CP) transition phase, the procuticle becomes flocculent or globular in appearance and (c) is converted to a simple electron-lucent amorphous or lamellate CP positioned outside the cell wall, subtended by a pectinaceous lamella. (d) Further construction of the amorphous or lamellate CP may involve accumulation of electron-dense globular lipids in the PCW beneath the CP. (e) The CP/CL (cuticular layer) transition. Within the primary cell wall polysaccharide matrix, globules of electron-dense lipids coated with electron-lucent shells construct the CP and the CP/CL transition zone.

maturity, the CL may contain either paradermal or more random lamellate structure closest to the CP, but lamellae may extend to any depth within the CL (Figure 2.22g). The lamellae apparently derive from the electron-lucent envelope that encloses the electron-dense globules. Soluble epicuticular lipids begin to accumulate and crystallise at the CP surface throughout the process of cutinisation (Figures 2.11a–d) while the leaf is still not fully expanded. The transport of wax to the surface occurs across the continuously lamellate or amorphous CP pores without disturbing the ordered appearance, and no pores can be detected by microscopy. The outer CL layers (ECL) undergo maturation of the polymer matrix (Figures 2.6e–h, 2.2g, 2.4a–e), involving intensive cross-linking of the existing cutin and its progressive conversion, together with any embedded polysaccharide to the aliphatic polymer cutan, which may be impregnated with SCL. Within thick SCW, further accumulation and accretion of CL-like material may occur at locations detached from the main CL deposit (Figures 2.1c and 2.2g) as cutin cystoliths, which may range from the basic size of the pre-cutin globules to several hundred nanometres in diameter. Later stages of development of the CW may involve deposition of inorganic material (silica and calcium salts) in and beneath the CL (Postek, 1981; Davis, 1987; Tenberge, 1989, 1992), and progressive lignification of the underlying SCW (as exemplified by *Picea*; Tenberge, 1989, 1992).

The EW layer is seen as forming a continuous basal film covering all of the cuticle surface that may be crystalline even if the crystallites cannot be resolved by microscopy. Crystallites of wax of various morphologies may emerge from the basal wax layer. They are capable of growth at their distal tips and edges, though other possibilities are not discounted. Crystallites form by self-assembly,

(**Figure 2.22**) The globules appear to assemble in situ beneath the CL, and are not observed traversing the PCW or SCW (secondary cell walls). Lamellae may become less regular within the PCW. An apparently amorphous epicuticular wax film is now present on the surface of the CP, which may be terminated by a single, continuous electron-lucent lamella. (f) Incorporation of PCW in the CL. The pectin lamella is no longer present. Reticulations are light in the outer CL and predominantly radial, reaching as far as the base of the CP. Lamellae may be absent, or occur to any depth and orientation within the CL, which is globular at its base. Epicuticular wax crystals begin to form before cell expansion has ceased. Thickness of the CP is maintained during rapid cell expansion. (g) Following the cessation of cell expansion, external and internal layers of the CL (ECL and ICL) may become chemically and structurally distinct. In the ECL, electron density of the matrix may diminish, and the boundary with the CP may lose contrast. Polysaccharide fibrils marking the CP/ECL boundary may also disappear. Reticulate polysaccharide formed in the PCW/SCW transition is usually more massive, denser and more three-dimensional than in the ECL. Crystalline epicuticular wax layer is well developed. In the mature cuticular membrane, the CP may be amorphous or any grade from weakly to strongly lamellate. The ECL may be non-reticulate, or contain reticulum throughout. The CL may contain lamellae extending to any depth from the CP. Cutinisation of the SCW may occur at points detached from the main body of the CM, forming cutin cystoliths (CYS). This developmental scheme, and variations on it, particularly with respect to the thickness of the various layers, appears to apply to the cuticles of all land plants. Further elaborations in mature cuticles include the impregnation of the CL with additional materials such as silica, calcium oxalate or lignin.

their morphology determined by their chemical composition. Individual crystallites may in some instances be pure crystal domains of an individual constituent of the EW. In other instances particular combinations of two or more compounds may collaborate to form a given crystal morphology. The orientation of wax crystallites may be controlled by their mutual interaction and by epitaxial growth on a CP in which the polymer structure has become aligned by stretching during cell expansion.

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3 The cutin biopolymer matrix

Ruth E. Stark and Shiyong Tian

3.1 Introduction: protective plant polymers

The leaves, fruits, and primary stems of higher plants are covered by a cuticular membrane that occupies approximately the outer 0.1–10 μm of the aerial plant surface. Chemically, the membrane consists of a variety of waxes that serve as waterproofing and the biopolyester cutin that functions as a densely networked structural support (Figure 3.1). As detailed in Chapter 4, the waxes are organized as both epicuticular lipids coating the outer surface of the cuticle and intracuticular lipids embedded within the cutin matrix. The cuticle controls the interactions of the plant with the environment, protects against water loss (Chapters 8 and 9), functions as the plant's primary protective barrier against pathogenic attack (Chapters 12 and 13), and is thought to be necessary for plant organ development (Chapter 10; Kolattukudy, 1980, 1984; Baker, 1982; Holloway, 1982; Kolattukudy and Espelie, 1989; Walton, 1990; Sieber *et al.*, 2000; Heredia, 2003). Moreover, the ω -hydroxy fatty acid monomeric constituents of cutin are thought to be involved in plant–pathogen interactions (Chapter 12; Heredia, 2003). In terms of agricultural impact, the breakdown of cuticular membranes by bacterial and fungal microorganisms contributes to an estimated 20% annual loss from crop damage worldwide (Oerke *et al.*, 1994).

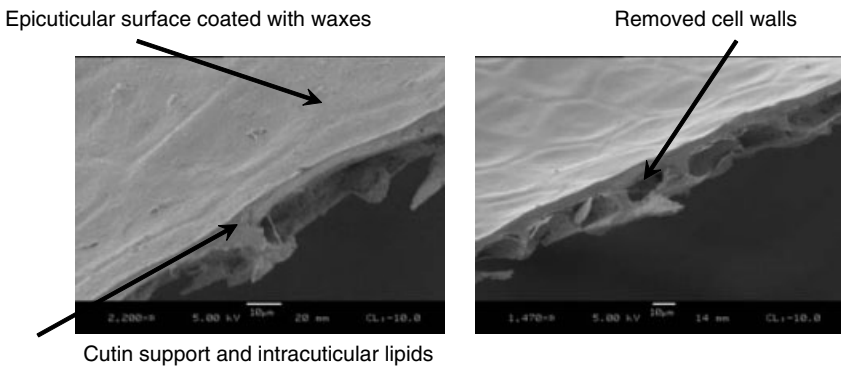


Figure 3.1 The architecture of the cuticular membrane and spatial organization of cuticular lipids, as illustrated by scanning electron micrographs of tomato fruit cuticle before and after dewaxing (left and right panels, respectively).

Among the protective plant polymers, cutin is distinguished from cutan, suberin, and lignin by several criteria. Lignins are the dominant biopolymers in vascular plants and woody tissues where their accumulation enhances the structural integrity of cell walls (Davies and Lewis, 1992); suberins share some protective and waterproofing functions with cutins but are found in underground roots, in cork, and at wound-healing tissue surfaces. Cutin is the polymeric support for membranes that cover the aerial parts (leaves, fruits, primary stems) of higher plants, whereas cutan refers to the resistant polymeric residue from dewaxing and saponification treatments (Boom *et al.*, 2005). Whereas lignin and suberin have substantial phenylpropanoid character, both cutin and cutan are primarily aliphatic polymers containing long methylene chains.

3.2 Biosynthesis

Both the biosynthesis of cutin monomers within cells and their extracellular assembly to form the cutin biopolymer have been the subject of longstanding study. Through studies of radio-labelled precursors and intermediates, the biochemical pathways for both C₁₆ and C₁₈ monomers were established (Figure 3.2). For instance, it was shown in *Vicia sativa* that oleic acid undergoes ω -hydroxylation and enantioselective epoxidation of the double bond and then hydroxylation of the latter functional group (Kolattukudy, 2001). Catalysis of the proposed transformations was shown to involve an epoxide hydrolase and a cytochrome P450-dependent ω -hydroxylase (Pinot *et al.*, 1992, Heredia, 2003). It has been three decades since the assembly of these monomers to form the cutin biopolymer was shown to be catalyzed by enzymes associated with epidermal cells and required both ATP and CoA (Kolattukudy, 1984), but the putative hydroxyacyl-CoA, cutin transacylase, that accomplishes the transformations has proven elusive. More recently, a new cutin acyltransferase and its corresponding gene from *Agave americana* leaves have been described in a preliminary report (Reina and Heredia, 2001).

3.3 Monomer composition

The monomeric constituents isolated from both leaf and fruit cutins consist predominantly of saturated hydroxylated aliphatic acids, usually a mixture of C₁₆ and C₁₈ homologues. In some cutins a single chain length is predominant: C₁₆ in tomato fruit (*Lycopersicon esculentum*) and broad bean leaf (*Vicia faba*), C₁₈ in spinach leaf (*Spinacia oleracea*). Other species such as apple fruit (*Malus pumila*) include primarily unsaturated C₁₈ monomers, similar to the suberin constituents of potato tubers and tree bark. No cutins have been detected in algae or fungi; cutins that lack C₁₈ monomers are in general characteristic of gymnosperms and lower plants. Surprisingly, similar chemical constituents may predominate in plants that belong to very different families.

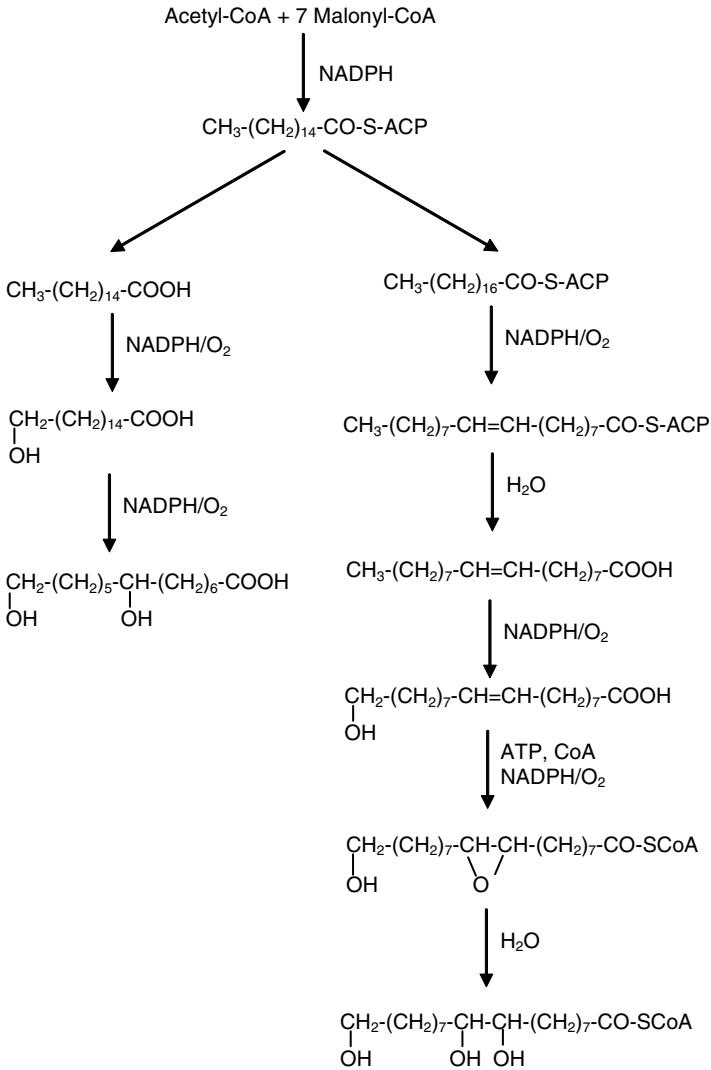


Figure 3.2 Biosynthetic pathways for the formation of C₁₆ and C₁₈ hydroxyacid families of cutin monomers. Adapted from Kolattukudy, 1984.

In both C₁₆ and C₁₈ acids ω-hydroxylation is very common, and additional hydroxy or epoxy substituents are often present at midchain position(s). For instance, common monomers include 9,16- and 10,16-dihydroxyhexadecanoic acids and 9,10-epoxy-18-hydroxyoctadecanoic acids. Moreover, aldehyde, ketone and carboxyl groups have been reported as additional substituents and chain lengths up to 20 have been observed (Holloway, 1982; Ray *et al.*, 1995; Tian, 2005). Small quantities of coumaric and ferulic acids have also been reported (Baker *et al.*, 1975;

Riley and Kolattukudy, 1975), and both glycerol and a series of glyceryl esters have been found recently in cutins from diverse leaf and fruit cuticles (Graca and Pereira, 2000; Graca *et al.*, 2002).

Cutin content may vary with the membrane thickness of the plant. Amounts range from approximately $10 \mu\text{g cm}^{-2}$ for membranes $0.1 \mu\text{m}$ thick to approximately $500 \mu\text{g cm}^{-2}$ for membranes $5 \mu\text{m}$ thick; typically 40–80% by weight of the cuticle consists of cutin (Kolattukudy, 1980; Walton, 1990; Heredia, 2003). Fruit cuticles in particular may exceed these amounts, for example, some apple cultivars can contain 1.5 mg cm^{-2} at maturity. Cutin chemical composition within a given plant can vary with organ (leaf or fruit) and location (adaxial or abaxial surfaces of the same leaf). Although many investigators have found only minor differences in cutin composition as leaves and fruits develop, others have noted the dominance of one or the another monomer at different stages of maturity, for instance, in *Vicia faba* leaves and *M. pumila* fruits (Holloway, 1982).

Although the trends above are well established, several pitfalls in the determination of cutin monomer composition should be noted. The typical chemical degradation reagents (methanolic KOH, LiAlH_4 , $\text{BF}_3\text{-CH}_3\text{OH}$, etc.) produce soluble products but also often leave behind at least 20% by weight of nondepolymerized residue, so that the resulting soluble monomers specify cutin composition in an incomplete and potentially misleading manner. For instance, it has been proposed that nonester cutin structures or heavily cross-linked regions are resistant to chemical degradation (Riederer and Schönherr, 1988; Ray *et al.*, 1998). Conversely, incomplete dewaxing of the cutin may lead to identification of monomers that do not come from the biopolymer at all. Moreover, the combination of gas chromatography and electron-ionization mass spectrometry that became customarily available in the 1970s to identify the soluble compounds may fail for species that are nonvolatile or prone to fragmentation (Holloway, 1982, 1984; Kolattukudy, 1984).

3.4 Polymeric structure of intact cutin

Plant cutins were identified as polyesters at least three decades ago based on their susceptibility to fungal degradation by hydrolytic enzymes (Kolattukudy, 1984) and consistent with the ester-forming ability of hydroxyacids. Even without direct evidence from oligomers or mature polymers, the fact that half of the C_{16} monomers contain a free midchain hydroxyl group suggests that they are involved in either cross-linking or branching (Holloway, 1982; Kolattukudy, 1984). Moreover, it has been suggested that ether bonds, serving as possible cutin cross-links, could protect plants from fungal invasion (Blée, 2002). Nonetheless, even the most complete information on monomer composition falls short of determining the polymeric architecture that is a likely key to the function of the cutin as a protective plant membrane.

For intact (insoluble) cutins, chemical functionalities may be identified using solid-state nuclear magnetic resonance (NMR) or infrared (IR) spectroscopies.

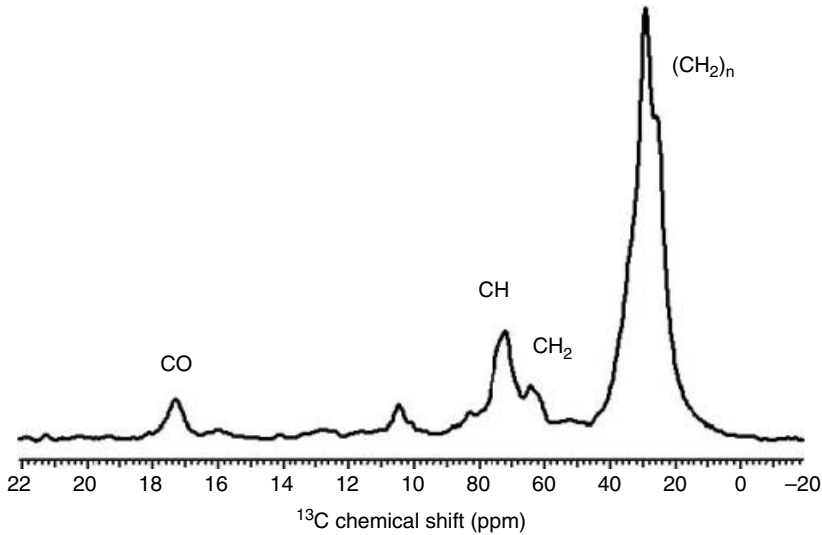


Figure 3.3 75 MHz cross polarization-magic-angle spinning (CPMAS) ^{13}C NMR spectrum of dry dewaxed tomato fruit cutin, obtained with an 8-kHz spin rate and referenced to tetramethylsilane via external hexamethylbenzene. The major NMR resonances are identified with the designated carbon functionalities in the biopolymer structure. From Batteas and Stark, 2005, by permission from Woodhead Publishing.

For instance, the cross polarization-magic angle spinning (CPMAS) ^{13}C NMR spectrum (Schaefer and Stejskal, 1979) of tomato fruit cutin (Figure 3.3) contains resonances from chain methylenes (30 ppm), CH_2O and CHO groups (64 and 72 ppm), and carboxyl groups (168–173 ppm), where the assignments are made provisionally from reference data on similar chemical compounds. Additional aromatics and/or olefinic signals appear at 105–150 ppm in the spectrum of lime (*Citrus aurantifolia*) fruit cutin (Zlotnik-Mazori and Stark, 1988), where the relative numbers of various carbon types have also been determined from NMR spectra acquired under different cross-polarization and decoupling conditions (Table 3.1). In both cases, the structural heterogeneity and amorphous nature of the biopolymers limit the resolution of the rather broad spectral lines, though cross-link sites may be identified because their motional restriction produces characteristically inefficient spin relaxation in the solid state. In a complementary fashion, Fourier transform infrared (FTIR) spectra also serve to identify and quantify molecular groupings in bulk cuticular samples (Luque *et al.*, 1995; Ramirez *et al.*, 1992). For instance, a recent report used this methodology to demonstrate augmented cross-linking in mature (ripe) tomato fruit cuticles (Benitez *et al.*, 2004).

The spectral assignments described above should be viewed as limited: as noted, they are made simply by analogy with similar chemical compounds; moreover they can only identify small molecular moieties. These shortcomings have been addressed using high-resolution magic-angle spinning (HR-MAS), a hybrid NMR technique

Table 3.1 Chemical composition and dynamics of lime fruit cutin from solid-state NMR¹

Carbon type	% Mobile ²	%Rigid ³	T ₁ (C) for rigid carbons (ms)
(CH ₂) _n	45	32	145
CH ₂ OCOR		1.7	122
CHOCOR		7.1	>7000
Aromatics, alkenes		3.8	~1000
CH ₂ O _C OR		0.9	~100
CHOCOR		4.9	~1700
C=O		0.7	

¹ Adapted from Zlotnik-Mazori and Stark, 1988 and Garbow and Stark, 1990.

² From comparison of integrated intensities for spectra with direct-polarization/low-power decoupling and cross-polarization/high-power decoupling (zero contact time), both acquired with magic-angle spinning.

³ From extrapolation of cross-polarization ¹³C NMR signal intensities to zero contact time.

in which solid samples are swelled in organic solvents to enhance molecular mobility and slow MAS produces high-resolution spectra (Keifer *et al.*, 1996; Millis *et al.*, 1997). It may then be possible to confirm directly or remotely bound ¹H–¹³C pairs within a functional group, establish covalent connectivities between monomer units, and discern which groupings are close to one another in space. This approach is illustrated for lime fruit cutin in Figure 3.4, where the MAS-assisted 2D ¹H–¹³C correlation (HMQC) NMR spectrum of the intact lime cutin polymer displays resolution approaching the soluble monomers and oligomers (discussed later) and reveals directly bonded C–H pairs that discriminate between resonances of, for example, CH₃, (CH₂)_n, CH₂C=O, and CH₂O groups in the various materials (Hurd and John, 1991; Fang *et al.*, 2001; Tian, 2005). Moreover, HMQC in conjunction with 2D ¹H–¹H Total Correlation Spectroscopy (TOCSY) has provided evidence in tomato fruit cutin for α -branched carboxylic acids and esters, midchain (secondary) alcohols, and olefinic groups (Deshmukh *et al.*, 2003). Thus, in addition to offering a direct assessment of cutin molecular structure, the HR-MAS NMR strategies confirm the presence of both expected monomeric building blocks and a small number of novel cross-linking elements that are likely resistant to chemical degradation.

3.5 Molecular structure of cutin fragments

3.5.1 Oligomeric degradation products

A complementary approach to understanding the protective functions of fruit cuticular polymers stakes out a middle ground between the identification of cutin hydroxylated fatty-acid building blocks from depolymerization products (Kolattukudy, 1984) and the examination of chemical functionalities and cross-link

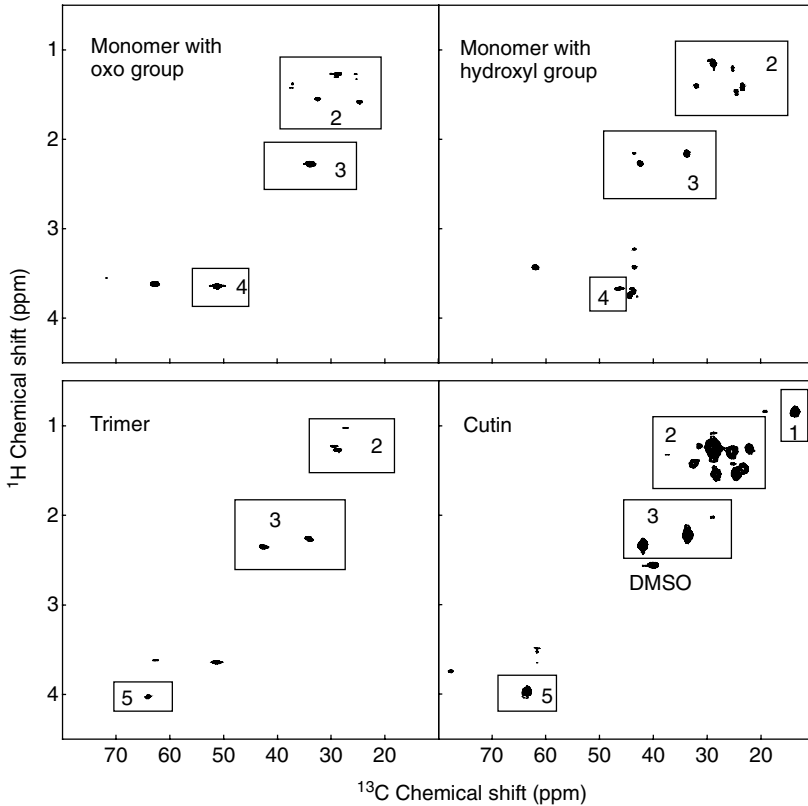


Figure 3.4 600 MHz MAS-assisted two-dimensional NMR of lime fruit cutin and its molecular building blocks, illustrating the characteristic spectral signatures of each material dissolved or swelled in DMSO at 50°C (Tian, 2005). These ^1H - ^{13}C heterocorrelated multiple-quantum coherence (gHMQC) data were obtained with 2-kHz spinning and referenced to tetramethylsilane. The spectral peaks in boxes 2, 3, and 5 of the contour plots signify single-bond interactions for three chemically distinct types of methylene groups.

constraints for intact materials: mild enzymatic and chemical procedures are used to produce soluble oligomers that nevertheless retain many essential covalent linkages within the cuticular support structure. The first report of such results (Osman *et al.*, 1995) used mild alkaline hydrolysis and proposed a primary ester of two ω -hydroxyhexadecanoic acid molecules but noted that dimers and higher oligomers are difficult to confirm by mass spectrometry (MS) because of their low abundance and modest volatility. In our laboratory, commercial lipases, iodotrimethylsilane, low-temperature HF, and methanolic KOH have each been used along with HPLC separations to generate oligomeric fragments from lime fruit cutin (Ray and Stark, 1998; Ray *et al.*, 1998; Stark *et al.*, 2000; Fang *et al.*, 2001). In addition to the need for specialized MS methodologies, all of these studies confront challenges that include crude product mixtures in which monomers dominate but the numerous

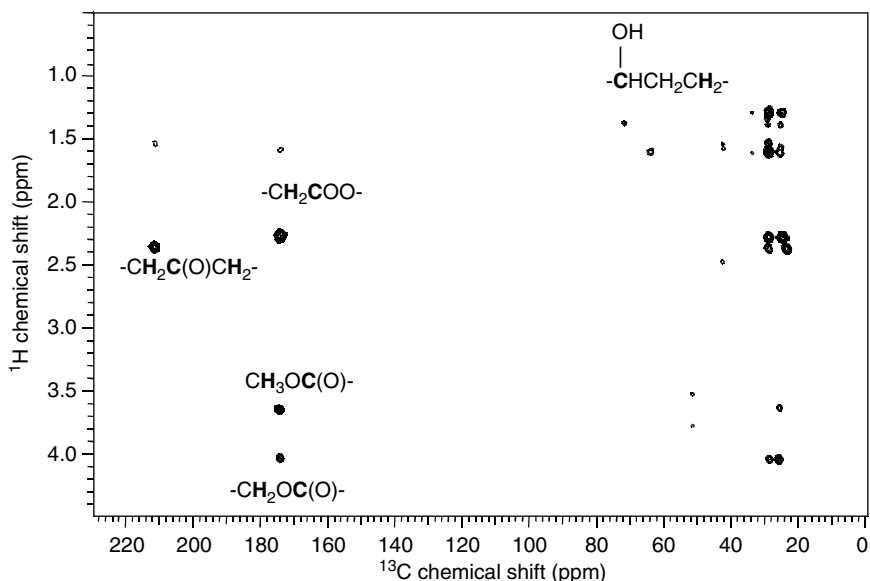


Figure 3.5 600 MHz two-dimensional NMR of a soluble lime fruit cutin trimer (**7**) from KOH hydrolysis, containing two units of 10,16-dihydroxyhexadecanoic acid and one unit of 10-oxo-16-hydroxydecanoic acid (Tian, 2005). The contour map from this ^1H - ^{13}C gradient-assisted heterocorrelated multiple-bond correlation (gHMBC) experiment (Rinaldi and Keifer, 1994) primarily displays three-bond interactions between carbon–proton pairs.

information-rich oligomers are rare, similar polarities that make separation of the various oligomers difficult, and functional groups that lack chromophores suitable for UV detection.

The NMR and MS spectroscopic identification protocols used for our lime cutin oligomers are illustrated in Figures 3.5 and 3.6. In contrast to the MAS-assisted 2D ^1H - ^{13}C correlation (gHMQC) NMR strategy described earlier, ^1H - ^{13}C multiple-bond correlation spectroscopy (gHMBC; Rinaldi and Keifer, 1994) carbon–hydrogen pairs identifies that are separated by three or four chemical bonds, making it an invaluable tool for the definitive identification of nonprotonated ester carbons and connections between monomer units. Either electrospray or atmospheric pressure chemical ionization MS (Smith, 1999) yield the molecular weight of each purified oligomer, confirming information from ^1H NMR integrations. The order in which the monomers are connected can occasionally be determined from fragments obtained by MS, and the presence of mixtures containing both C_{16} and C_{18} units may be deduced.

Whereas a unique pentamer linked exclusively by secondary esters (**1**) (Figure 3.7) was obtained from reaction of lime fruit cutin with porcine pancreatic lipase, most of the isolated oligomers have primary ester linkages and midchain hydroxyl or oxo groups; all possible combinations of these units are found in the dimers and trimers (Figures 3.7 and 3.8). Although it is possible to obtain more and

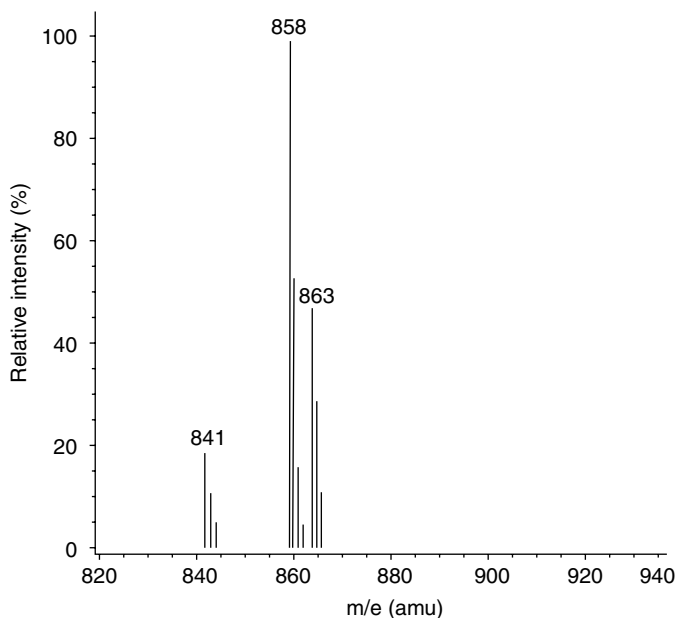


Figure 3.6 ESI/MS spectrum of **7**, showing molecular ions corresponding to $M + \text{NH}_4^+$ (m/e 858), $M + \text{Na}^+$ (m/e 863), and $M + \text{H}^+$ (m/e 841) (Tian, 2005). The ^{13}C isotopic peak intensity is about 55%, consistent with a trimer structure containing 49 carbons.

larger oligomers using an apparatus that permits quenching of the degradative reaction (Tian, 2005), the resulting products are numerous and similar in polarity, thus difficult to purify in amounts sufficient for full structural characterization. Only the products of alkaline hydrolysis have been found to contain any secondary hydroxyl groups or secondary esters, despite prior reports of such functional groups in intact cutin from lime and tomato fruits, respectively (Zlotnik-Mazori and Stark, 1988; Deshmukh *et al.*, 2003). The overall scarcity of such connectivities among all oligomers could stem from either light cross-link density or inefficient hydrolysis of the densest polymeric regions.

3.5.2 Polymeric residues from chemical degradation procedures

After exhaustive degradation of leaf cutin by alkaline hydrolysis or transesterification there remains a cutan residue thought to consist of cutin monomers connected by nonester bonds. Application of a variety of physical methods has suggested that this nonsaponifiable material contains an ether-linked network of methylene chains, double bonds, and carboxyl groups (Villena *et al.*, 1999). However, treatment of lime fruit cutin with iodotrimethylsilane or with HF at low temperature leaves behind unreacted residues in which esters of secondary alcohols are evidenced by resonances in their respective NMR spectra (data not shown).

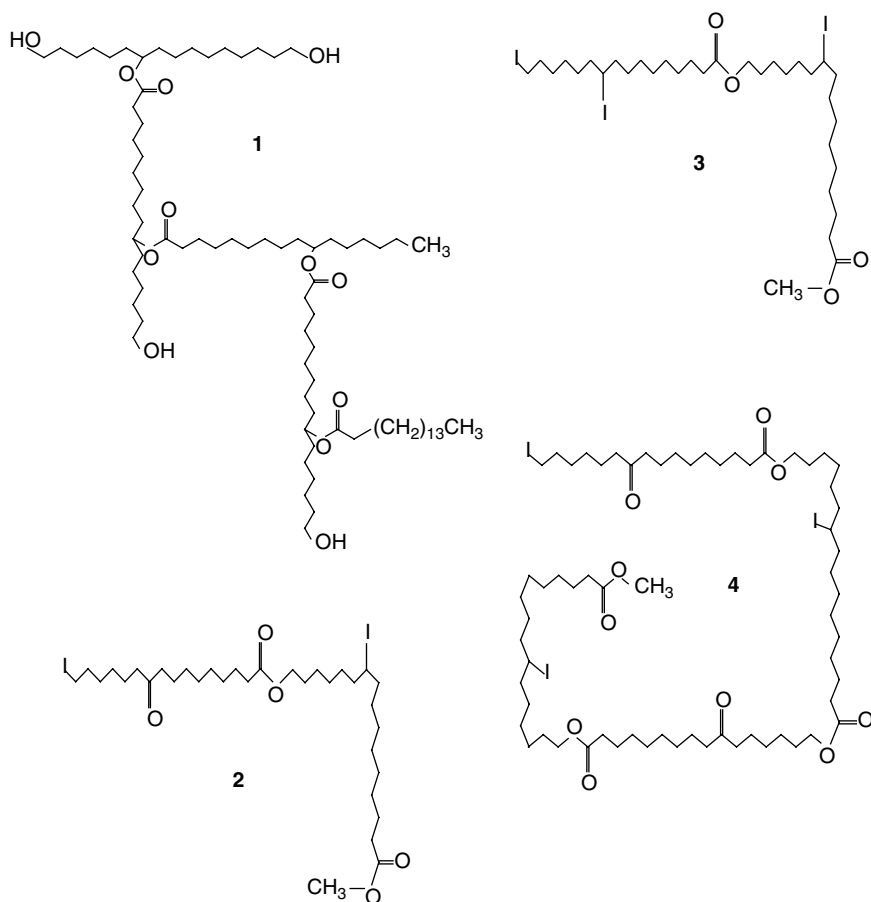


Figure 3.7 Oligomers obtained from partial degradation of lime fruit cutin with porcine pancreatic lipase (**1**, Ray and Stark, 1998) and with iodotrimethylsilane (**2–4**, Ray *et al.*, 1998), respectively.

3.6 Mechanical properties

3.6.1 Methodology

As for synthetic polymeric materials, it is expected that the mechanical behavior of plant cuticular biopolymers will influence adsorption, diffusion, and cracking – with practical agricultural consequences for the foliar application of chemicals, control of water loss, and maintenance of fruit appearance, respectively. Various physical methods offer complementary views of the surface and bulk mechanical properties of plant cuticular membranes. As discussed in Chapter 1, atomic force microscopy (AFM) provides the opportunity for *in situ* characterization of the surface – regarding both topology and mechanical response of plant tissues – but it lacks true chemical

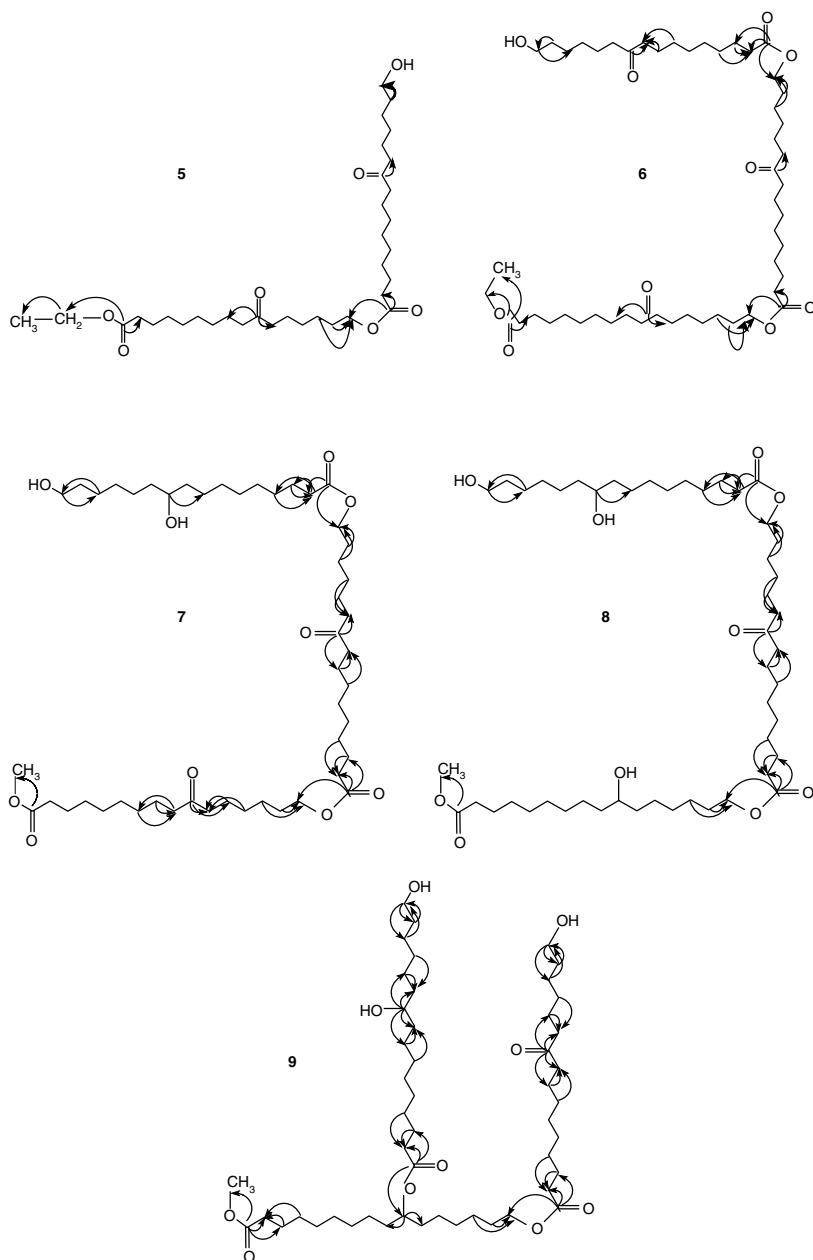


Figure 3.8 Oligomers obtained from partial degradation of lime fruit cutin: a dimer (**5**, Fang *et al.*, 2001) and a trimer (**6**) from low-temperature HF treatment; trimers esterified through primary alcohols (identified provisionally as **7** and **8**); a trimer from methanolic KOH that displays mid-chain esterification through a secondary alcohol (**9**) (Tian, 2005). Arrows designate ^1H - ^{13}C connectivities deduced from HMBC NMR experiments.

specificity. Applying NMR provides only average (bulk) properties but can reveal detailed information on molecular composition and architecture (described earlier) and offer insight into the dynamics of the component molecules of plant tissues, which may be related in turn to the bulk mechanical behavior of the system. Both of these techniques can complement and extend the stress-strain approaches reported by Petracek and Bukovac (1995), as illustrated for tomato and lime fruits later.

Although AFM is becoming well established as a tool to provide the three-dimensional nanoscale architecture of biological materials directly under physiological conditions (Kirby *et al.*, 1996; Mechaber *et al.*, 1996; Round *et al.*, 2000; Batteas and Stark, 2005) its different imaging modes also yield mechanical parameters such as surface viscoelastic response. In contrast to bulk rheology, which reflects elastic response parallel to a membrane, the AFM tip in a nanoindentation experiment senses the mechanical resistance to deformation in a direction perpendicular to the surface, the route taken by many pathogens. The Hertz model of contact mechanics (Hertz, 1882) is then used to deduce the Young's modulus of elasticity from plots of resistive force versus indentation distance, plotted for both the sample and an ideal hard surface (silicon). Alternatively, lateral force microscopy probes the torsional response of a cantilever as it is scanned over the surface of interest, reflecting both tip-sample adhesive interactions and shear stress. Finally, surface viscoelasticity may be examined by setting the modulation amplitude and frequency of the AFM tip against a surface and then mapping changes in these settings with position.

Whereas the NMR spectra described earlier for solid cutin polymers and the corresponding monomers and oligomers in solution can identify chemical groupings and establish molecular architecture, it is NMR spin relaxation that adds complementary molecular-level detail to AFM-based assessments of cuticular surface mechanical properties. Spectral linewidths and spin-relaxation rates reflect supra-molecular organization and site-specific polymer dynamics on several timescales, both of which serve to link molecular structure and bulk mechanical properties (North, 1975; Schaefer and Stejskal, 1979; Bovey and Mirau, 1996). For instance, ^1H NMR linewidths in a two-dimensional wide-line-separation (WISE) experiment (Schmidt-Rohr *et al.*, 1992) measure the extent of motional narrowing for each carbon type, whereas rotating-frame relaxation rates probe cooperative motions and cuticular resiliency as well as the organization of cutin and wax domains (Schaefer and Stejskal, 1979; Garbow and Stark, 1990).

3.6.2 *Measurements of surface elastic modulus*

In order to examine how the elasticity of the cuticular membrane changes as a function of environmental variables such as humidity, force-distance measurements were conducted on dewaxed and native tomato cuticle samples under controlled hydration conditions (Round *et al.*, 2000). The resulting elastic moduli (resistances to deformation) are summarized in Table 3.2. For dry samples, the lipid-covered cuticle surface has an elastic modulus five times smaller than the dewaxed cutin

Table 3.2 Surface elastic moduli of tomato fruit cuticles from atomic force microscopy

Relative humidity	Dewaxed cuticle (E, MPa) ¹	Native cuticle (E, MPa)
0%	32 ± 11	6.2 ± 1.7
30%	9.2 ± 1.5	—
60%	5.5 ± 1	—
Water	5.8 ± 2	5.7 ± 2.5

¹ From Round *et al.*, 2000.

polymer. Whereas the modulus of cutin drops fivefold upon hydration (Round *et al.*, 2000), the elasticity is invariant to hydration state for native cuticle. There are several plausible explanations for this last observation: AFM does not sense the underlying polymeric support; wax mitigates the effects of hydration on the cuticle; or the moduli cannot be determined precisely for heterogeneous materials such as lipid-covered cuticular surfaces.

As reported previously for amorphous synthetic polymers (Galuska *et al.*, 1997; Lee *et al.*, 1997), AFM-based lateral force measurements also provide a means to evaluate the impact of temperature on the mechanical properties of the cuticular surface *in situ* (Round, *et al.*, unpublished observations). The ‘drag’ of the AFM tip as it slides across the surface is found to increase with temperature as described previously (Batteas and Stark, 2005). The onset temperature of these changes (approximately 28°C) is consistent with a phase transition observed previously at 30°C for extracted epicuticular lipids (Luque and Heredia, 1997), though the surface becomes too soft above 45°C to make additional AFM measurements.

3.6.3 Measurements of bulk molecular dynamics

NMR relaxation measurements on intact lime and tomato fruit cuticles have also proven to be a rich source of molecular-level dynamic information, which is linked in turn to (bio)polymer mechanical properties (North, 1975) and complementary to determinations of bulk and surface rheology. Each of the major polyester functional groups identified in the CPMAS ¹³C solid-state NMR spectrum of dry tomato cutin (Figure 3.3) may be characterized in terms of its molecular motion. One measure of the dynamic properties of the various carbon segments uses the WISE experiment described earlier (Schmidt-Rohr *et al.*, 1992) and illustrated in Figure 3.9. Chain methylenes resonating at 30 ppm have motionally narrowed lines as compared with ester-linked methylenes at 72 ppm; even among the bulk methylene groups the ¹H linewidths discriminate between mobile and rigid components that may correspond to chains and cross-links, respectively.

In parallel with the surface elasticity determinations made under stress conditions involving hydration, abrasion, and temperature, NMR relaxation has been used to understand the AFM trends in terms of dynamic behavior at specific molecular

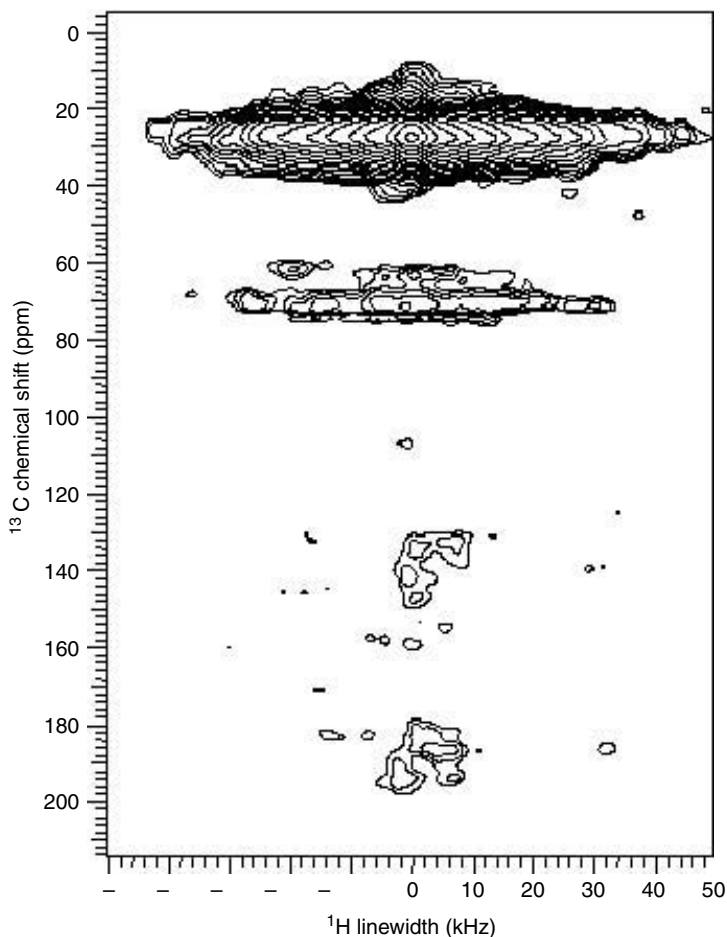


Figure 3.9 Contour plot from a 2D ^1H - ^{13}C WISE NMR spectrum of dry tomato fruit cutin. The chain methylenes (30 ppm ^{13}C) exhibit superimposed ^1H spectral patterns with linewidths of 5 and 35 kHz, whereas the ester-linked methylenes (72 ppm ^{13}C) have a corresponding ^1H linewidths of ~ 25 kHz.

sites of the cutin biopolymer. Table 3.3 shows how the linewidths and spin relaxation rates vary with relative humidity and temperature for both dewaxed and native tomato cuticle samples. Upon hydration of the dewaxed samples, both the diminished linewidths of the $(\text{CH}_2)_n$ resonances and greater proportion of the narrow spectral component indicate enhanced segmental motions at rates ≥ 50 kHz, linked previously to a corresponding decline in the resistance to deformation (North, 1975). An analogous trend of increasing segmental motions with rising temperature is evident from the ^{13}C spin-lattice relaxation rates. These motional trends are also in accordance with the drops in surface and bulk moduli observed with AFM and rheological methods. Moreover, even though water may displace hydrogen-bonded

Table 3.3 Flexibility of tomato fruit cuticle from solid-state NMR spectral measurements

Conditions	Dewaxed cuticle			Native cuticle		
	Room Temperature ^1H Linewidth (kHz) ¹					
	Broad component	Narrow component	% Narrow	Broad component	Narrow component	% Narrow
Dry (0% RH)	38.2 ± 3.8^2	5.3 ± 0.5^2	9 ± 2^2	30 ± 5	5.5 ± 0.5	25 ± 5
Saturated	31.3 ± 3.1^2	4.7 ± 0.5^2	18 ± 4^2	27 ± 5	5.0 ± 0.5	15 ± 3
		Room temperature $\langle R_1(C) \rangle$ (sec ⁻¹) ³				
	Cutin (CH ₂) _n	Wax (CH ₂) _n		Cutin (CH ₂) _n	Wax (CH ₂) _n	
Dry (0% RH)	3.6 ± 0.1	—		3.2 ± 0.2	3.0 ± 0.2	
Saturated	3.68 ± 0.02	—		1.5 ± 0.2	3.1 ± 0.1	
Ambient (50% RH)		Variable temperature $\langle R_1(C) \rangle$ (sec ⁻¹) ⁴				
253 K	3.00 ± 0.2					
263 K	3.38 ± 0.2					
273 K	3.66 ± 0.2					
283 K	3.86 ± 0.2					

¹ Proton linewidths corresponding to the bulk methylene groups resonating at 30–35 ppm.

² From Round *et al.*, 2000.

³ $\langle R_1(C) \rangle \equiv 1/\langle T_1(C) \rangle$ is the average spin-lattice relaxation rate for each carbon type. Values were derived from the short-time behavior (10–320 ms) of ^{13}C magnetization observed after a CP inversion-recovery pulse sequence (Torchia, 1978). The line broadening used to apodize the NMR data was reduced to 15 Hz in order to resolve cutin and wax resonances at 30 and 33 ppm, respectively.

⁴ Values of $\langle R_1(C) \rangle$ were determined as described above using a nominally identical tomato cutin sample.

cross-links within the cutin polyester, the NMR results show that remote chain segments become more flexible in hydrated tomato cutin. But as deduced from AFM measurements, no water-induced plasticizing effects are evident in the NMR data for native cuticles; in fact, both the proportion of narrow ^1H components and rates of ^{13}C spin-lattice relaxation evidence less efficient segmental dynamics for the chain methylenes. Physically, it is likely that strong acyl-chain interactions between the cutin and wax constituents both block the plasticizing effects of water and attenuate the chain segmental motions.

3.7 Thermodynamic properties

As noted above, phase transitions have been monitored for cuticular lipids as well as intact cuticle (Luque and Heredia, 1997; Casado and Heredia, 2001b; Matas *et al.*, 2004). Glass transition temperatures (T_g) were found to be 23°C for both tomato cuticle membranes and the cutin itself, but saturation of the membranes with water reduced T_g to about 16°C (Matas *et al.*, 2004). Differential scanning calorimetry (DSC) also revealed an anomalously high value of specific heat (2.0–2.5 J K⁻¹ g⁻¹) for tomato cutin (Luque and Heredia, 1997; Casado and Heredia, 2001a,b), suggesting a thermoregulatory role in leaves and fruits. Given the potentially deleterious effects of heat waves and frosts on fruit viability, it is

important to develop a molecular understanding of associated changes in cuticular surface structure, segmental motion, and cutin-wax domain architecture that may degrade the mechanical behavior of the cuticle so that cracking of the polymeric veneer is promoted and the tissue below becomes susceptible to pathogenic attack (Garbow and Stark, 1990).

3.8 Summary and prospectus

Beginning with the identification of plant cutin monomers and the investigation of their biosynthesis in the 1970s, our molecular-level understanding of these protective biopolymers has been augmented by recent spectroscopic studies of intact cutins and their oligomeric degradation products. With these latter investigations, which rely on specialized methods including FT-IR, solid-state NMR, HR-MAS NMR, and electrospray MS, the ester linkages typical of extended cutin chains and several types of cross-links in tomato and lime fruit cutins have been established. A more comprehensive picture of the cross-link elements and their prevalence within the biopolymer structure would deepen our understanding of how cutin functions as a support structure for the coverings of aerial plant surfaces.

A complementary physical view of the cutin biopolymer and its embedded waxes is also being assembled using DSC, AFM, and NMR relaxation methods. Importantly, these assessments of bulk and surface cuticular mechanical behavior may be made under environmental stress conditions including hydration, abrasion and extreme temperatures. For instance, in tomato fruit cuticles it has been possible to correlate temperature-induced changes in surface elastic modulus with the phase behavior of the epicuticular waxes and to associate hydration-induced plasticization of dewaxed cutin with the flexibility identified in acyl chain segments of the cutin biopolymer. Such studies can lay the groundwork for future crop protection strategies based on molecular and micromechanical principles.

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4 Composition of plant cuticular waxes

Reinhard Jetter, Ljerka Kunst and A. Lacey Samuels

Plant cuticles contain, beside cutin (Chapters 2 and 3), wax as the second major chemical component. Cuticular wax plays pivotal physiological and ecological roles in the interactions between plants and their abiotic (Chapters 5 and 8) and biotic environments (Chapters 10–12), respectively. Usually, the mixture of compounds that can be obtained by surface extraction of intact plant organs with organic solvents of low polarity is designated as ‘wax’. This is typically a mixture with characteristic composition for each species and organ. Thus, in this chapter, to distinguish between diverse mixtures originating from different sources, we will use the plural ‘waxes’, while the singular ‘wax’ will refer to the mixture from one particular source. It should be noted that our use of the term wax differs from the definition used in purely chemical contexts, where it is usually restricted to long-chain alkyl esters.

A number of reviews have previously addressed the chemical composition of plant cuticular waxes. They mostly emphasised selected aspects, such as analytical procedures (Walton, 1990), qualitative wax composition (Tulloch, 1976), epicuticular compounds (Baker, 1982) or the critical assessment of experimental approaches (Riederer and Markstädter, 1996). Much new information on the composition and structure of plant cuticular waxes has accumulated over the last decade. Here we attempt to summarise our knowledge on the chemical structure of plant cuticular waxes, integrating aspects from all these previous reviews and the literature published since they appeared. To this end, we will briefly describe the experimental procedures used for wax analysis (Section 4.1), summarise the qualitative composition of waxes (Section 4.2) and review our knowledge on spontaneous reactions occurring within waxes (Section 4.3). Then the quantitative chemical composition of wax mixtures (Section 4.4) and their dynamic changes will be described (Section 4.5). Due to space constraints, this review of chemical structures and of quantitative compositions, as well as the corresponding references, cannot be comprehensive, but must instead be attempted by depicting overall patterns and using individual examples. Finally, we will summarise our current understanding of the arrangement of wax constituents in epicuticular and intracuticular layers within the cuticle (Section 4.6).

4.1 Methods used for the chemical analysis of plant cuticular waxes

4.1.1 Wax extraction

Plant cuticular waxes are typically prepared for chemical analysis by surface extraction of intact organs. Extraction has to be carried out under controlled conditions

in order to give reproducible results. The choice of solvent, the solvent volume, its temperature and the duration of contact between the solvent and the plant surface have to be chosen carefully. In most cases, an exhaustive extraction of wax is desirable, as the total amounts present will be the best basis for comparisons, for example, between species, organs, developmental stages and tissues grown under varying conditions.

Solvents of intermediate polarity should be used to maximise solubility of all wax constituents, including the extremely hydrophobic hydrocarbons and the much more polar compounds containing (multiple) functional groups (Riederer and Schneider, 1989; Stammitt *et al.*, 1996). Chloroform proved to have ideal properties and gave particularly high, reproducible wax yields for various species (Holloway, 1984). In some studies, other solvents, for example, methylene chloride (Arrendale *et al.*, 1988) or petrol ether (Salasoo, 1983), have been used. In all of these solvents, most wax compounds have solubilities in the range of 1–10 mg/ml, and hence the volume needed to cover the tissue entirely is generally sufficient to dissolve all the wax present. In a number of investigations, *n*-hexane has been used to extract cuticular waxes and has given satisfactory results (Rashotte *et al.*, 2001). However, it should be noted that this solvent, due to its very low polarity, can dissolve only limited amounts of the more polar wax constituents, so that relatively large volumes have to be applied for exhaustive extraction.

The extraction efficiency can be improved by increasing the extraction temperature. This is generally not necessary if suitable solvents are used, and therefore extraction is usually performed at room temperature. To the authors' knowledge, only in one case where oligomeric and/or polymeric constituents (derived of aldehyde monomers) were present, was it necessary to extract with warm solvent to release the wax compounds (Riedel *et al.*, 2003).

The duration of extraction has varied widely in previous studies, in part to distinguish between wax exposed near the surface or embedded deep inside the cuticle (Silva Fernandes *et al.*, 1964; Holloway, 1974; Baker *et al.*, 1975; Baker and Procopiou, 1975). The time required for solvent molecules to enter into the cuticle, to mobilise wax and to wash it out must depend on the thickness and molecular structure of the cuticle, depending in turn on the plant species and organ investigated. Therefore, the minimum time necessary for exhaustive extraction would have to be determined prior to each investigation. For a large number of tissues, however, it was found that extraction is complete after 5–20 s (Holloway, 1984). Therefore, extraction is usually not optimised for new objects, but instead standard protocols comprising two extraction steps of 30 s are employed and assumed to yield total wax mixtures. It should be noted that for some plant species it was shown that, even though the superficial extraction appeared exhaustive, the cuticle contained more soluble wax components that could only be released by soxhlet extraction of isolated cuticular membranes (Riederer and Schneider, 1989).

In many cases extraction of cuticular wax has been performed by immersion of entire organs into large volumes of solvent. While this method of preparation is adequate for unifacial tissues, it is by nature unable to distinguish between different

sides of the same organ, for example, the adaxial and abaxial sides of leaves, which may have diverse compositions. Extraction of single tissue sides has been attempted by running solvent over the surface (Holloway *et al.*, 1977b; Eigenbrode *et al.*, 1998), by sweeping the tissue with solvent-saturated glass wool (Wen and Jetter, unpublished results) and by containing the solvent in glass cylinders pressed onto the surface that is to be extracted (Jetter *et al.*, 2000). Only the last procedure allows control of extraction conditions, such as extraction time, solvent volume and temperature. All these methods must be validated by repeated extraction to prove exhaustive yields, and by comparison of single-side yields with the multi-side yields determined by dipping experiments.

Special care has to be taken to avoid contamination of samples by internal lipids, possibly extracted by solvent entering through surface lesions or the cut areas created during harvest of the material. Extraction of internal lipids is usually indicated by the presence of chlorophyll, visible as green colour of the extract, and by phospholipids detected chemically. Extraction of fresh and healthy tissue in most cases yields wax preparations that are free of internal lipids. It has not been systematically studied whether the solvent might also enter through stomata, and therefore the extracts might contain soluble lipids originating from substomatal cavities or intercellular spaces.

For comparisons of chemical data between studies, and correlations of wax composition with functional and structural parameters, the wax quantities have to be expressed in absolute values. Although amounts have sometimes been given in units of wax mass per tissue dry weight, it is more useful to quantify wax mass per surface area. This value corresponds to a layer thickness, hence allowing direct interpretation together with microscopic results and data on the transport of water and organic compounds across the cuticle. Wax quantities have to be determined by comparison with known amounts of an internal standard. An artificial compound that is similar to the most common wax constituents should be chosen as standard, and must be added to the sample shortly before or after extraction.

Further sample processing is straightforward and little sample loss usually occurs before instrumental analysis. Sensitivity of analytical instrumentation has gradually improved by the introduction of gas chromatography (GC), capillary GC, and flame ionisation detectors (FID) or mass spectrometers (MS). Consequently, for most plant species and organs, extraction of 1–10 cm² of surface area will yield sufficient wax for single analyses. In some instances, even the wax mixtures from areas smaller than 1 cm² have been used for complete compound identification and quantification.

4.1.2 Instrumental analysis

Due to the complexity of mixtures and the relatively low amounts available, cuticular wax extracts can best be analysed by GC. To improve peak shape and thus give better resolution, functional groups containing active hydrogen should be derivatised. To this end, hydroxyl groups of alcohols and carboxylic acids are

typically transformed into the corresponding trimethyl silyl (TMSi) ethers and esters, employing silylation reagents like bis-*N,O*-trimethylsilyltrifluoroacetamide (BSTFA). It has been shown that this derivatisation reaction goes to completion, and that the resulting compounds can be very easily identified and accurately quantified (Deas *et al.*, 1974; Little, 1999). Using these derivatives, compounds with different functional groups and/or varying carbon numbers have, in all cases, been separated by GC (Figure 4.1a).

After GC separation, individual compounds in most studies have been quantified using FID detection. The FID has especially high sensitivity and broad range of proportionality. Selected aliphatic compounds are usually employed as internal standards for quantification. Unfortunately, the FID is destructive and does not yield chemical information; so separate GC-MS analyses have to be performed for compound identification. Currently, the spectra of only a few wax constituents are represented in the published electronic MS libraries. Authentic standards of many wax constituents are commercially available, or can easily be prepared in single-step semi-syntheses, and are important for structure confirmation by co-elution experiments and spectral comparisons. Mass spectral fragmentation patterns are very distinct for most compound classes present in waxes, especially for TMSi derivatives (Figures 4.1b and d). Under normal conditions, electron impact MS also gives information on the molecular weight of the compound, thus allowing assignments of the chain lengths of aliphatic compounds. Therefore, a qualitative analysis of a cuticular wax mixture, including the assignment of all the previously known compounds, can usually be performed within a few hours.

In many instances, isomers could also be baseline separated by GC. This is true for skeletal isomers of diverse triterpenoids and for positional isomers of alkanediols (Franich *et al.*, 1979). Baseline separation cannot be achieved for isomeric alkyl esters, i.e. compounds with the same overall carbon numbers but with differences in the alcohol and fatty acid moieties (Sümmchen *et al.*, 1995b). Also, secondary alcohols with subtle differences can typically not be separated, for example, the C₂₉ isomers nonacosan-14-ol and nonacosan-15-ol in the stem wax of *Arabidopsis thaliana* (Figure 4.1c; Jenks *et al.*, 1995). In contrast, the more pronounced geometrical differences between asymmetric alcohols like the C₃₃ isomers tritriacontane-10-ol, -12-ol and -14-ol suffice for partial GC separation (Figure 4.1e).

To circumvent problems caused by decomposition of aldehydes during derivatisation with BSTFA, an additional step in sample preparation has been employed in one study (Nass *et al.*, 1998). In this protocol, solid phase extractions on short silica columns were used to separate wax mixtures into two fractions that were analysed independently. More sophisticated pre-separation procedures have also been employed to prepare larger quantities of individual compound classes for structure elucidation. These included one- and two-dimensional thin-layer chromatography (TLC) as well as (repeated) column chromatography (Tulloch, 1975; Holloway, 1984). While these techniques are of great value for qualitative investigations, special care has to be taken when employing them in quantitative analyses (e.g. the use of multiple internal standards).

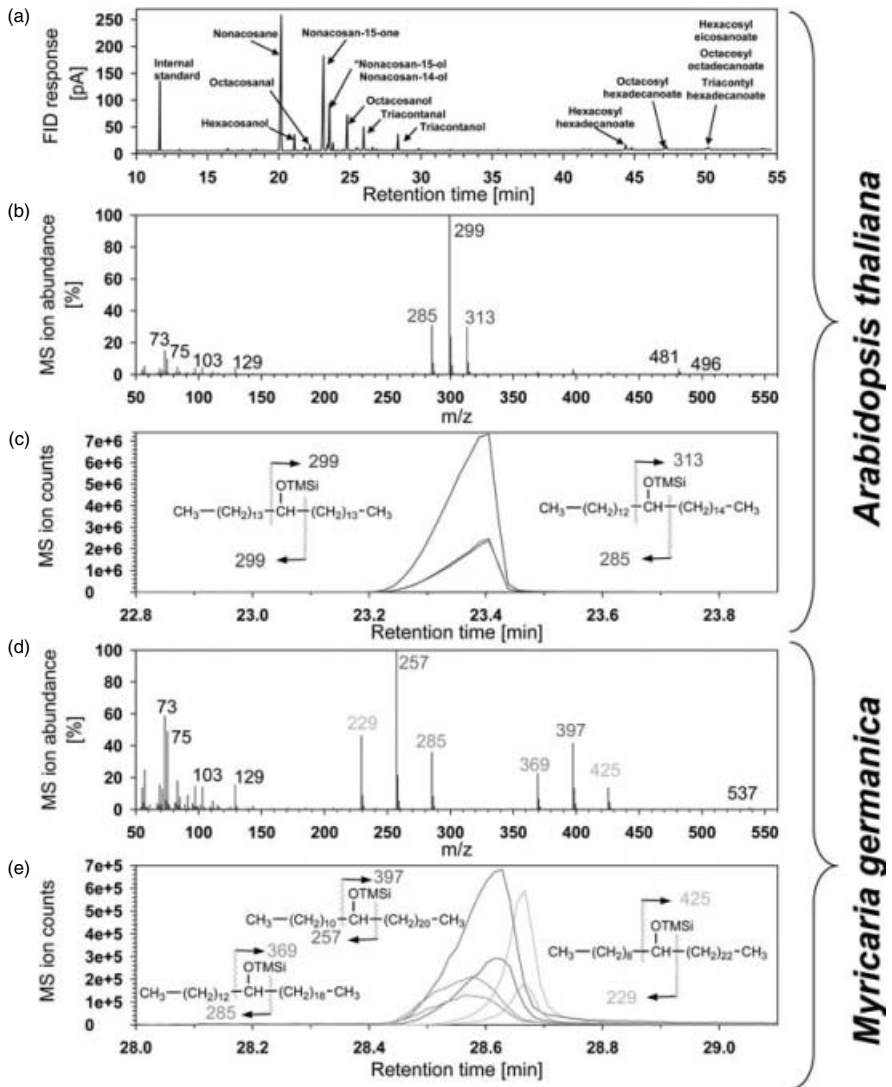


Figure 4.1 Gas chromatographic (GC) separation and mass spectrometric (MS) identification of plant cuticular wax components. (a) GC-FID trace of the wax mixture extracted from inflorescence stems of *Arabidopsis thaliana* ecotype Columbia. (b) Mixed mass spectrum of trimethyl silyl (TMSi) derivatives of nonacosan-14-ol and nonacosan-15-ol [corresponding to the GC peak at 23.5 min in (a)] showing characteristic α -fragments at $m/z = 285, 299, 313$. (c) GC-MS traces of the α -ions, demonstrating that the isomeric 14- and 15-alcohols cannot be separated under the GC conditions used. (d) Mixed mass spectrum of TMSi derivatives of tritriacontan-10-ol, tritriacontan-12-ol and tritriacontan-14-ol (from leaf wax of *Myricaria germanica*) showing characteristic α -fragments at $m/z = 229, 257, 285$ and $m/z = 369, 397, 425$, respectively. (e) GC-MS traces of the α -ions, demonstrating that the isomeric 10-, 12- and 14-alcohols can be partially separated under the GC conditions used. This figure is produced in colour in the colour plate section, which follows page 249.

4.2 Chemical profiles of plant cuticular waxes

4.2.1 Ubiquitous constituents of cuticular waxes

A number of aliphatic compounds have been identified in the wax mixtures of virtually all the plant species investigated to date (Figure 4.2). This standard set of compounds is characterised by unbranched, fully saturated hydrocarbon backbones that may carry one primary oxygen-containing functionality, i.e. a terminal hydroxyl, carbonyl or carboxyl group. Therefore, plant cuticular waxes are typically mixtures of primary *n*-alcohols, *n*-aldehydes and fatty acids as well as *n*-alkanes, each of these compound classes comprising a homologous series of chemicals with chain lengths ranging from 20 to almost 40 carbons. A species-specific array of these compounds alone may thus comprise more than 50 different chemical structures.

Additionally, esters of C₁₆–C₃₄ fatty acids and C₂₀–C₃₆ primary alcohols have been identified, giving rise to homologous compounds with chain lengths ranging from C₃₆ up to C₇₀, and to dozens of isomers for each chain length (Gülz *et al.*, 1994; Shepherd *et al.*, 1995; Sümmchen *et al.*, 1995a). In many instances the esters contain alkyl and acyl moieties with chain lengths similar to the free alcohols and acids present in the wax mixture (Purdy and Truter, 1963; Allebone and Hamilton,

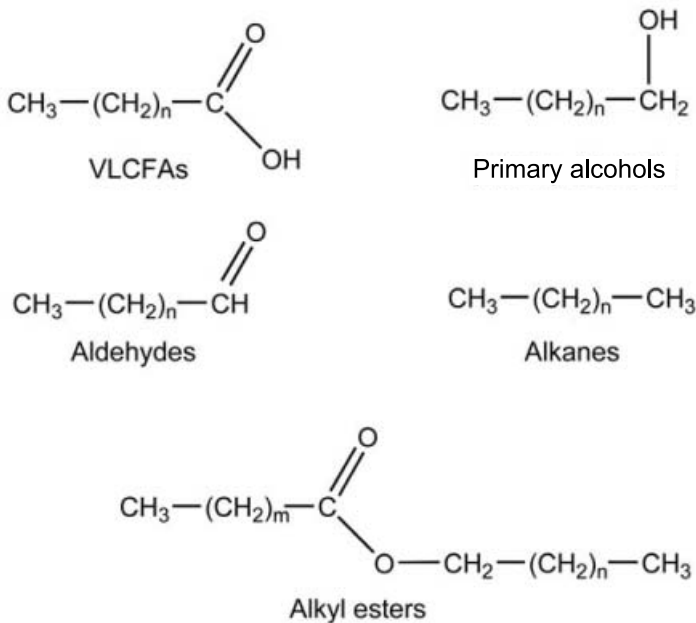


Figure 4.2 Structures of components ubiquitously occurring in plant cuticular wax mixtures.

1972). Consequently, these alkyl esters can be classified as dimeric products, while the corresponding alcohols and acids (as well as aldehydes and alkanes) represent monomeric wax constituents.

4.2.2 Taxon-specific major constituents of cuticular waxes

In addition to the ubiquitous constituents, the wax mixtures of individual plant taxa may contain relatively high percentages of specific compounds or compound classes. The majority of structures involved may be summarised as follows: the compounds mostly have unbranched, fully saturated aliphatic chains with 29 or 31 carbons and alcohol or keto groups (Table 4.1). In contrast to the standard wax constituents, the taxon-characteristic components frequently have secondary functional groups, giving rise to the possibility of positional isomerism. Some of the taxon-specific compounds also contain two or more secondary functional groups. Both the chain lengths and the positions of the functional groups can be easily determined by GC-MS, by interpreting the characteristic patterns of α -fragmentation (Ubik *et al.*, 1975; Holloway *et al.*, 1976).

Secondary alcohols have been identified in a number of plant waxes, and can be classified into three groups (Table 4.1; Figure 4.3). The first is characterised by homologous series of secondary alcohols with a relatively broad chain length distribution and a vast array of positional isomers, containing hydroxyl groups typically between positions C-4 and C-12. Typical examples have been described for the cuticular waxes from strawberry leaves (Baker and Hunt, 1979), apple fruits (Verardo *et al.*, 2003) and rose flowers (Mladenova and Stoianova-Ivanova, 1977).

A second class of secondary wax alcohols is defined by the very strong predominance of a single chain length (in most cases C₂₉ or C₃₁) and the presence of one isomer that has the hydroxyl group at the central carbon of the chain. It is usually accompanied by a second isomer carrying the functionality on the carbon adjacent to centre. Accordingly, the leaf wax of *Pisum sativum* contains large amounts of the C₃₁ secondary alcohols hentriacontan-16-ol and hentriacontan-15-ol, together with traces of homologous secondary alcohols (Macey and Barber, 1970; Kolattukudy, 1970; Holloway *et al.*, 1977b). The waxes from leaves of *Clarkia elegans* (Hunt *et al.*, 1976), *Brassica oleracea* (Baker, 1974) and *Brassica napus* (Holloway *et al.*, 1977a), as well as from stems of *A. thaliana* (Rashotte *et al.*, 1997) contain the C₂₉ secondary alcohols nonacosan-15-ol and nonacosan-14-ol, together with small amounts of secondary alcohols with other chain lengths.

Finally, a third class of secondary alcohols from plant cuticular waxes, again dominated either by C₂₉ or by C₃₁ homologues, is characterised by the position of the hydroxyl function exclusively on carbon-10 or -12. Nonacosan-10-ol is the predominant constituent of leaf waxes from Papaveraceae (Jetter and Riederer, 1996), Gymnospermae (Riederer, 1989), Rosaceae (Wollrab, 1969) and other diverse taxa of vascular plants (Holloway *et al.*, 1976), as well as on the sporophytes of some moss species (Neinhuis and Jetter, 1995). Hentriacontan-12-ol is found at high concentration in the leaf wax of *Myricaria germanica* (Jetter, 2000). These asymmetric

Table 4.1 Typical composition of prominent compound classes with secondary functional groups in plant cuticular waxes

Compound class	Homologous series		Isomer mixture		Examples		
	Range of chain lengths	Dominating chain length	Position of 1st functional group	Position of 2nd functional group	Plant taxon	Organ	References
<i>sec.</i> Alcohols	C ₂₁ –C ₃₅	C ₂₆ –C ₃₀	C-4 to C-12	—	<i>Rosa damascena</i>	Petals	Mladenova and Stoianova-Ivanova, 1977
	C ₂₇ –C ₃₁	C ₂₉	C-14 and C-15	—	<i>Arabidopsis thaliana</i>	Inflor, stems	Rashotte <i>et al.</i> , 1977
	C ₂₉ –C ₃₃	C ₃₁	C-15 and C-16	—	<i>Brassica oleracea</i>	Leaves	Baker, 1974
Ketones	C ₂₇ –C ₃₁	C ₂₉	C-10	—	<i>Pisum sativum</i>	Leaves	Hollway <i>et al.</i> , 1977b Macey and Barber, 1970
	C ₂₉ –C ₃₃	C ₂₉	C-12	—	Gymnospermae	Needles	Kolatukudy, 1970
	C ₂₇ –C ₃₁	C ₂₉	C-15	—	Papaveraceae	Leaves	Riederer, 1989
	C ₂₉ –C ₃₃	C ₃₁	C-12	—	<i>Myricaria germanica</i>	Leaves	Jetter and Riederer, 1996
	C ₂₇ –C ₃₁	C ₂₉	C-15	—	<i>Arabidopsis thaliana</i>	Leaves	Jetter, 2000
<i>sec.</i> Alkanediols	C ₂₉ –C ₃₃	C ₃₁	C-16	—	<i>Brassica oleracea</i>	Leaves	Rashotte <i>et al.</i> , 1977
	C ₂₇ –C ₃₁	C ₂₉	C-10	—	<i>Aristolochia gigantea</i>	Leaves	Baker, 1974
	C ₂₇ –C ₃₁	C ₂₉	C-14	—	<i>Osmunda regalis</i>	Leaves	Meusel <i>et al.</i> , 1999
	C ₂₇ –C ₃₁	C ₂₉	C-10	C-15	<i>Brassica oleracea</i>	Fronds	Jetter and Riederer, 2000
	C ₂₇ –C ₃₁	C ₂₉	C-10	C-3 to C-16	<i>Nelumbo nucifera</i>	Leaves	Hollway and Brown, 1977
<i>sec.</i> Ketols	C ₂₉ –C ₃₃	C ₃₁	C-12	C-4 to C-16	<i>Myricaria germanica</i>	Leaves	Barthlott <i>et al.</i> , 1996
	C ₂₇ –C ₃₁	C ₂₉	C-14	C-15	<i>Brassica oleracea</i>	Leaves	Jetter, 2000
β-Diketones	C ₂₇ –C ₃₁	C ₂₉	C-10	C-4 or C-5		Leaves	Hollway and Brown, 1977
	C ₂₇ –C ₃₃	C ₃₁	C-14,16	—	Gymnospermae	Needles	Hollway <i>et al.</i> , 1977a
Hydroxy-β-diketones	C ₂₉ –C ₃₅	C ₃₁	C-8,10	—	Poaceae	Leaves	Riederer, 1989
	C ₂₇ –C ₃₃	C ₃₁	C-14,16	C-4 to C-9 or C-25	<i>Eucalyptus</i> sp.	Leaves	Tulloch, 1981
	C ₂₇ –C ₃₃	C ₃₁	C-14,16	C-4 to C-9 or C-25	<i>Buxus sempervirens</i>	Leaves	Hom <i>et al.</i> , 1964
Oxo-β-diketones	C ₂₇ –C ₃₃	C ₃₁	C-14,16	C-4 to C-9 or C-25	Poaceae	Leaves	Dierckx, 1973 Tulloch <i>et al.</i> , 1980 Tulloch <i>et al.</i> , 1980



Figure 4.3 Examples of compounds with secondary functional groups, accumulating to high concentrations in the wax mixtures of certain plant taxa.

alcohols contain a stereogenic centre, and an excess of the *S* enantiomer has been reported for nonacosan-10-ol from *Picea pungens* (Jetter and Riederer, 1994).

Wax secondary alcohols of the second and third categories described earlier are frequently accompanied by the corresponding ketones, at widely varying concentrations. In leaf wax of *C. elegans* (Hunt *et al.*, 1976), *B. oleracea* (Baker, 1974) and *B. napus* (Holloway *et al.*, 1977a), as well as on inflorescence stems of *A. thaliana* (Rashotte *et al.*, 1997) the C₂₉ ketone nonacosan-15-one is present in relatively high concentrations. The symmetric C₃₁ ketone hentriacontan-16-one (=palmitone), but not the corresponding symmetric secondary alcohol hentriacontan-16-ol, has been reported in the leaf waxes of *Allium porrum* (Maier and Post-Beittenmiller, 1998) and diverse dicots including *Aristolochia gigantea* and *Paeonia officinalis* (Meusel *et al.*, 1999). It is noteworthy that *Asparagus officinalis* leaf wax has been found to contain the C₂₇ ketone heptacosan-14-one, a symmetric ketone with relatively short chain length (Scora *et al.*, 1986), while *Centhrantus ruber* wax contains nonacosan-14-one (Meusel *et al.*, 1999). Finally, corresponding to the class of asymmetric secondary alcohols with high regio- and chain-length specificity, nonacosan-10-one has been described as a constituent of the wax on leaves of *Encephalartos spp.* (Osborne and Stevens, 1996), as well as on fronds of *Osmunda regalis* (Jetter and Riederer, 2000).

Also associated with secondary alcohols, alkanediols and ketols have been described as cuticular wax constituents. They are characterised by a strong predominance of the C₂₉ homologue, and can be classified into two groups according to the position of functionalities. In those diols and ketols typically detected together with corresponding symmetric secondary alcohols, one functionality is located on the central carbon and the second on the adjacent carbon. Thus, nonacosane-14,15-diol, 15-hydroxynonacosan-14-one and 14-hydroxynonacosan-15-one have been identified in leaf wax of *Brassica* species (Holloway and Brown, 1977; Holloway *et al.*, 1977a). A second group of diols and ketols, associated with the corresponding asymmetric alcohols, has one functional group on carbon number 10 and a second group on one of the carbons between C-3 and C-16. Respective nonacosanediol isomers have been described for wax mixtures from diverse Gymnospermae (Franich *et al.*, 1979), Papaveraceae (Jetter and Riederer, 1996), and are especially abundant in the wax of *Nelumbo nucifera* (Barthlott *et al.*, 1996). In some of these species, 10-hydroxynonacosan-4-one and 10-hydroxynonacosan-5-one have been reported together with the diols.

It is noteworthy that all the ketones, ketols and diols thus identified as important components of cuticular waxes showed a chain length or isomer pattern closely matching those of the secondary alcohols present either in the same species or in other taxa. Based on these purely chemical observations it has been suggested that the secondary alcohols are biosynthesised first and that the other compounds are derivatives formed by ensuing pathway steps (Franich *et al.*, 1979; Jetter and Riederer, 1996).

In the leaf waxes of numerous species of the Poaceae (Tulloch, 1981) and of the genus *Eucalyptus* (Horn *et al.*, 1964), large percentages of aliphatic β -diketones have

been described. This compound class is characterised by unbranched hydrocarbon chains with 27–33 carbons and two mid-chain carbonyl groups in 1,3-position to each other. The β -diketones usually show strong predominance of single homologues and isomers in the wax of a given species, but can vary substantially between taxa. The most widely distributed representative, the C_{31} compound hentriacontane-14,16-dione, is the dominant compound in the waxes of various organs of well-investigated species such as *Hordeum vulgare* (Jackson, 1971; von Wettstein-Knowles and Netting, 1976a) and *Triticum aestivum* (Bianchi *et al.*, 1979). In contrast, leaf wax of *Buxus sempervirens* was reported to contain mainly the isomeric hentriacontane-8,10-dione (Dierickx, 1973).

In many cases further derivatives of β -diketones have been described, including hydroxy- or keto- β -diketones with an additional alcohol or carbonyl function on carbons C-4 to C-9 or C-25 (Tulloch *et al.*, 1980). These derivatives occur as species-specific mixtures including various isomers that all share the same basic chain length and position of the β -diketo functionality. Thus, similar to the relation between secondary alcohols and alkanediols and ketols, the chemical structures suggest that β -diketones are the parent compounds from which further derivatives are biosynthesised through hydroxylation and hydroxyl oxidation.

4.2.3 Cuticular triterpenoids

In the cuticular wax mixtures of many plant species, triterpenoid constituents have been detected. While they occur at trace levels in most cases, including model species such as *A. thaliana* (Jenks *et al.*, 1995) and *B. oleracea* (Baker and Holloway, 1975), they can accumulate to very high concentration in the waxes of specific taxa. Cuticular triterpenoids can be classified according to the carbon backbone structure, according to the number, nature, and position of functional groups and according to the derivatives formed through bonding between the triterpenoid and other compounds.

Out of the more than 200 basic triterpenoid carbon skeletons described to date (Xu *et al.*, 2004), only a small number has been detected in plant cuticular waxes (Figure 4.4). The large majority of these cuticular constituents are pentacyclic triterpenoids, characterised by the presence of five condensed carbon rings. Triterpenoids with fernane structures and related carbon backbones have been detected mostly on the surfaces of fern fronds (Tanaka *et al.*, 1992). In the cuticular wax mixtures of seed plants, lupane, oleanane and ursane derivatives have been encountered most frequently (Walton, 1990). For example, in wax from *Prunus laurocerasus* leaves, oleanane and ursane structures co-occur as major components (Jetter *et al.*, 2000), while oleananes predominate in waxes on grapes (*Vitis vinifera*; Radler, 1970), plums (*Prunus domestica*; Ismail *et al.*, 1977) and olives (*Olea europaea*; Bianchi *et al.*, 1992), on shoots of *Euphorbia lathyris* (Hemmers *et al.*, 1989b), and on leaves of *Tilia tomentosa* (Gülz *et al.*, 1988). Reports on single species from other taxa documented diverse variations of this standard set of triterpenoids. For instance, in wax of tomato fruits (*Lycopersicon esculentum*),

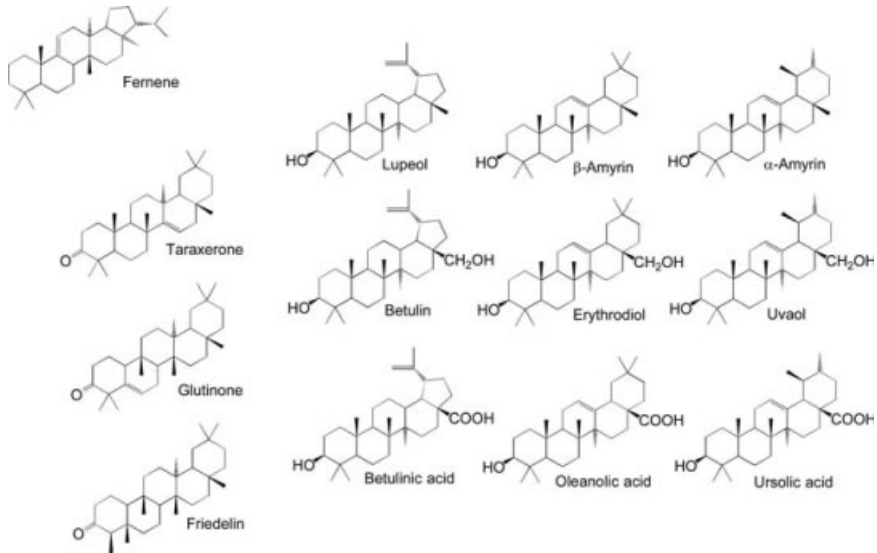


Figure 4.4 Examples for triterpenoids occurring in plant cuticular waxes.

oleanane and ursane structures have been found associated with equivalent amounts of δ -amyrin (Vogg *et al.*, 2004). In relatively few taxa, including *Cirsium arvense* (Tulloch and Hoffman, 1982) and *Ricinus communis* (Guhling and Jetter, unpublished results), ursane or lupenane derivatives predominate, respectively. In other groups of plants, wax mixtures dominated by other triterpenoid skeletons including taraxerane in *Macaranga spp.* (Markstädter *et al.*, 2000) and glutinane in *Euphorbia cyparissias* (Hemmers *et al.*, 1989a) have been described.

The large majority of the triterpenoids identified in cuticular waxes of various plant species to date contain a hydroxyl function in the 3β position. This finding can be explained with the generally accepted concepts for triterpenoid biosynthesis (Chapter 5), according to which 3β -alcohols are the immediate products of the first steps of the pathways, and are hence ubiquitously present in triterpenoids of higher plants. In many cases, the 3β -triterpenols are accompanied by the corresponding ketones, sometimes accumulating to relatively high percentages. For example, in stem cuticular waxes of several *Macaranga* species taraxerone was found (Markstädter *et al.*, 2000), while glutinone was one of the prominent compounds of *Euphorbia cyparissias* wax (Hemmers *et al.*, 1989a) and friedelin was reported for grapefruit (*Citrus paradisi*) waxes (Nordby and McDonald, 1994).

Whereas thousands of triterpenoid structures with different numbers of functional groups on the carbon backbone have been identified from internal plant tissues, only very few of them have so far been detected in cuticular waxes. For the lupenane, oleanane and ursane structures, some of the C-28 oxidation products have been reported, including the diols erythrodiol, uvaol (Griffiths *et al.*, 2000) and betulin (Wollenweber *et al.*, 1999) as well as the hydroxyacids oleanolic and ursolic acids

(Jetter *et al.*, 2000). It may be speculated that other oxygen-containing triterpenoid derivatives might not be present in cuticular waxes because they are too polar to partition into such a lipophilic mixture.

Finally, various derivatives of the triterpenoids described above have been identified in cuticular waxes. These include alkyl ethers and acyl esters of the triterpenoid alcohols as well as alkyl esters of the triterpenoid acids. Hence, the triterpenols lupeol, β - and α -amyrin tend to be associated with the corresponding methyl ethers (Smith and Martin-Smith, 1978), acetate esters (Manheim and Mulroy, 1978) or long-chain (C_{16} and C_{18}) as well as very long-chain ($>C_{20}$) fatty acid esters (Gülz *et al.*, 1988). Ursolic and oleanolic acid have been found accompanied by their methyl esters (Bakker *et al.*, 1998).

While these examples demonstrate some emerging patterns in the distribution of triterpenoid structures in cuticular wax mixtures, our knowledge is still very sketchy. It relies on relatively few analyses of species selected with a strong bias towards certain plant taxa, and was performed by only a few research groups. The identification of cuticular triterpenoids is largely based on GC-MS data used to separate and delineate isomers with relatively subtle differences in structure. Fragmentation patterns and MS peak intensities are less predictable than for aliphatic wax constituents. Hence, it is very important that triterpenoid structures, after MS interpretation, be confirmed by comparison with authentic standards. Unfortunately, only a few triterpenoids, representing little diversity of carbon backbones, are commercially available or can easily be prepared and authenticated from plant material. This hampers structure identification of novel cuticular triterpenoids, creates a bias towards repeated description of known structures, and might thus give the misleading impression of only a few structures being present in the cuticular waxes of diverse plant taxa. Our chemical knowledge of cuticular triterpenoid diversity and distribution, bearing important biological implications on cuticle structure and function, will likely change substantially as more species are investigated and more standards for identification become available.

4.2.4 *Other minor constituents of cuticular waxes*

Beside the compounds described earlier, a large number of wax constituents have been identified over the past decades, and the following description can only highlight a few examples for specialty components of plant cuticular waxes. Some structural diversity arises from modifications in the hydrocarbon chains of aliphatic wax constituents. In a few cases, a double bond is present, giving rise to unsaturated compounds that are analogous to the predominating saturated wax constituents. Double bonds with *Z*-configuration seem to predominate, and they have frequently been located between carbon numbers 2 and 3 or 9 and 10. As a second modification, methyl branches have been detected on the hydrocarbon chain of wax alkanes, primary alcohols, fatty acids and their alkyl esters. Typically, homologous series of both iso- and anteiso-isomers are present, that is, compounds with methyl branches located either one or two carbons away from the alkyl chain terminus, respectively.

One example for the relatively prominent presence and wide arrays of branched structures is the leaf wax of *Brassica* species, where primary alcohols and fatty acids with iso- and anteiso-branching have been reported as free compounds and in the corresponding alkyl esters (Baker and Holloway, 1975). Branched hydrocarbons have been identified in petal waxes of various species, including *Antirrhinum majus* (Goodwin *et al.*, 2002) and *Papaver rhoeas* (Stransky and Streibl, 1969).

Aliphatic acids may be found esterified with methanol, resulting in series of homologous fatty acid methyl esters with acyl chain lengths from C₂₀ to C₃₄. In a similar way, C₂₀–C₃₀ wax primary alcohols are found esterified with acetic acid in some cases. Another class of trace compounds worth mentioning are esters formed between very long chain fatty acids and secondary alcohols. In the wax mixtures of some plant species that were otherwise characterised by the presence of β -diketones, such esters were formed by medium-chain alkan-2-ols. Their composition and distribution in the waxes of various plant organs has been intensively studied for *Hordeum vulgare* (von Wettstein-Knowles and Netting, 1976b; Mikkelsen, 1984; von Wettstein-Knowles and Madsen, 1984) (Chapter 5).

Relatively recently, a vast range of aliphatics with novel structures containing two functional groups has been identified. They are generally characterised by C₂₂–C₃₂ hydrocarbon chains, the presence of a primary oxygen-containing functional group on one chain terminus and of a second oxygen-functionality at specific distances inside the chain. Variations of these primary and secondary functional groups give rise to homologous series of alkanediols in the wax of *Papaver* species (Jetter *et al.*, 1996b), *Osmunda regalis* (Jetter and Riederer, 1999b), *M. germanica* (Jetter, 2000) and *R. communis* (Vermeer *et al.*, 2003), together with hydroxyaldehydes for *R. communis* (Vermeer *et al.*, 2003) and ketoalcohols for *O. regalis* (Jetter and Riederer, 1999b). Corresponding hydroxy fatty acids were found in the form of δ -lactones on the leaves of *Cerintho minor* (Jetter and Riederer, 1999a). Most remarkably, the substitution was in all cases on odd-numbered carbons, resulting in bifunctional compounds of 1,3-, 1,5-, 1,7- and so on. It was suggested that this geometry of functional groups might arise from polyketide-type elongation, explaining the mid-chain functionality as a product of modified fatty acid elongation cycles.

Various reports have described cuticular wax constituents that combine structural features of the standard aliphatic wax structures with those of other plant metabolites. Most notably, the latter may contain aromatic ring structures mainly originating from the phenyl propanoid metabolism. For example, mixed aliphatic–aromatic esters may be formed by wax fatty acids and cinnamyl alcohol in wax of *Vicia faba* flowers (Griffiths *et al.*, 1999), as well as hydroxyphenyl propanol, and hydroxyphenyl butanol in *Taxus baccata* (Jetter *et al.*, 2002). Other aromatic compounds have been detected in plant cuticular waxes, including homologous series of very long-chain fatty acids esterified with benzyl alcohol or phenylethyl alcohol (Gülz and Marnier, 1986), as well as of alkyl benzoates (Gülz *et al.*, 1987). Finally, series of 5-alkyl resorcinols accumulate to relatively high amounts in the wax on seeds of *H. vulgare* (Garcia *et al.*, 1997).

The waxes of various gymnosperms, but also of a few other plant species, contain two classes of characteristic compounds. The first of them, called estolides, are oligomeric structures formed by ester linkages between two or more hydroxy fatty acids, α,ω -diols, as well as primary and secondary alcohols (Tulloch and Bergter, 1981). The second class of compounds, frequently associated with estolides, are characteristic triglycerides containing two normal fatty acyl moieties esterified to the glycerol positions C-1 and C-3, while having a hexanoic or octanoic acyl moiety bonded to C-2 (Tulloch and Hoffman, 1982).

Finally, it should be noted that in a number of plant species, tocopherols have been reported as wax constituents (Gülz *et al.*, 1992). The three most abundant representatives of these compounds in internal lipids, α -, γ - and ϵ -tocopherol, are usually occurring together in the plant cuticle (Griffiths *et al.*, 2000).

4.2.5 Other compounds located at or near the plant surface

In surface extracts of many plants, a number of phytosterols, in most cases including β -sitosterol and stigmasterol, have been identified (Holloway, 1971). These compounds have, for example, been reported in leaf waxes of Poaceae species (Avato *et al.*, 1990). Based on their overall polarity, but also based on structure similarities with the pentacyclic triterpenoids commonly found in cuticular waxes, it seems plausible that the phytosterols might be actively exported towards the cuticle and/or passively partitioned into the cuticular waxes. On the other hand, it should be noted that the phytosterols reportedly accumulate in specific membrane compartments such as plasma membranes (Borner *et al.*, 2005). This must lead to relatively concentrated pools of internal phytosterols, which might cause substantial contamination of surface extracts when solvent molecules accidentally reach inner parts of the tissue even if only locally. Consequently, it cannot be judged at present whether phytosterols are in fact present in cuticular wax mixtures, and if so what their cuticular concentrations are.

Similarly, other compounds like alkaloids and free C₁₆ and C₁₈ fatty acids might be extraction artefacts. To date, the alkaloids detected in surface extracts of fruit capsules of diverse *Papaver* species are the only nitrogen-containing cuticle constituents (Jetter and Riederer, 1996). It has been pointed out that for each poppy species only those alkaloids carrying methoxy groups instead of hydroxyls, that is, with the lowest polarities, were found in the extracts. Even though this observation suggests passive partitioning of alkaloids into the cuticular wax, direct proof for their presence in the cuticle is missing. The same holds true for palmitic and stearic acids, abundant fatty acids in all tissues that have repeatedly been reported in plant surface extracts (Walton, 1990). Even though it seems plausible that these compounds accumulate in cuticular waxes, their localisation in or on the cuticle needs to be confirmed by *in situ* investigations.

Finally, in some special cases, compounds other than waxes may be present at the plant surface. The diterpenoids exuded by glandular trichomes on leaves of *Nicotiana* species may serve as one example (Severson *et al.*, 1984). Even though

these compounds are produced locally, they spread across the entire leaf surface and cover it with a fairly thick, homogenous film, probably consisting of polymerised oxidation products. As a second example, the surface of some fern fronds (Wollenweber, 1982; Wollenweber *et al.*, 1993) and of diverse angiosperm leaves, especially in the Primulaceae and Lamiaceae (Wollenweber and Dietz, 1981), are dusted over by a thick farinose layer of flavonoids, deposited as relatively large particles. Due to their comparatively high polarity and the molecular structure that is clearly differing from typical wax compounds, it seems unlikely that these flavonoids are exported together with wax.

4.3 Spontaneous reactions of cuticular wax constituents

Large portions of the wax mixtures consist of hydrocarbon chains, constituting the alkyl moieties of the diverse compound classes described earlier. As the reaction repertoire of these parts of the cuticular waxes is very limited, virtually no spontaneous chemical changes are to be expected on the hydrocarbon chains. On the other hand, the functional groups of the wax constituents might engage in spontaneous reactions within the cuticle, and could thus lead to gradual modifications of the wax composition.

Based on purely chemical arguments, a number of reactions seem feasible for wax constituents. These include ester bond formation or cleavage, photodimerisation of compounds with C=C double bonds, hemiacetal and acetal formation, aldol and Claisen condensations, as well as redox transformations involving alcohols, aldehydes/ketones and fatty acids. As plant cuticles are in some cases exposed to extreme climatic conditions for long periods of time during the lifetime of individual tissues, speculations on such chemical reactions have repeatedly been put forward. It is a well-documented fact that plant surfaces frequently experience relatively high temperatures, intense irradiation of (partially high energy) light and substantial concentrations of singlet oxygen as an oxidizing agent (Jones, 1992). On the other hand, various other parameters necessary to describe the chemical environment, for example, the concentration of water and other reagents, or the presence of catalysts like metal ions, acids or bases have not been monitored accurately to date. It is therefore impossible to predict whether spontaneous reactions of cuticular waxes are likely to occur.

From a practical point of view, spontaneous wax transformations seem to be rare. In the numerous wax analyses performed to date, systematic changes in wax composition as a function of time have scarcely been reported. Instead, fairly constant amounts of waxes and their constituents were found when the same species and organ was repeatedly investigated, in many cases using mature tissue of likely widely varying age. This is especially true for model species used in cuticle research, such as *T. aestivum*, *H. vulgare*, and *A. thaliana*. It has to be added, though, that due to the complex mixtures and substantial biological variability in their quantitative

composition (see Section 4.4), subtle changes caused by chemical reactions would likely have been missed in these studies.

Self-reaction products of very long-chain aldehydes have been reported for the wax mixtures of *Nepenthes alata* (Riedel *et al.*, 2003), *Oryza sativa* and *Saccharum officinarum* (Haas *et al.*, 2001), and flower wax of some decorative roses (Mladenova *et al.*, 1976). Both indirect evidence employing Fourier Transform Infrared (FTIR) spectroscopy and direct chromatographic evidence showed that these wax aldehydes are present as oligomeric or polymeric acetal forms. Although the corresponding monomers are readily released in hot solvent, it has not been possible to date to transform them back into acetals (Riedel *et al.*, 2003). This result suggests that special conditions exist during formation, export or accumulation of wax in the plant cuticle that allow reaction between aldehyde molecules. One key prerequisite for this reaction might be that adjacent molecules in the wax mixture are sufficiently aligned to bring the carbonyl groups into close contact.

To test the hypothesis of (possibly subtle) spontaneous reactions between wax components and the atmosphere, either analyses closely monitoring wax composition over time or artificial reaction set-ups mimicking the natural situation have to be performed. The first approach has been taken in only a few studies. As an example, the wax on upper surfaces of *P. laurocerasus* leaves was found to change dramatically during organ development (Jetter and Schäffer, 2001). To distinguish between ontogenetic changes and possible chemical reactions, in this case, the second approach was also used. The wax mixture was removed from young leaves without altering the molecular arrangement, deposited on a glass surface and exposed to weathering for two weeks. While the native plant surface experienced dramatic modifications in this period of time, the artificial set-up remained unchanged.

In two other investigations, artificial set-ups were also used to test specific reactions of individual wax compounds. In the first study, asymmetric secondary alcohols, being the major constituents in the (epi-)cuticular wax of gymnosperm needles, were exposed to various concentrations of air pollutants such as ozone, NO₂ and SO₂ (Jetter *et al.*, 1996a). Even though these gases are strong oxidizing agents, implied in direct oxidative damage to the plant surface, they did not react with the wax alcohols under ambient conditions. Only when applied in more than 1000-fold higher doses, could the expected oxidation products be detected (ketones and carboxylic acids generated by oxidative α -bond cleavage). In the second study, possible UV-induced photoreactions of cuticular phenylpropanoids were monitored using homologous series of *cis*- and *trans*-coumarates from beech leaf wax (Riederer and Markstädter, personal communication). The expected 2 + 2 cyclisation products were detected in the resulting mixture in very small concentrations, but only upon irradiation with high doses of UV radiation. In summary, all the experimental evidence currently available argues against the hypothesis that spontaneous reactions occur within the bulk of cuticular wax mixtures at any substantial scale.

In a TOF-SIMS (Time-Of-Flight Secondary Ion Mass Spectrometry) analysis of the top layer of molecules on *P. laurocerasus* leaves, some signals could not be explained with the known epicuticular wax constituents (Perkins *et al.*, 2004).

This raised the possibility that spontaneous transformations might be occurring in a very thin layer near the cuticle surface. Reactions thus restricted to the outermost surface would be of great biological importance, but have yet to be confirmed in more detailed studies.

4.4 Quantitative composition of cuticular waxes

4.4.1 Chain length distributions within compound classes

Most of the wax constituents described in the previous sections contain an alkyl moiety with one or more functional groups. Even in early chemical analyses of plant cuticular waxes, it was noticed that compounds within one compound class, for example, the primary alcohols and fatty acids, comprise complete series of compounds differing only in the number of methylene units (Chibnall *et al.*, 1931; Kreger, 1958; Juniper, 1959). These homologous series had characteristic patterns in the relative amounts of constituents. The compositional patterns have two aspects, relating to the ratio between structures with odd and even carbon numbers, on one hand, and to the overall chain length distribution on the other. Similar patterns have been found in the waxes of all plant species investigated to date and for all alkyl-containing wax compound classes alike (Baker, 1982).

In all the homologous series of wax constituents, a strong predominance of compounds with either odd or even carbon numbers has been found. Compound classes with preference for even carbon numbers are fatty acids, aldehydes and primary alcohols. Similarly, the acyl moiety of diverse esters (e.g. methyl, phenyl propyl, benzyl esters and alkyl esters) as well as the alkyl moiety of diverse other esters (e.g. alkyl acetates) is largely dominated by even-numbered homologues (for references see Section 4.2). Compound classes with preference for odd carbon numbers include alkanes, secondary alcohols and ketones as well as β -diketones and their hydroxy- and keto-derivatives. In summary, compound classes with a terminal functional group have a predominance of even-numbered homologues, whereas those lacking the primary functionality tend to have odd-numbered chains. This chemical classification likely reflects the biosynthetic relationships between wax compound classes, grouping at least fatty acids and primary alcohols along one pathway (Chapter 5). Loss of the terminal functional group together with its carbon atom leads to alkanes, secondary alcohols and ketones along another pathway.

Interestingly, the same patterns are found for multifunctional compound classes. For example, alkanediols and ketols were reported to have either even or odd carbon numbers depending on the position of their functional groups. Where both functionalities are located on mid-chain carbons, diols and ketols are largely dominated by odd-chain compounds (Holloway and Brown, 1977; Franich *et al.*, 1979). In contrast, diol and ketol series with one primary and one secondary functional group show a strong predominance of homologues with even carbon numbers (Jetter *et al.*, 1996b; Vermeer *et al.*, 2003).

The overall chain length distribution of wax compound classes is best summarised giving the range of chain lengths, the most abundant chain length and its relative portion in the class. The characteristics of homologous series in cuticular waxes can vary widely, to the extreme with one compound largely predominating and several other homologues being present only in trace amounts. As an example for this situation, the wax on inflorescence stems of *A. thaliana* shows a strong predominance of C₂₉ homologues of alkanes, secondary alcohols and ketones (Rashotte *et al.*, 1997). In other cases, relatively even distributions of five and more chain lengths have been reported. For example, the seed coat wax of *Fagus sylvatica* contains an especially wide array of alkane, primary alcohol and fatty acid homologues, each of them accounting for less than 35% of the fractions, respectively (Gülz *et al.*, 1989). In many plant species, the chain length distribution was found to match between compound classes, both in their chain length range and in relative homologue contributions. Consequently, the overall wax composition is in many cases dominated by a single chain length. Most frequently, wax mixtures with a pronounced preference for C₂₆, C₂₈, C₂₉, C₃₀ or C₃₂ compounds have been encountered.

The homologue pattern within compound classes and the resulting chain length profile of the overall wax mixture play important roles in the models for the physical structure of the cuticle. Two hypotheses have been put forward that describe the crystalline arrangement of wax molecules, either inside the cuticle or on its surface. The first hypothesis predicts that the chain length distribution of aliphatics influences the size and geometry of crystalline domains in the wax, and consequently limits the water flow across the cuticle (Riederer and Schreiber, 1995; Chapter 6–8). The primary physiological function of the cuticle is thus linked to the chain length distribution in the homologous series of wax constituents. The second hypothesis proposes that the accumulation of individual compounds near the cuticle surface (Baker, 1982), triggered by the strong predominance of a single compound class and homologue, causes the formation of epicuticular wax crystals (see Section 4.6.1). The ecological functions associated with these surface crystals (Chapters 10–12) are therefore also linked to the chain length distribution in the wax. In conclusion, the homologue pattern is a central parameter in our current understanding of the causal relationship between the chemical composition, the physical properties and the biological functions of the cuticle.

4.4.2 *Distribution of compound classes*

To further summarise wax chemical datasets beyond homologue distributions, the relative amounts of compound classes within the wax mixture are usually given as percentages. To this end, all the known compounds, as described in Section 4.2, can be categorised according to their functional groups. The wax constituents that remain unidentified should also be quantified and given as a separate class of compounds to complete the analysis. For most plant species, more than 80% of the mass of compounds within the cuticular wax mixture have been identified.

Relatively broad ranges of compound classes have been reported for cuticular waxes from some species, with no class predominating. For example, the wax on leaves of *Zea mays* was found to contain 9–42% each of alkanes, aldehydes, primary alcohols, fatty acids and alkyl esters (Bianchi *et al.*, 1984). It should be noted that waxes containing such a broad range of compounds, all at relatively small percentages, are exclusively found on tissues that do not have epicuticular wax crystals on their surface. In contrast, the wax mixtures from many other plant species were reported to contain high percentages of a single compound class. This situation may be illustrated by the composition of *H. vulgare* leaf wax, as it was found to contain 89% of primary alcohols together with only 0.2–9.2% of alkanes, aldehydes, fatty acids and alkyl esters (Giese, 1975). Interestingly, the leaf surfaces of this species are covered with epicuticular wax crystals, leading to the assumption that the high percentage of alcohols causes the formation of the surface structures.

Our current knowledge relies on analyses of relatively few plant species, and a description of compound classes grouped by plant taxa must necessarily be biased. Only a few selected examples may therefore suffice to illustrate the specific predominance of individual compound classes in diverse plant groups. Alkanes are the major constituents on leaves of Cactaceae [e.g. *Opuntia engelmannii* (Wilkinson and Mayeux, 1990)], and Brassicaceae [e.g. *B. oleracea* (Baker, 1974)], and in the abaxial leaf wax of *P. sativum* (Holloway *et al.*, 1977b). Asymmetric secondary alcohols dominate the wax composition of all gymnosperm needles (Riederer, 1989), of Papaveraceae leaves and fruit capsules (Jetter and Riederer, 1996) and of diverse Liliaceae, Ranunculaceae and Rosaceae (Holloway *et al.*, 1976). Triterpenoids tend to accumulate in the wax mixtures of a range of taxa, including Euphorbiaceae, Crassulaceae and Rosaceae (Walton, 1990). Diverse species of Fabaceae have leaf waxes with high percentages of primary alcohols (Baker, 1982), *Eucalyptus* leaf waxes are dominated by β -diketones (Horn *et al.*, 1964) and many Poaceae species have large quantities of either of these compound classes on various organ surfaces (Baum and Tulloch, 1982). The particularly well-studied grass species in the genera *Triticum*, *Hordeum* and *Secale* may thus serve as fine examples to illustrate the complexity of cuticular wax compositions, varying widely between surfaces of awns, fruits, stems, leaf sheaths and blades, and even depending on the position and side of the leaf. Finally, it should be noted that some compound classes, even though they are ubiquitous constituents of waxes, including the free fatty acids and aldehydes, have rarely been found in high percentages.

Analyses of single compound classes that lack information on the relative contribution of these compounds to the overall wax mixture have frequently been published. As the percentages of individual compound classes can vary widely, it cannot be determined whether these reports deal with major wax constituents or only with trace compounds. The resulting data therefore cannot contribute to our knowledge on cuticular wax composition, even though substantial effort was invested on these analyses of various interesting species.

4.4.3 Wax amounts

On a third level of the chemical datasets, absolute quantities of cuticular wax loads have to be considered. They are usually given in units of micrograms per square centimetre referring to the mass of wax per area of cuticle surface. Corresponding values have the advantage that they can be directly compared between species and organs. It has been shown that solvent molecules can enter deep into the cuticle within short times of immersion (Jetter *et al.*, 2000). Consequently, both epi- and intracuticular waxes are likely released, and the resulting mixtures must contain the total waxes rather than only the epicuticular portion. Even though many previous reports stated loads of epicuticular waxes, they should now be re-interpreted as giving the loads of total cuticular waxes instead.

Depending on the plant species and organ analysed, wax loads range from one to several hundred micrograms per square centimetre of surface area. In many cases, fruit cuticles have been found to contain higher amounts of waxes than the corresponding leaf waxes of the same species. For example, tomato fruits showed a wax coverage of $15 \mu\text{g}/\text{cm}^2$, while leaves had only $3 \mu\text{g}/\text{cm}^2$ of total cuticular wax (Vogg *et al.*, 2004). Numerous analyses of cuticular waxes also revealed that tissues possessing epicuticular wax crystals tend to have especially high wax loads. In many of these cases, $50\text{--}100 \mu\text{g}/\text{cm}^2$ of wax have been reported, and it must be concluded that a large portion of the wax mass is located in the surface crystals of these species.

Implicitly, values for the wax loads also give information on the thickness of the wax layer. This interpretation is based on the fact that all major wax constituents consist mainly of methylene units, causing fairly constant densities of approximately $0.8\text{--}1.0 \text{ g}/\text{cm}^3$ for the different compounds as well as the resulting mixture. A load of $1 \mu\text{g}/\text{cm}^2$ of pure wax would consequently correspond to a layer thickness of 10 nm. However, this estimate is directly applicable only to epicuticular wax films, as these are the only compartments containing pure wax. For species and on organs where a large portion of the wax is localised in the epicuticular wax film, the comparison of wax loads gives a direct comparison of layer thickness. For all other tissues, wax loads have to be interpreted in perspective with the amounts of cutin present. For a number of species, both wax and cutin amounts were quantified, and cutin was found to make up 40–80% of the cuticle mass (Schreiber and Riederer, 1996).

4.5 Dynamics of wax composition

In the previous sections of this chapter, the composition and quantity of plant cuticular waxes has been described as a fairly constant character of given plant species and tissues. This static view seems overly simplistic and has frequently been questioned. As plant cuticular waxes play pivotal physiological and ecological roles, it might be advantageous to adapt their composition and properties to fluctuating environmental

conditions. Such dynamic changes might occur on a number of levels, including individual compounds, compound classes or entire wax mixtures, and affect their relative percentages or absolute amounts. Variations might for example be visible in individual leaves according to the growth conditions during organ formation, or induced by environmental conditions after organogenesis. These possible dynamic effects must be distinguished from changes in wax composition caused by ontogenetic programs, that is, differential regulation of biosynthetic pathways during development.

Before dynamic effects can be evaluated, the intraspecific variability has to be assessed. In diverse studies, tissues have been systematically harvested from various plant individuals and analysed. Sample sizes typically ranged from $n = 3$ to 10, and in most cases the reported standard deviations were 5–20% of the average absolute amounts of individual wax compounds. Similar variabilities have been reported for the surface loads of compound classes as well as entire wax mixtures for a number of plant species.

A few studies have investigated the ontogenetic development of plant cuticular waxes using organs of different age on the same plant. For example, a comparison of leaves along a single branch of *Hedera helix* showed that the cuticular wax contained high percentages of alkanes, aldehydes, primary alcohols and fatty acids early on, and increasing amounts of alkyl esters during seasonal development (Hauke and Schreiber, 1998). In the most detailed ontogenetic study available to date, the epicuticular wax film on adaxial surfaces of *Prunus laurocerasus* leaves was investigated (Jetter and Schäffer, 2001). While the overall amount of waxes increased steadily over the 60 d period of leaf growth and maturation, the relative composition changed three times very drastically (Figure 4.5). In the first 10 d of leaf development alkyl acetates dominated the mixture and then declined gradually. They were replaced by larger amounts of primary alcohols that in turn diminished after approximately 10 d, when finally alkanes started accumulating to result in the mature surface composition. Very similar chemical developments have been found in *P. laurocerasus* plants grown in different years under various conditions. It must be concluded that the dynamic changes seen in this system reflect an ontogenetic programme that strictly regulates the biosynthesis and accumulation of surface compounds. Unfortunately, comparable data for other plant species are lacking, and it is therefore not clear whether *P. laurocerasus* shows exceptional dynamic changes during development, or whether similar effects are more widespread.

Further evidence for dynamic changes comes from experiments in which cuticular waxes on living tissue have been disturbed at one time and investigated again later. Due to the pronounced developmental effects of its leaf cuticular waxes, *P. laurocerasus* was again used as a model species. When the epicuticular wax film was removed from the leaves at various stages of development, it was in all cases regenerated within a few days and was found to have the composition typical for that next time in development (Jetter and Schäffer, unpublished results). This shows that regeneration is more than mere re-organisation of preformed compounds, but involves *de novo* biosynthesis of compounds according to the preset ontogenetic

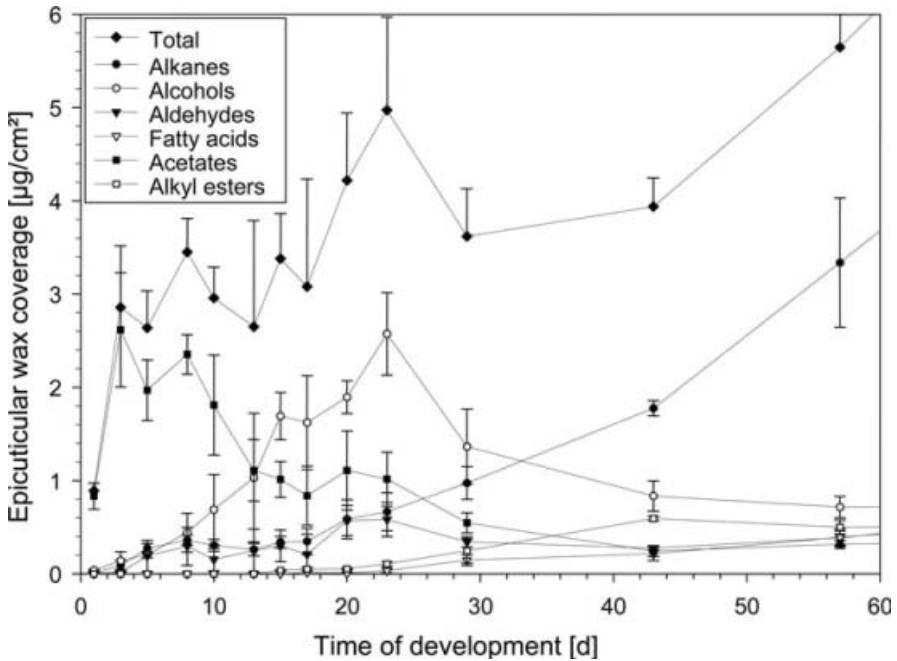


Figure 4.5 Development of the epicuticular wax layer on adaxial *Prunus laurocerasus* leaf surfaces. In the course of leaf growth and maturation, the composition of the epicuticular wax changes repeatedly. Initially alkyl acetates dominate, whereas primary alcohols accumulate to high concentrations between days 14 and 28 of the time course, and alkanes reach highest amounts much later.

program. In an SEM survey, regeneration of surface waxes has also been observed for certain plant species, either restricted to the still growing tissues or continued even after expansion had ceased (Neinhuis *et al.*, 2001). This might point to similar ontogenetic programmes acting in species other than *P. laurocerasus*. But this generalisation has to be cautious, because the microscopic results are only qualitative. They must be supported by quantitative information, e.g. chemical analyses comparing wax composition before the experiment, immediately after wax removal and after regeneration.

Finally, dynamic effects in cuticular wax composition induced by environmental conditions can be discussed. Extensive studies have been carried out to test the effects of light, temperature and air humidity during growth of leaves of *B. oleracea* (Baker, 1974). In this model system, growth conditions largely influenced the wax composition of the growing tissue. Most interestingly, both increased temperature and decreased relative humidity caused an increase in overall wax amounts, and a shift in percentages from alkanes and ketones to aldehydes and primary alcohols. Changes in irradiant light energy had comparatively little effect. These results are of great interest, as they suggest a feedback mechanism, maybe causing increased

wax accumulation under transpiration stress. Unfortunately, the transpiration barrier in *B. oleracea* leaves has not been characterised, and therefore the hypothesis that the physiological properties of the waxes of this species are affected by changes in composition cannot be tested at present.

Additional studies have addressed the effects of growth conditions on cuticular wax composition in other species as well. In one example, it was reported that drought caused a slight decrease in the percentage of alkanes in leaf wax of *Rosa x hybrida*, but no change in the overall wax amounts (Jenks *et al.*, 2001). Even though this effect was only very small, it is similar to the results for *B. oleracea* and might hence reflect a more general phenomenon. In another study, reduced light was found to decrease average chain lengths in various wax compound classes of *H. vulgare* leaves (Giese, 1975). Overall, relatively small effects of growth conditions have been found for plant species other than the *Brassica* species.

As summarised in Section 4.3, the wax compositions and quantities found on surfaces of diverse species are fairly stable characters, even though plants had been grown under various (uncontrolled) conditions. It therefore seems that, under normal circumstances and for many species, wax properties are largely controlled by genetic programmes rather than by environmental factors. The very limited information available to date suggests that the *Brassica* leaf wax composition is exceptionally responsive to changes in environmental conditions. It will be most interesting to study the effect of growth conditions on the regulation of wax biosynthesis once the necessary molecular tools become available, focusing on comparisons between *Brassica* and other model species.

4.6 Arrangement of plant cuticular waxes

In recent years, much progress has been made in combining chemical (see Sections 4.4 and 4.5) with structural (Chapter 2) information on plant cuticles, so that we can now clearly distinguish three layers within the cuticular wax defined as intracuticular wax, epicuticular wax film, and epicuticular wax crystals protruding from this film. All the available evidence suggests that molecules of wax components spontaneously self-arrange and segregate into the three layers. In the following section, we will summarise our current knowledge on (1) the composition of epicuticular wax crystals, (2) the processes leading to separation of epicuticular film and intracuticular wax and (3) the crystalline arrangement of wax molecules within all three cuticular compartments.

4.6.1 Formation of epicuticular crystals

Scanning electron microscopy surveys of various plants revealed a fascinating diversity in the shape, size and arrangement of epicuticular wax crystals (Jeffree, 1986; Barthlott *et al.*, 1998). Due to this diversity, questions arose regarding the composition, organisation, biogenesis and function of these surface structures. In an

initial effort to answer these questions, comparative studies were performed over more than three decades. They revealed good correlations between characteristic shapes and the predominance of certain compounds in the wax mixture. For example, the presence of platelet-shaped crystals in many cases coincided with high concentrations of primary alcohols in the total wax, while epicuticular tubules were correlated with nonacosan-10-ol, and threads with triterpenoids (Figure 4.6).

In a second approach, *in vitro* crystallisation experiments were performed with solutions of pure compounds or wax fractions containing them. They yielded structures that closely matched those on the plant surfaces in shape, size and arrangement (Jeffree *et al.*, 1975; Jetter and Riederer, 1994; Meusel *et al.*, 1999), showing that the presence of these components in high concentrations was the only and sufficient prerequisite for formation of the epicuticular wax crystals. These studies also confirmed that the characteristic shapes of crystals were due to the presence of certain compounds (Jeffree, 1986). Alternative hypotheses, explaining the shapes of epicuticular wax by its extrusion through pores of characteristic geometries (Hall and Donaldson, 1962), were ruled out.

In order to gain direct evidence for composition of surface wax crystals, methods for the selective sampling of epicuticular material had to be developed. Most of the previous experiments had employed surface extraction of the intact tissue with organic solvents. The solvents, however, do not only mobilise epicuticular wax, but also enter into deeper layers of the cuticle where they release intracuticular wax (Jetter *et al.*, 2000). Thus, extractive methods can only give information on total wax composition, and it was not clear whether the composition of the epicuticular wax structures differed from the intracuticular wax layer underneath. Jeffree (1996) and Ensikat *et al.* (2000) reported that frozen droplets of polar liquids can be used to mechanically remove epicuticular wax crystals from plant surfaces and transfer them onto artificial substrates. This new sampling method was then employed to probe the smooth epicuticular wax film on *P. laurocerasus* leaves (see Section 4.6.2), and for the first time epi- and intracuticular wax constituents could be quantified separately (Jetter *et al.*, 2000).

To date, mechanical sampling has been applied only in one study of epicuticular crystals – focusing on surfaces of *N. alata* pitchers (Riedel *et al.*, 2003). Parts of the inner walls of these pitchers are covered with wax platelets, creating a microscopically rough surface that is assumed to be slippery for insect feet and helps to catch prey in these carnivorous plants (Knoll, 1914). Previous analyses had been based on extraction of total waxes, and consequently did not allow the identification of the wax constituents involved in the formation of these crystals (Juniper *et al.*, 1989). The selective removal of epicuticular wax with frozen water as a cryo-adhesive demonstrated that the wax platelets consisted predominantly of the C₃₀ aldehyde triacontanal (Riedel *et al.*, 2003). This investigation presents the first direct evidence that the accumulation of a single compound is responsible for the formation of unique surface wax structures. At the same time, the results showed that wax platelets that are virtually identical in shape can be formed on different plant species either by primary alcohols or by aldehydes (Figure 4.6).

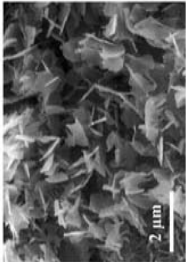

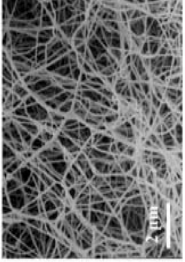
Crystal shape	Species	Main crystal component	Selected references
 <p>Platelets</p>	<i>Pisum sativum</i>	$\text{CH}_3-(\text{CH}_2)_{24}-\overset{\text{OH}}{\text{C}}-\text{H}$ Hexacosanol	Macey and Barber, 1970 Giесе, 1975
	<i>Hordeum vulgare</i>		
	<i>Allium porrum</i>	$\text{CH}_3-(\text{CH}_2)_{14}-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)_{14}-\text{CH}_3$ Hentriacontan-16-one	Rhee <i>et al.</i> , 1998
 <p>Tubules</p>	<i>Nepenthes alata</i> *	$\text{CH}_3-(\text{CH}_2)_{28}-\overset{\text{O}}{\parallel}{\text{C}}-\text{H}$ Triacontanal	Riedel <i>et al.</i> , 2003†
	<i>Papaver somniferum</i>		
	<i>Picea pungens</i>	$\text{CH}_3-(\text{CH}_2)_8-\overset{\text{OH}}{\text{C}}-\text{H}$ Nonacosan-10-ol	Jeffree <i>et al.</i> , 1975‡ Jetter and Riederer, 1994* Barthlott <i>et al.</i> , 1996
	<i>Nelumbo nucifera</i>		Neinhuis and Jetter, 1995
	<i>Pogonatum umigerum</i>		Wen and Jetter (unpublished)
 <p>Threads</p>	<i>Taxus baccata</i> *		Horn <i>et al.</i> , 1964 von Wettstein-Knowles and Netting, 1976a
	<i>Eucalyptus globulus</i>	$\text{CH}_3-(\text{CH}_2)_{12}-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)_{14}-\text{CH}_3$ Hentriacontane-14,16-dione	
	<i>Hordeum vulgare</i>		
	<i>Dudleya brittonii</i>	$\beta\text{-Amyrin acetate}$	Manheim and Mulroy, 1978
	<i>Macaranga tanarius</i> *	$\beta\text{-Amyrin}$	Markstädtler <i>et al.</i> , 2000
	<i>Ficus communis</i> *	Lupeol	Hobl, Guhling and Jetter (unpublished)‡

Figure 4.6 Examples for characteristic shapes of epicuticular wax crystals caused by their main components. Platelets are formed either by primary alcohols, ketone or aldehydes, tubules are formed by asymmetric secondary alcohols (nonacosan-10-ol) or by β -diketones, while threads are formed by triterpenoids. The SEMs shown are examples from the species marked by an asterisk (*). Evidence for crystal-forming compounds is provided by correlation (unmarked references), by *in vitro* reconstitution experiments (references marked by †), or by selective crystal sampling and analysis (references marked by ‡).

In the epicuticular wax of *N. alata* pitchers, small amounts of primary alcohols with a broad chain length distribution and tetracosanoic acid (C₂₄ fatty acid) were also detected. The same compounds were present in the underlying intracuticular wax layer, albeit in much higher concentrations. In contrast, aldehydes were found at much lower concentration in the intracuticular wax than in the epicuticular crystals of *N. alata* (Riedel *et al.*, 2003). In summary, gradients of aldehydes and counter-gradients of alcohols and fatty acids exist between intra- and epicuticular wax of this species.

All the current results taken together, crystal formation can be interpreted as a spontaneous physical process of phase separation within the cuticular wax mixture (Jetter and Riederer, 1994). Once the crystal-forming compound accumulates above a critical concentration in the mixture, it starts to form a separate solid phase. From comparative studies of related plant species with/without epicuticular wax crystals, the threshold concentrations for some crystal-forming compounds can be assessed. Jetter and Riederer (1996) accordingly estimated that nonacosan-10-ol must be accumulated beyond 40% before tubular crystals start to form. Above that threshold composition, crystal-forming compounds will accumulate in the crystals. The crystals consequently differ dramatically from the bulk wax composition.

In a number of cases, a clear correlation between single dominating wax constituents and the shape of epicuticular crystals could not be established (Figure 4.7). Conflicting evidence has, for example, been reported

- (1) for *B. oleracea*, where three different compound classes together form the surface structures,
- (2) for *B. oleracea* and *Arabidopsis thaliana*, as they have very distinct surface features but share similar wax composition,
- (3) for transversely ridged rodlets that have very similar appearance on the surfaces of diverse plant species, even though they consist either of alkanes, or of symmetrical or asymmetrical ketones and
- (4) for one of these compounds, hentriacontan-16-one, as it can alternatively also form platelet-shaped crystals on *Allium porrum* (Rhee *et al.*, 1998).

Based on this information, it seems likely that crystals with the characteristic shapes of dendrites, longitudinal bundles of rodlets and transversely ridged rodlets are formed as a consequence of local crystallisation conditions together with chemical composition. To date, there is no direct evidence for the composition of these epicuticular structures, and it cannot be judged whether they differ from the underlying intracuticular layer.

4.6.2 Chemical differences between epicuticular film and intracuticular wax

The compositional gradients between surface crystals and the underlying wax mixture led to speculations that similar differences might exist between epicuticular wax films and the intracuticular layer. Therefore, a distinction between intra- and

Crystal shape	Species	Main crystal component	Selected references
Dendrites	<i>Brassica oleracea</i> *	$\text{CH}_3-(\text{CH}_2)_{13}-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)_{15}-\text{CH}_3$ Nonacosan-15-one	Baker, 1974 Holloway <i>et al.</i> , 1977a
		$\text{CH}_3-(\text{CH}_2)_{27}-\text{CH}_3$ Nonacosane	
Longitudinal bundles of rodlets	<i>Arabidopsis thaliana</i> *	$\text{CH}_3-(\text{CH}_2)_{15}-\overset{\text{OH}}{\mid}{\text{C}}-(\text{CH}_2)_{15}-\text{CH}_3$ Nonacosan-15-ol	Jenks <i>et al.</i> , 1995 Rashotte <i>et al.</i> , 1997 Rashotte <i>et al.</i> , 2001
		$\text{CH}_3-(\text{CH}_2)_{27}-\text{CH}_3$ Nonacosane	
Transversely ridged rodlets	<i>Aristolochia gigantea</i> <i>Liriodendron tulipifera</i>	$\text{CH}_3-(\text{CH}_2)_{13}-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)_{13}-\text{CH}_3$ Nonacosan-15-one	
		$\text{CH}_3-(\text{CH}_2)_{13}-\overset{\text{OH}}{\mid}{\text{C}}-(\text{CH}_2)_{13}-\text{CH}_3$ Nonacosan-15-ol	
Transversely ridged rodlets	<i>Gypsophila acutifolia</i>	$\text{CH}_3-(\text{CH}_2)_{14}-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)_{14}-\text{CH}_3$ Hentriacontan-16-one	Meusel <i>et al.</i> , 1999 [§] Gülz <i>et al.</i> , 1992
		$\text{CH}_3-(\text{CH}_2)_{25}-\text{CH}_3$ Hentriacontane	Meusel <i>et al.</i> , 1999 [§]
Transversely ridged rodlets	<i>Osmunda regalis</i> *	$\text{CH}_3-(\text{CH}_2)_{18}-\overset{\text{O}}{\parallel}{\text{C}}-(\text{CH}_2)_{18}-\text{CH}_3$ Nonacosan-10-one	Jetter and Fiederer, 2000
		$\text{CH}_3-(\text{CH}_2)_{25}-\text{CH}_3$ Hentriacontane	

Figure 4.7 Examples for characteristic shapes of epicuticular wax crystals caused by their main components together with the (local) crystallisation conditions. The same compounds form different crystal shapes on *Brassica oleracea* leaves and *Arabidopsis thaliana* inflorescence stems, while various compounds form similar crystals in the shape of transversely ridged rodlets on the leaf surfaces of *Aristolochia gigantea*, *Gypsophila acutifolia* and *Osmunda regalis*. The SEMs shown are examples from the species marked by an asterisk (*). Evidence for crystal-forming compounds is provided by correlation (unmarked references), or by *in vitro* reconstitution experiments (references marked by †).

epicuticular wax was attempted, initially by variations of solvent extraction methods (Silva Fernandes *et al.*, 1964; Baker and Procopiu, 1975). Wax released by very brief (surface) extraction of the intact tissue was considered as epicuticular, while thorough extraction of isolated cuticular membranes was performed in order to analyse the intracuticular wax constituents. Later, films of cellulose acetate or nitrocellulose were employed to remove waxes mechanically from the plant surface (Baker *et al.*, 1983; Haas and Rentschler, 1984). In all these studies, gradients in the percentages of individual compounds were detected, suggesting chemical differences between the intracuticular wax and the epicuticular film. But, unfortunately, even the mechanical probing protocols involved organic solvents that probably led to the (partially) mixed extraction of intra- and epicuticular waxes. Therefore, none of the methods was sufficiently selective to allow a reliable quantification of the compositional gradients between wax layers.

The development of the cryo-adhesive technique (see Section 4.6.1) allowed for the first time the sampling of epicuticular wax films from plant surfaces with high selectivity. This new method was employed to probe the smooth epicuticular wax film on *P. laurocerasus* leaves and to quantify its constituents (Jetter *et al.*, 2000). Steep gradients between the epi- and intracuticular wax layers were detected, proving that the mechanical sampling technique has a much higher selectivity than the previously used extractive protocols. Based on these quantitative results, the selectivity of other mechanical sampling techniques were tested, and aqueous solutions of gum arabic established as a second adhesive for the selective removal of epicuticular waxes (Jetter and Schäffer, 2001).

Employing cryo-adhesive sampling, the epicuticular wax load on adaxial cuticles of mature *P. laurocerasus* leaves was found to be $13 \mu\text{g}/\text{cm}^2$ (Jetter *et al.*, 2000). With a total wax coverage of $28 \mu\text{g}/\text{cm}^2$ and a cutin matrix of $333 \mu\text{g}/\text{cm}^2$ (Schreiber and Riederer, 1996), the epicuticular wax film accounted for less than 4% of the cuticular material. The thickness of the epicuticular layer could be estimated based on the amount of material removed (approximately 130 nm), and the result agreed well with the thickness measured using SEM (<200 nm). The epicuticular wax mixture consisted exclusively of aliphatic constituents in a characteristic pattern of alkanes, alcohols, aldehydes, fatty acids and alkyl acetates. In sharp contrast, the intracuticular wax contained large amounts of triterpenoids together with small percentages of aliphatics. Consequently, two chemically and morphologically distinct layers are present within the *P. laurocerasus* leaf cuticle. In tomato fruits (Vogg *et al.*, 2004) and several other species (Vermeer and Jetter, unpublished results) a similar segregation of triterpenoids within the intracuticular layer has been found, while epicuticular wax mixtures again consisted exclusively of aliphatics.

The layered structure of cuticular waxes might be explained by the sequential formation and/or deposition of the different constituents. Accordingly, the aliphatic compounds found at the surface of mature leaves should accumulate first, that is, early during leaf development, while the triterpenoids would be later deposited in a lower layer. To test this hypothesis, a detailed investigation into the ontogenetic development of *P. laurocerasus* leaf surfaces was undertaken (Jetter and

Schäffer, 2001). In the course of leaf development, the epicuticular wax composition changed continuously (Figure 4.5), while the composition of the intracuticular wax did not change, the triterpenoids being constantly present in very high amounts. These results showed that in *P. laurocerasus* chemical differences between epi- and intracuticular waxes are not due to sequential addition of layers during development, but must arise from diffusion of compounds and spontaneous phase separation. The self-diffusion of very long-chain aliphatic molecules in wax mixtures (Schreiber and Schönherr, 1993) is fast enough to account for the developmental changes observed in the *P. laurocerasus* leaf cuticle.

4.6.3 Crystalline arrangement of epi- and intracuticular wax molecules

Investigations using FTIR and nuclear magnetic resonance (NMR) spectroscopy, X-ray diffraction and Differential Scanning Calorimetry (DSC) have revealed that in mixtures of (synthetic) long-chain aliphatic compounds most of the methylene groups are packed in a dense lattice with crystalline order (Le Roux, 1969; Basson and Reynhardt, 1992). In contrast, the chain ends of the hydrocarbon molecules are in an amorphous state with little lattice order, giving rise to typical wax characteristics like softness and broad melting ranges. As plant cuticular waxes consist of very long-chain aliphatic mixtures with (in some cases) extremely broad chain length distributions, it seems plausible that they should be amorphous in large parts and exhibit properties of soft material. This general notion has to be evaluated using quantitative information on the relative portions of crystalline and amorphous zones of plant waxes at physiological temperatures.

Early X-ray powder diffraction studies showed that plant cuticular aliphatics are in an all-*trans* conformation (linear zig-zag backbones) and aligned in parallel (Piper *et al.*, 1931, 1934; Kreger and Schamhart, 1956). As sharp diffraction signals were obtained for all the plant waxes investigated, it became clear that at least portions of the mixtures had crystalline order (Kreger, 1948). Unfortunately, the relative percentages of crystalline and amorphous wax parts were not assessed in these investigations. DSC, X-ray diffraction, solid state NMR and FTIR techniques have since been employed to quantify their relative amounts. For cuticles that are devoid of surface crystals, hence containing relatively large portions of intracuticular wax, the aliphatic components showed crystallinities between 20% (*Citrus aurantium*), 30% (*F. sylvatica*), 58% (*Juglans regia*) and 74% (*Hedera helix*) (Reynhardt and Riederer, 1991, 1994; Merk *et al.*, 1998). Thus, the intracuticular wax can be described as a mosaic of amorphous and crystalline zones. Diffusion of molecules and spontaneous phase separation suffice to explain the segregation into these domains. Currently nothing is known about the geometry and relative arrangement of these (likely submicroscopic) zones within the intracuticular layer.

The molecular order in epicuticular waxes can be assessed using overlapping sets of compositional data and X-ray diffractograms for diverse plant species representing several of the types of surface shapes, for example, platelets, tubules and threads (Kreger, 1948; Meusel *et al.*, 2000). It can be assumed that in these cases

the epicuticular wax layer contains by far the largest portion of the wax mixture, and, therefore, the crystallographic data will largely reflect the molecular arrangement in respective surface wax structures. In most cases these epicuticular structures contain predominant single components and exhibit relatively little chain length variation (see Section 4.6.1). This leads to the conclusion that the structures must have high lattice order even in the regions of chain termini and consequently relatively large crystalline domains. Comparatively small amorphous domains are likely due to the co-crystallising admixtures that are present only in low percentages. To appropriately describe the high degree of molecular order in all these surface structures, they may be called 'epicuticular wax crystals'. The same holds true for epicuticular wax structures formed by multiple compounds.

In summary, spontaneous self-arrangement of wax molecules leads to substantial crystalline order both within the epicuticular crystals and the intracuticular layer. This conclusion raises the question whether continuous crystalline zones exist between both cuticle compartments. To date, no studies have been performed that separately address the crystalline nature of intracuticular wax, epicuticular film and surface crystals of one species. It would be interesting to compare the molecular arrangement in these layers, possibly employing wax biosynthetic mutants, to judge the continuity between crystalline domains in the different cuticle compartments.

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5 Biosynthesis and transport of plant cuticular waxes

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5.1 Introduction

Our current knowledge of the wax biosynthetic pathways is a result of almost 80 years of research during which major discoveries arose in several distinct stages, usually coupled with the development of new analytical techniques or scientific approaches. Identification of the first wax components dates back to the 1920s when Clenshaw and Smedly-Maclean (1929) reported the isolation of the C₃₁ alkane *n*-hentriacontane from spinach (*Spinacia oleracea*), and Channon and Chibnall (1929) discovered the C₂₉ alkane *n*-nonacosane and the C₂₉ ketone nonacosan-15-one in cabbage (*Brassica oleracea*). Isolation and identification of additional wax constituents from several sources followed, and by the mid 1930s, first pathways for their biosynthesis were proposed [reviewed by Kolattukudy (1970a)]. After the initial flurry of activity, this area of research lay dormant for over 30 years until the mid-1960s. At that time systematic biochemical investigations by Kolattukudy and co-workers using radiolabelled precursors and a variety of chemical inhibitors led to elucidation of a number of biosynthetic steps involved in the formation of aliphatic wax components, and a more complete general picture of the wax biosynthetic pathways started to emerge. In the next phase, significant contributions to the field came from genetic and biochemical investigations of barley (*Hordeum vulgare*) mutants by von Wettstein-Knowles. This research most notably provided experimental evidence for the involvement of multiple chain elongating systems in wax biosynthesis and the existence of the β -ketoacyl elongation pathway for the synthesis of β -diketones and alkan-2-ols primarily in monocots. Finally, during the last decade, substantial progress in our understanding of this subject is due to concerted biochemical and genetic research efforts in *Arabidopsis thaliana* and *Zea mays*, and most recently the use of powerful genomic tools available in *A. thaliana*. In this chapter, we summarise the current view of cuticular wax biosynthesis and its transport to the plant surface.

5.2 Synthesis of very long-chain fatty acid wax precursors

Saturated very long-chain fatty acids (VLCFAs; Kolattukudy, 1966), with chain lengths between C₂₀ and C₃₄, are the precursors for the biosynthesis of aliphatic cuticular wax components, such as primary and secondary alcohols, aldehydes,

alkanes, ketones and alkyl esters (Chapter 4). Formation of VLCFAs is a complex process that requires the coordinated activity of several enzyme complexes in different cellular compartments (plastid, cytoplasm and endoplasmic reticulum; Section 5.6). The first phase, the *de novo* fatty acid synthesis of C₁₆ and C₁₈ acyl chains is carried out by the well-characterised soluble fatty acid synthase complex (FAS) localised in the plastid stroma (Figure 5.1) (Ohlrogge *et al.*, 1993; Ohlrogge and Browse, 1995). Fatty acid synthesis proceeds through a cycle of four reactions: condensation of a C₂ moiety originating from malonyl-ACP to acyl-ACP, followed by the reduction of β -ketoacyl-ACP, the dehydration of β -hydroxyacyl-ACP and the reduction of *trans*- Δ^2 -enoyl-ACP. In each cycle the acyl chain, attached to acyl carrier protein, is extended by two carbons. Three different FAS complexes participate in the production of 18-carbon fatty acids in the plastid. They differ in their condensing enzymes which have strict acyl chain length specificities: KASIII (C₂ to C₄) (Clough *et al.*, 1992), KASI (C₄ to C₁₆) and KASII (C₁₆ to C₁₈) (Shimakata and Stumpf, 1982). Thus, KASIII initiates fatty acid biosynthesis with acetyl-CoA as a substrate, KASI extends the chain to C₁₆, and KASII completes the chain elongation to C₁₈. The two reductases and the dehydratase have no apparent acyl chain length specificity and are shared by all three plastidial elongation complexes (Stumpf, 1984).

The second phase, the extension of the ubiquitous C₁₆ and C₁₈ fatty acids to VLCFA chains used for the production of cuticular wax components, is catalysed by fatty acid elongases (FAE) (von Wettstein-Knowles, 1982), multienzyme complexes bound to the endoplasmic reticulum (ER) (Xu *et al.*, 2002; Kunst and Samuels, 2003; Zheng *et al.*, 2005). To reach the fatty acid elongation sites at the ER, saturated C₁₆ and C₁₈ acyl groups first have to get hydrolysed from the ACP by an acyl-ACP thioesterase, then exported from the plastid by an unknown mechanism, and esterified to CoA. Based on amino acid sequence similarity and substrate specificity, two classes of acyl-ACP thioesterases, designated FATA and FATB, have been described in plants. The FATA class exhibits a strong preference for 18:1-ACP *in vitro*, while the FATB thioesterases predominantly use saturated fatty acids (Voelker *et al.*, 1996). The *in planta* role of the FATB class was assessed in an *A. thaliana* mutant disrupted in the *FATB* thioesterase gene (Bonaventure *et al.*, 2003). Analyses of leaf and stem cuticular wax in the *fatb* line exhibited 20 and 50% reductions in total wax load, respectively, indicating that the FATB thioesterase supplies saturated fatty acids for the synthesis of wax precursors in epidermal cells (Figure 5.1).

During export from the plastid, free fatty acids are esterified to CoASH by a long-chain acyl-CoA synthetase (LACS). This increases their solubility in the aqueous compartment of the cytoplasm and prevents resorption into the plastid. The fatty acyl-CoA thioesters can then be used for fatty acid elongation or glycerolipid biosynthesis. A family of nine LACS isozymes has been detected in *A. thaliana* (Shockey *et al.*, 2002). One of the isozymes, LACS9, was shown to be localised in the plastid envelope and suggested to be the major plastidial acyl-CoA synthetase (Schnurr *et al.*, 2002). Surprisingly, a T-DNA insertion in the *LACS9* gene in

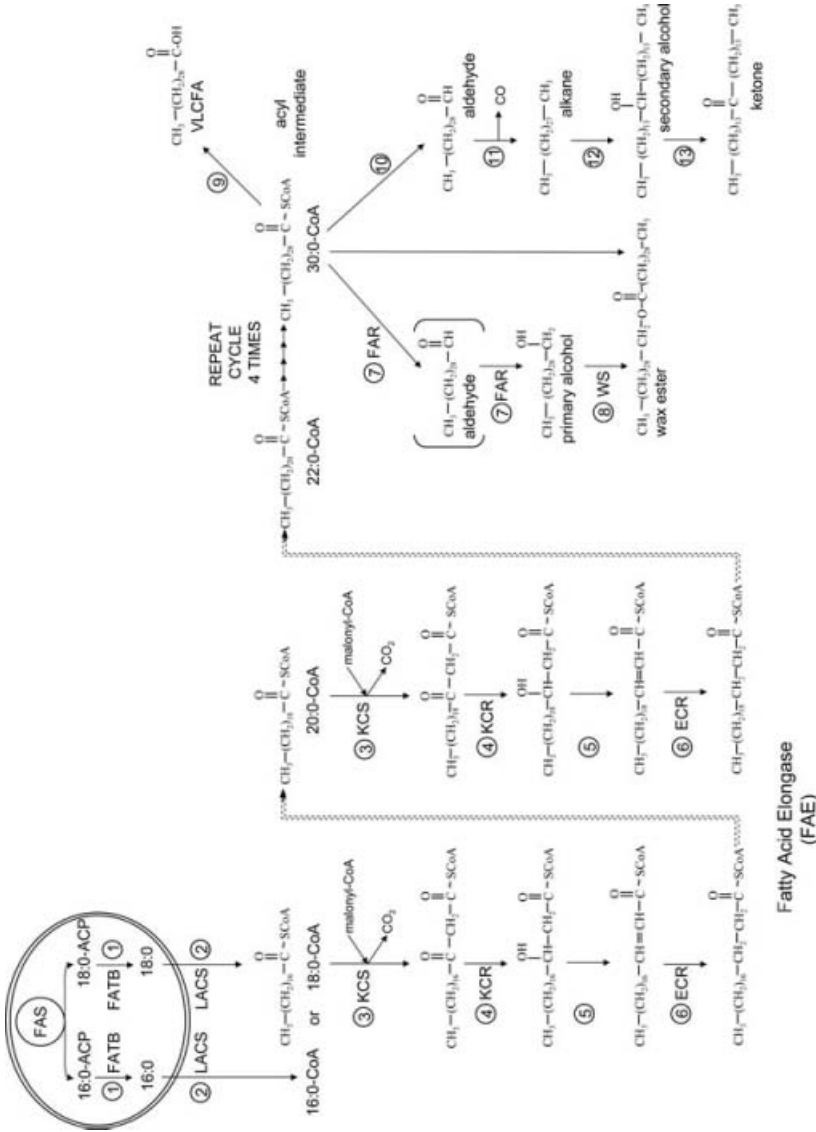


Figure 5.1 Biosynthetic pathways for the formation of very long-chain acyl-CoAs (VLCFAs), and their conversion into aliphatic wax constituents, C₁₆ and C₁₈ fatty acyl-ACPs formed by the FAS in the plastid are hydrolysed by an (1) acyl-ACP thioesterase (FATB), and during transport through the plastid envelope esterified to CoASH by a (2) long-chain acyl-CoA synthetase (LACS). C₁₆ and C₁₈ fatty acyl-CoAs undergo successive elongations, each extending the acyl chain by C₂ units, carried out by the enzymes of the fatty acyl elongase (FAE): (3) β-ketoacyl-CoA synthase (KCS), (4) β-ketoacyl-CoA synthase (KCR), (5) putative β-hydroxyacyl-CoA dehydrase and (6) enoyl-CoA reductase (ECR). The VLCFA wax precursors, C₂₀–C₃₄ long, are then used for the production of primary alcohols and esters by the (7) fatty acyl reductase (FAR) and (8) wax synthase (WS), respectively, as well as aldehydes, alkanes, secondary alcohols and ketones by currently uncharacterised enzymes (10–13). Enzymatic steps (1)–(13) shown in this figure are described in more detail in Table 5.1. Chemical formulas for one representative compound from each lipid class are shown.

the *lacs9-1* mutant did not affect the export of acyl groups from the plastid or cause any changes in plant growth or development. Analysis of another gene, *LACS2*, showed that its expression in leaves was limited to the epidermis. Characterisation of the *lacs2* null mutant revealed altered cuticle permeability and reduced thickness of the cutin layer on the abaxial surface of leaves, but the mutation had no effect on the wax load or composition. These data, together with evidence that *LACS2* shows good activity with 16-hydroxypalmitate substrate *in vitro* led to a proposal that this enzyme was involved in the production of ω -hydroxy fatty acyl-CoA intermediates for cutin biosynthesis (Schnurr *et al.*, 2004). Thus, even though two *LACS* candidates have been characterised to some extent, these studies failed to identify the isozyme involved in the CoA activation of acyl precursors required for wax production.

Elongation of long-chain fatty acids (C_{16} , C_{18}) to VLCFA involves four consecutive enzymatic reactions analogous to those of the FAS (Figure 5.1), and results in a two carbon extension of the acyl chain per cycle. However, unlike the FAS where the C_2 units for the condensation reaction are donated by malonyl-ACP, the C_2 donor for FAE is malonyl-CoA, generated by the multifunctional extraplastidial acetyl-CoA carboxylase (ACCase). In *A. thaliana* two genes, *ACC1* and *ACC2*, encode the multifunctional ACCases. Even though these genes appear to be ubiquitously expressed, molecular genetic studies of mutants disrupted in the *ACC1* gene unequivocally established that *ACC1* is involved in supplying malonyl-CoA for VLCFA elongation (Baud *et al.*, 2003, 2004).

The chain lengths of aliphatic wax components are typically in the range of 20–34 carbons; thus multiple elongation cycles are needed to extend the acyl chain to the final length. The differential effects of inhibitors on incorporation of radiolabelled precursors into wax components of various chain lengths, and analyses of mutants with defects in fatty acid elongation, demonstrated that sequential acyl chain extensions are carried out by several distinct elongases with unique substrate chain length specificities (von Wettstein-Knowles, 1993). Specificity of each elongation reaction resides in the elongase condensing enzyme (Lassner *et al.*, 1996; Millar and Kunst, 1997). Consistent with the requirement for fatty acyl precursors of various chain lengths for the synthesis of cuticular wax lipids, a large family of 21 elongase condensing enzyme-like sequences has been identified in the *A. thaliana* genome (Dunn *et al.*, 2004). An unrelated *ELO*-like gene family of putative condensing enzymes, related to the *Saccharomyces cerevisiae* condensing enzymes ELO1, ELO2 and ELO3, was also annotated (Dunn *et al.*, 2004). It is not known how many of these putative condensing enzymes participate in wax production and how many different condensing enzymes are needed for the elongation of a C_{18} to a C_{34} fatty acyl CoA, since elongase condensing enzymes can catalyse multiple elongation steps. The only wax-specific condensing enzyme characterised to date is CER6 (Millar *et al.*, 1999; Fiebig *et al.*, 2000; Hooker *et al.*, 2002) that catalyses the elongation of fatty acyl-CoAs longer than C_{22} (Zheng *et al.*, 2005) and is expressed in the epidermis, which is the site of wax biosynthesis (Kolattukudy, 1968).

Table 5.1 Summary of enzymatic steps involved in cuticular wax biosynthesis. Current knowledge of the enzymes catalysing individual reactions is provided, and the information on cloned genes encoding these enzymes, wherever available, is given.

Reaction in Figure 5.1	Enzyme and abbreviation	Biochemical evidence	Molecular evidence	Genes cloned	References
①	Acyl-ACP thioesterase FATB class (FATB)	<i>In vitro</i> substrate preference of the <i>A. thaliana</i> FATB 16:0 > 18:1 > 18:0 > 14:0; <i>A. thaliana</i> mutant phenotype	Demonstrated role in supplying saturated fatty acids for wax biosynthesis in <i>A. thaliana</i>	<i>FATB</i> (<i>A. thaliana</i>)	Bonaventure <i>et al.</i> , 2003 Voelker 1996
②	Long chain acyl-CoA synthetase (LACS)		Suggested role in activation of fatty acids to acyl CoAs during their export from the chloroplast in <i>A. thaliana</i>	<i>LACS9</i> (<i>A. thaliana</i>)	Schnurr <i>et al.</i> , 2002
③	β -Ketoacyl-CoA synthase (KCS)		Demonstrated in vivo role in elongation of fatty acyl chains for wax biosynthesis in <i>A. thaliana</i>	<i>CER6</i> (<i>A. thaliana</i>) <i>KCS1</i> (<i>A. thaliana</i>)	Millar <i>et al.</i> , 1999 Fiebig <i>et al.</i> , 2000 Todd <i>et al.</i> , 1999
④	β -Keto-acyl-CoA reductase (KCR)		Demonstrated in vivo role in elongation of fatty acyl chains for wax biosynthesis in <i>Z. mays gl8</i> mutants	<i>GL8A</i> (<i>Z. mays</i>) <i>GL8B</i> (<i>Z. mays</i>)	Xu <i>et al.</i> , 1997 Xu <i>et al.</i> , 2002 Perera <i>et al.</i> , 2003 Dietrich <i>et al.</i> , 2005
⑤	Putative β -hydroxyacyl-CoA dehydratase	Identification of the metabolic intermediates of fatty acyl CoA elongation demonstrated the requirement for a dehydratase	Molecular identity of the proposed dehydratase enzyme/gene is unknown		Fehling and Mukherjee, 1991
⑥	Enoyl-CoA reductase (ECR)		Demonstrated in vivo role in elongation of fatty acyl chains for wax biosynthesis in <i>A. thaliana cer 10</i> mutants	<i>CER10</i> (<i>A. thaliana</i>)	Zheng <i>et al.</i> , 2005

⑦	Alcohol-forming fatty acyl reductase (FAR)	Solubilised 58 kDa protein from <i>P. sativum</i> microsomes catalyses (C ₁₆) alcohol formation <i>in vitro</i> ; expression of the jojoba FAR gene in <i>B. napus</i> and <i>E. coli</i> resulted in alcohol formation	Demonstrated <i>in vivo</i> role of NADPH-dependent alcohol-forming reductase in biosynthesis of fatty alcohols in <i>S. chinensis</i> (jojoba) embryos	FAR (<i>S. chinensis</i>)	Metz <i>et al.</i> , 2000 Vioque and Kolattukudy, 1997
⑧	Wax synthase (WS)	Expression of the jojoba WS gene in <i>A. thaliana</i> embryos resulted in the formation of alkyl esters	Demonstrated <i>in vivo</i> role in biosynthesis of alkyl esters in <i>S. chinensis</i> (jojoba) embryos	WS (<i>S. chinensis</i>)	Lardizabal <i>et al.</i> , 2000
⑨	Putative thioesterase	The presence of a relatively constant proportion of free VLCFAs in wax mixtures suggests a requirement for a thioesterase	Molecular identity of the proposed thioesterase enzyme/gene is unknown		
⑩	Putative aldehyde-forming fatty acyl reductase	Solubilised 28 kDa protein from <i>P. sativum</i> microsomes catalyses (C ₁₆) aldehyde formation <i>in vitro</i>	Molecular identity of the proposed reductase is unknown		
⑪	Putative decarboxylase	Conversion of aldehydes to alkanes with release of CO demonstrated <i>in vitro</i> using <i>P. sativum</i> microsomes	Molecular identity of the proposed decarboxylase is unknown		Cheesbrough and Kolattukudy, 1984
⑫	Putative hydroxylase	Enzyme activity shown in leaf tissue of <i>B. oleracea</i> by assay for hydroxylation of radiolabelled alkane yielding secondary alcohols	Molecular identity of the proposed hydroxylase is unknown		Kolattukudy <i>et al.</i> , 1973
⑬	Putative oxidase	Enzyme activity shown in leaf tissue of <i>B. oleracea</i> by assay for oxidation of radiolabelled secondary alcohols yielding ketones	Molecular identity of the proposed oxidase is unknown		Kolattukudy <i>et al.</i> , 1973

Unlike the condensing enzymes, the other three enzyme activities of the FAE are presumably identical in all plant VLCFA elongases. They have been suggested to have broad substrate specificities and generate acyl products of diverse chain lengths used to make different classes of lipids (Millar and Kunst, 1997), including cuticular waxes, seed triacylglycerols (TAGs) and sphingolipids. This hypothesis has not been tested directly until recently because the genes encoding the two reductases and the dehydratase have not been available.

Although the dehydratase remains unknown, the cloning and characterisation of the β -ketoacyl reductase (Beaudoin *et al.*, 2002) and enoyl reductase (Kohlwein *et al.*, 2001) genes from *S. cerevisiae* allowed the identification of the corresponding genes from plants. The maize *GLOSSY8* (*GL8*) gene was previously suggested to encode a reductase involved in fatty acid elongation (Xu *et al.*, 1997). Evidence presented by Beaudoin *et al.* (2002) and additional characterisation of the maize *glossy8* mutant (Xu *et al.*, 2002) confirmed that the maize *GL8* functions as a β -ketoacyl-reductase that is required for wax production. Further analyses of the maize genome revealed that there is another *GL8*-like gene present in maize (Perera *et al.*, 2003; Dietrich *et al.*, 2005). Both genes, named *GL8A* and *GL8B*, are not expressed exclusively in the epidermis, but also in internal tissues of a number of organs, suggesting that they may not be involved only in wax production. In addition, attempts to generate double mutants by crossing *gl8a* \times *gl8b* failed because embryos carrying both mutations were not viable. This information that β -ketoacyl-reductase activity is essential for normal development of maize kernels demonstrates that it has a more general role than wax biosynthesis (Perera *et al.*, 2003). It was suggested that reduced sphingolipid levels and altered composition of sphingolipids detected in the double mutant may be responsible for embryo lethality (Dietrich *et al.*, 2005).

A BLAST query of the *A. thaliana* genome database using the β -ketoacyl reductase sequence from *S. cerevisiae* (Beaudoin *et al.*, 2002) resulted in the identification of two putative homologues. One of these *A. thaliana* sequences was previously reported to be an orthologue of the maize *GLOSSY8* (*GL8*) gene, and therefore suggested to encode a reductase involved in fatty acid elongation (Xu *et al.*, 1997). Further molecular and biochemical characterisation of these genes/enzymes is required to determine their biochemical and biological functions.

An *A. thaliana* single-copy gene was identified as an enoyl-CoA reductase (*ECR*) candidate also based on its similarity to the yeast *tsc13* gene encoding the *ECR* (Kohlwein *et al.*, 2001). Heterologous expression of the putative plant *ECR* gene rescued the temperature-sensitive lethality of yeast *tsc13-1elo2* Δ cells (Gable *et al.*, 2004) demonstrating that it functions as an *ECR*. The *A. thaliana* *ECR* gene is ubiquitously expressed, and the protein physically interacts with the *ELO2P* and *ELO3P* condensing enzymes when expressed in yeast (Gable *et al.*, 2004). The *A. thaliana* *ECR* was shown to be identical to *CER10* (Zheng *et al.*, 2005) – the gene defective in one of the original *eceriferum* *A. thaliana* mutants identified by Koorneef *et al.* (1989). Biochemical analysis of the *cer10* mutant demonstrated that the *ECR* gene product is involved in VLCFA elongation required for the synthesis

of all the VLCFA containing lipids, including cuticular waxes, seed TAGs and sphingolipids (Zheng *et al.*, 2005). Surprisingly, knockout mutations in the single *A. thaliana* *ECR* gene are not lethal. Furthermore, in the absence of the *ECR* gene product, *cer10* mutants still accumulate 40% of wild-type cuticular wax, as well as VLCFAs in sphingolipids and seed TAGs. It is possible that the *ECR* identified on the basis of sequence similarity to the yeast *TSC13P* is not the only *ECR* in *A. thaliana*. Alternatively, unknown enzymes functionally similar to the *ECR* may complement the *CER10* deficiency by supporting VLCFA synthesis (Zheng *et al.*, 2005).

5.3 Biosynthetic pathways to monofunctional aliphatics

5.3.1 Synthesis of primary alcohols and wax esters

Primary alcohols are found in cuticular wax mixtures either as free alcohols or in the form of esters of fatty alcohols and fatty acids (Figure 5.1). From the time they were discovered in cuticular wax, primary alcohols were assumed to be produced by the reduction of fatty acyl-CoA. However, evidence for such a reduction was not available until the 1970s when the biosynthesis of alcohol from the fatty acyl-CoA using cell free preparations from *Euglena gracilis* was demonstrated (Kolattukudy, 1970b). Although the alcohol-generating reduction presumably proceeds through an aldehyde intermediate, a free aldehyde was not released, but could be demonstrated by chemical trapping (Kolattukudy, 1970b). In higher plants, the reduction of fatty acyl-CoAs to fatty alcohols was first reported for *B. oleracea* (Kolattukudy, 1971). Partial purification of reducing activities from *B. oleracea* leaves led to a proposal that primary alcohol production is a two-step process carried out by two separate enzymes – an NADH-dependent acyl-CoA reductase required for a reduction of fatty acids to aldehydes, and an NADPH-dependent aldehyde reductase required for a further reduction of aldehydes to primary alcohols (Kolattukudy, 1971). Initially, support for the involvement of two reductases in the conversion of fatty acids to alcohols came from the analyses of the *gl5* mutant of *Z. mays* in which a block in production of primary alcohols results in high accumulation of aldehydes (Bianchi *et al.*, 1978). However, further analyses of the *gl5* maize line revealed that it actually carried two mutations at different loci, subsequently designated *GL5* and *GL20* (von Wettstein-Knowles, 1995), and only lines homozygous for both mutations were glossy. Thus far wax analyses of the *gl5* and *gl20* single mutant lines have not been performed, and the mutated genes have not been identified. Without this information it is not possible to rationalise the biochemical phenotype observed in the original *gl5gl20* double mutant.

Important contributions to the study of alcohol forming fatty acyl-CoA reductases (FAR) have come from the study of developing jojoba seeds (*Simmondsia chinensis*). This is an unusual plant which produces wax esters comprised of long-chain alcohols and fatty acids as a seed lipid energy reserve. Biochemical studies by Pollard *et al.* (1979) indicated that in jojoba embryos alcohol formation from

fatty acid precursors requires a single FAR, and that it proceeds without the release of the free aldehyde. In support of this notion, Vioque and Kolattukudy (1997) purified two distinct reductase activities from pea (*Pisum sativum*) and demonstrated that the 58 kDa enzyme generates fatty alcohols without any detectable accumulation of fatty aldehyde intermediates, as first described in *E. gracilis* (Kolattukudy, 1970b). These results were verified by solubilisation and biochemical characterisation of a FAR from the jojoba embryos and cloning of the corresponding cDNA. Expression of the jojoba FAR cDNA in *Escherichia coli* and *Brassica napus* resulted in a functional FAR enzyme and the accumulation of fatty alcohols in *E. coli*, as well as fatty alcohols together with wax esters in transgenic *B. napus* oil (Metz *et al.*, 2000). The pea and the jojoba alcohol-forming fatty acyl reductases are both integral membrane proteins, which appear to be associated with the ER and have similar molecular masses in the range of 56–58 kDa (Vioque and Kolattukudy, 1997; Metz *et al.*, 2000). FAR-related sequences from maize (*Z. mays*), rice (*Oryza sativa*), cotton (*Gossypium hirsutum*) and *B. napus* found in the public databases, and a family of eight FAR-like proteins in *A. thaliana* (Kunst and Samuels, 2003) suggest that alcohol-generating FARs are ubiquitous in plants. One of the eight FAR-like *A. thaliana* sequences is likely the *CER4* gene, given that the *cer4* mutant exhibits major decreases in primary alcohols and wax esters, and slightly higher levels of aldehydes, alkanes, secondary alcohols and ketones (Hannoufa *et al.*, 1993; Jenks *et al.*, 1995). The *cer4* biochemical phenotype has been attributed to a lesion in an aldehyde reductase of the acyl-reduction pathway (Hannoufa *et al.*, 1993; Jenks *et al.*, 1995). In view of the new evidence indicating that primary alcohols are produced from fatty acids without release of an aldehyde intermediate, the *cer4* phenotype is most likely due to a mutation in the alcohol-forming acyl-CoA reductase, not the aldehyde reductase. The observed increase in aldehyde, alkane, secondary alcohol and ketone levels in the *cer4* mutant can then be explained by an increased flux of fatty acyl precursors into the alkane biosynthetic pathway.

The primary alcohols generated in the epidermal cells are further used for the synthesis of wax esters (Figure 5.1). Biochemical studies of wax ester formation in *B. oleracea* leaves (Kolattukudy, 1967a) and jojoba (*S. chinensis*) seeds (Wu *et al.*, 1981) suggested that this reaction involves the transfer of an acyl chain from fatty acyl-CoA to a fatty alcohol that is catalysed by a membrane-bound acyltransferase (wax synthase, WS). Partial purification of a WS polypeptide from jojoba embryos allowed the identification of a cDNA encoding the WS enzyme (Lardizabal *et al.*, 2000). The identity of the cloned cDNA was demonstrated by activity assays in developing *A. thaliana* embryos, expressing the jojoba WS cDNA. The transgenic *A. thaliana* embryos also accumulated high levels of wax esters. Hydrophathy analysis of the deduced protein sequence revealed seven to nine transmembrane domains (TMD) suggesting that jojoba WS is an integral membrane protein (Lardizabal *et al.*, 2000). The jojoba WS has considerable similarity with twelve *A. thaliana* sequences (Lardizabal *et al.*, 2000; Kunst and Samuels, 2003). Several of these twelve putative genes are differentially expressed in inflorescences and seeds of

A. thaliana but to date their enzymatic functions have not been assessed, nor have their biological roles been determined.

5.3.2 *Synthesis of alkanes, secondary alcohols and ketones*

Alkanes present in cuticular wax are approximately twice as long as the fatty acids of the cellular membranes. This finding, together with the discovery that alkanes, secondary alcohols and ketones of identical chain lengths and same location of their functional groups within their carbon chain occur in wax mixtures isolated from a certain tissue, led to a proposal that alkane molecules are generated by a head-to-head condensation between two fatty acids (Channon and Chibnall, 1929). Such a condensation reaction with decarboxylation of one of them would yield a ketone, which would be reduced to produce a secondary alcohol, then dehydrated and further reduced to generate an alkane. However, labelling experiments with ^{14}C and/or ^3H showed that head-to-head condensation reaction is not involved in alkane production in *B. oleracea* (Kolattukudy, 1966), *P. sativum* or *S. oleracea* (Kolattukudy, 1968), and that the entire carbon chains of C_{16} fatty acids were incorporated into alkanes without decarboxylation, that is, without the loss of the carboxyl carbon. This result was further substantiated by the demonstration that fatty acids of various chain lengths (C_{10} – C_{18}) were all incorporated into C_{29} alkane *n*-nonacosane with the longer ones being incorporated much more efficiently (Kolattukudy, 1966). Furthermore, the exogenously added labelled C_{29} ketone nonacosan-15-one could not be converted into C_{29} secondary alcohol (nonacosan-15-ol) or alkane (*n*-nonacosane) by *B. oleracea* leaf disks, peeled epidermis or leaf homogenates (Kolattukudy, 1966). On the basis of this new experimental evidence, Kolattukudy (1966, 1967b) proposed an elongation–decarboxylation pathway for alkane biosynthesis. According to this pathway, C_{16} fatty acid is elongated by the addition of C_2 units and, when it reaches the appropriate length (C_{30} – C_{32}), it gets decarboxylated. The generated alkane can then be hydroxylated to secondary alcohol, which can give rise to a ketone by oxidation. Conversion of ^3H -labelled C_{29} alkane to C_{29} secondary alcohol and ketone, and ^3H -labelled secondary alcohol to C_{29} ketone by *B. oleracea* leaves confirmed that this sequence of reactions was feasible (Kolattukudy and Liu, 1970). Similarly, ^3H -labelled C_{30} fatty acid was incorporated into C_{29} alkane by *B. oleracea* leaves (Kolattukudy *et al.*, 1972), ^3H -labelled C_{32} fatty acid into C_{31} alkane by cell free extracts from *P. sativum* leaves (Khan and Kolattukudy, 1974) and ^3H -labelled C_{24} fatty acid (tetracosanoic acid) to C_{23} alkene (*n*-tricosane) by *Allium porrum* (Cassagne and Lessire, 1974). These data, together with the isolation of mutants with lesions in elongation or decarboxylation that also inhibit alkane production (von Wettstein-Knowles, 1993), provided additional support for the elongation–decarboxylation hypothesis of alkane synthesis.

Progress leading to proposals for the biochemical steps involved in the production of alkanes, secondary alcohols and ketones has not been matched by identification and biochemical characterisation of enzymes catalysing the implicated reactions. Early experiments, designed to examine the mechanism of fatty acid conversion to

alkane, demonstrated that cell-free preparations from *P. sativum* required oxygen and ascorbate for alkane synthesis and that alkane production was strongly inhibited by metal ion chelators (Khan and Kolattukudy, 1974). Subsequent work showed that C₁₈–C₃₂ fatty acids can serve as substrates for alkane formation, and that all of these substrates generated alkanes *two* carbons shorter than the parent fatty acid. The discrepancy between this result and the observed predominance of alkanes with odd numbers of carbons found in cuticular wax mixtures was suggested to be due to differences between alkane generating reactions occurring *in vitro* and *in vivo*. *In vitro* α -oxidation of fatty acids gives rise to *n*-2 alkanes, whereas *in vivo* aldehydes formed from an acyl-CoA by a reductase could be decarbonylated to *n*-1 alkane (Bognar *et al.*, 1984). The aldehyde had not been previously recognised as an intermediate in alkane biosynthesis because a chemical mechanism for conversion of an aldehyde to an alkane was not known until the discovery that porphyrin-coordinated ruthenium complexes can catalyse such a decarbonylation reaction (Domazetis *et al.*, 1981). That an analogous reaction can generate alkane and CO from an aldehyde was then shown using *P. sativum* microsomes (Cheesbrough and Kolattukudy, 1984). However, in these experiments the decarbonylation was not assayed using the natural C₃₀ aldehyde as substrate, but the conversion of C₁₈ aldehyde to C₁₇ alkane was monitored instead. In addition, because the amount of CO released was too low to allow direct chemical detection, the CO was trapped using a rhodium complex, which forms a stable adduct with CO. The recovery of CO from C₁₈ aldehyde was slightly lower than the stoichiometric amount of alkane formed. In order to verify the involvement of the decarbonylase in alkane biosynthesis, dissect the mechanism of conversion of an aldehyde to an alkane and determine the subcellular site of alkane production, it is necessary to isolate the enzyme catalysing this reaction. The purification of the decarbonylase was attempted from *P. sativum* leaves (Schneider-Belhaddad and Kolattukudy, 2000). However, only partial purification was achieved and the quantity of purified protein was so low that reliable characterisation of the enzyme was not possible. For example, it had not been determined if a porphyrin was present, and the nature of the metal ion, believed to be involved in the synthesis of alkanes because of its sensitivity to metal chelators, remains uncertain. Similarly, mechanistic details of the conversion of aldehydes to alkanes still need to be elucidated.

If aldehydes are indeed direct precursors of alkanes, their production requires a FAR. A 28-kDa enzyme that catalyses the conversion of fatty acyl-CoA to an aldehyde *in vitro* has been solubilised and purified to homogeneity from *P. sativum* leaves (Vioque and Kolattukudy, 1997). The biochemical identity of this putative reductase, however, needs to be confirmed *in planta*, and its substrate specificity and cellular compartmentation remain to be determined.

Radioactive labelling studies (Kolattukudy and Liu, 1970; Kolattukudy *et al.*, 1972; Khan and Kolattukudy, 1974) and genetic evidence indicating that a block in alkane synthesis results in an absence of secondary alcohols and ketones (McNevin *et al.*, 1993) support the biochemical pathway for the synthesis of secondary alcohols and ketones from alkanes (Kolattukudy and Liu, 1970). At present, however,

the individual steps in this pathway remain hypothetical, as the enzymes involved in catalysing the proposed reactions have not been identified and characterised. The only available information pertaining to these enzymatic steps dates back to the labelling experiments of Kolattukudy *et al.* (1973), who showed that $^3\text{H-C}_{29}$ alkane *n*-nonacosane was incorporated into two different isomers of C_{29} secondary alcohol (nonacosan-14-ol and nonacosan-15-ol) in *B. oleracea* in a ratio similar to that found in the natural mixture, whereas the ketone was almost exclusively nonacosan-15-one. This work also revealed that these reactions require molecular oxygen and are severely inhibited by phenanthroline, a metal chelator. Curiously, in the presence of phenanthroline, incorporation of ^3H into ketones was much higher than into the secondary alcohols. Based on the fact that this inhibition was reversed by Fe^{2+} , it was suggested that hydroxylation of the C_{29} alkane *n*-nonacosane may involve a mixed function oxidase (Kolattukudy *et al.*, 1973), but in the absence of the purified enzyme, this hypothesis could not be experimentally tested. It has also not been determined if hydroxylation *in vivo* occurs at the alkane level, or whether a hydroxyl group can also be inserted into a fatty acyl or aldehyde precursor.

From the earlier discussion, it is clear that progress in our understanding of the biosynthetic pathway leading to the formation of alkanes, secondary alcohols and ketones awaits identification of the enzymes catalysing the formation of these major cuticular wax constituents. Attempts using traditional biochemical approaches have had limited success since wax biosynthetic enzymes are membrane-bound, and because the sufficient amount of epidermal tissue and the substrates required for enzyme activity assays are difficult to obtain. Even though genetic approaches in *A. thaliana* and *Z. mays* seem better suited for such a task, they have not yet been fruitful in identifying the genes encoding these enzymes. Only three *A. thaliana* mutants, *cer1*, *cer22* and *wax2*, have been reported to have altered levels of alkanes, secondary alcohols and ketones in their stem wax (Hannoufa *et al.*, 1993; McNevin *et al.*, 1993; Lemieux *et al.*, 1994; Jenks *et al.*, 1995; Chen *et al.*, 2003; Kurata *et al.*, 2003; Rashotte *et al.*, 2004). *Cer1* and *cer22* both exhibit increases in aldehyde levels and dramatic reductions in the accumulation of alkanes, secondary alcohols and ketones suggesting a block in the conversion of aldehyde to alkane. Based on this biochemical phenotype, it was proposed that *CER1* may encode an aldehyde decarbonylase (Hannoufa *et al.*, 1993; McNevin *et al.*, 1993). This suggestion was subsequently questioned, because *cer1* mutation also decreased the primary alcohol levels in stem wax (Jenks *et al.*, 1995). A simple block in the aldehyde decarbonylase step of the pathway could conceivably result in a greater flux of carbon towards primary alcohols, but reduced accumulation of primary alcohols would not be expected. The cloning of the *cer1* gene (Aarts *et al.*, 1995) did not reveal the biochemical identity of the *CER1* protein, but the deduced amino acid sequence of *CER1* showed that it contains three histidine-rich motifs common with fatty acyl desaturases, alkane hydroxylase and xylene monooxygenase – three enzymes that catalyse similar reactions (Fox *et al.*, 1994; Shanklin *et al.*, 1994). *CER1* also has some sequence similarity with proteins of unknown function: EPI23, a *Kleinia odora*

epidermis-specific protein, GLOSSY1 protein of *Z. mays* (Hansen *et al.*, 1997; Sturaro *et al.*, 2005) and WAX2 of *A. thaliana* (Chen *et al.*, 2003).

CER22 was also predicted to be the C₃₀ aldehyde decarbonylase, or a protein regulating its activity in *A. thaliana* stems (Rashotte *et al.*, 2004), because the mutation in *CER22* blocks the conversion of the C₃₀ aldehyde triacontanal to C₂₉ alkane *n*-nonacosane. Unlike *cer1*, the *cer22* line does not have substantially altered primary alcohol levels, but does exhibit a dramatic increase in the levels of wax esters [300% of the wildtype (WT); Rashotte *et al.*, 2004], presumably due to a redirection of carbon towards primary alcohols. The *CER22* gene has not yet been isolated; so the hypothesis that it may encode a protein involved in the conversion of aldehydes to alkanes remains to be confirmed.

Wax2/yore-yore/faceless pollen1 mutant of *A. thaliana*, identified independently by three research groups (Ariizumi *et al.*, 2003; Chen *et al.*, 2003; Kurata *et al.*, 2003), has a glossy bright green stem due to an approximately 80% decrease in total wax load. Aldehydes, alkanes, secondary alcohols and ketones that jointly comprise up to 90% stem wax in WT *A. thaliana* were severely reduced in this mutant, with corresponding increases detected in the levels of fatty acids, primary alcohols and esters. Cloning of the *WAX2* gene indicated that the predicted *WAX2* polypeptide is an integral membrane protein sharing the highest sequence similarities with the GLOSSY1 of *Z. mays* (Sturaro *et al.*, 2005), a rice sequence annotated as a GLOSSY1 homologue (Hansen *et al.*, 1997), *K. odora* EPI23 (Hansen *et al.*, 1997) and *A. thaliana* CER1 (Aarts *et al.*, 1995). The authors (Chen *et al.*, 2003; Kurata *et al.*, 2003) speculate that *WAX2* may function as an aldehyde-generating acyl-CoA reductase; however, based on the limited amount of information currently available, it is not possible to deduce the biochemical role of *WAX2* in wax biosynthesis.

Our inability to identify genes encoding enzymes involved in the production of alkanes and their derivatives using genetic approaches may be due to several reasons. First, *A. thaliana* genome sequencing revealed that only 35% of the predicted protein encoding sequences are unique, while 37% belong to families with more than five members (The Arabidopsis Genome Initiative, 2000). The lack of obvious phenotypes in the vast majority of single gene knockout mutants suggests that this genetic redundancy might be a serious obstacle in the functional characterisation of *A. thaliana* genes. Second, the standard visual screens for wax-deficient mutants that rely on a change in epicuticular wax crystal density, generating an alteration in surface reflectance, will consistently detect only mutants with pronounced phenotypes, most likely those with lesions in regulatory functions, or early wax biosynthetic steps. Mutants with defects causing more subtle changes in wax load or composition that do not result in changes in surface reflectance (see Chapter 6) would escape visual detection. Therefore, more accurate screening methods, such as gas chromatography, will need to be employed to identify them (Rashotte *et al.*, 2004). Third, it is conceivable that some of the enzymes catalysing the biosynthetic reactions proposed on the basis of radioactive tracer and inhibitor studies are known, but that their involvement in these reactions has not been established,

because the type of reaction carried out by these enzymes and the mechanism of catalysis is not understood. Rigorous biochemical work on proteins implicated in the production of alkanes, secondary alcohols and ketones, such as CER1 or WAX2, is therefore required to elucidate their precise biochemical function and the nature of the chemical reaction that they catalyse. Finally, the suggested biosynthetic steps are based on studies of alkane, secondary alcohol and ketone formation in leaves of several plant species and may not reflect the situation in all plants and tissues. Verification of this pathway in additional plant species is clearly an important future goal.

5.3.3 Biosynthesis of β -diketones and alkan-2-ols

β -diketones are a major component of the cuticular wax in some *Eucalyptus*, *Festuca* and *Agropyron* species, and are also found in *Dianthus* and *Rhododendron* species as well as in cereals including barley (*H. vulgare*), wheat (*Triticum aestivum*), oats (*Avena sativa*) and rye (*Secale cereale*) (von Wettstein-Knowles, 1972; Tulloch, 1976; Walton, 1990). Occasionally they are accompanied by short esterified alkan-2-ols, as in *Agropyron*, *Eucalyptus* and barley. In *Sorghum bicolor*, however, alkan-2-ols have been detected in the absence of β -diketones (von Wettstein-Knowles, 1995). The biosynthesis of these lipids is carried out by enzyme complexes with features of both FAE and polyketide synthases. Our present understanding of the β -diketone pathway is based on genetic and biochemical studies of *cer-cqu* mutants of barley (reviewed by von Wettstein-Knowles, 1993, 1995).

Initial genetic analyses of *cer-c*, *cer-q* and *cer-u* mutants showed that these three complementation groups were very tightly linked and that double and triple mutants were present relatively frequently (von Wettstein-Knowles and Sjøgaard, 1980). Subsequent reversion studies demonstrated that these double and triple mutants could revert to WT as a result of single mutational events (von Wettstein-Knowles, 1992). These results are consistent with a single *CER-CQU* gene coding for a polypeptide with three functional domains, CER-C, CER-Q and CER-U (von Wettstein-Knowles and Sjøgaard, 1981).

Wax analyses of the spikes of the *cer-c*, *cer-q* and *cer-u* barley mutant revealed that the *CER-CQU* gene product was involved in the production of β -diketones, hydroxy- and oxo- β -diketones, methyl ketones and alkan-2-ols (von Wettstein-Knowles, 1976, 1993). *CER-Q* mutations blocked the synthesis of β -diketones and all their derivatives, indicating that the *CER-Q* activity was required early in the pathway, and was likely involved in β -ketoacyl elongation (Mikkelsen and von Wettstein-Knowles, 1978; Mikkelsen, 1979). Later biochemical investigations demonstrated that the *cer-q* domain has β -ketoacyl synthase activity (Mikkelsen, 1984). *CER-C* mutations blocked β -diketone formation, and resulted in elevated levels of alkan-2-ols, but the activity specified by the *CER-C* domain is not known, although it is probably involved in two successive rounds of elongation (von Wettstein-Knowles, 1992). *CER-U* mutations caused the decrease in hydroxy- β -diketone accumulation with a corresponding increase in the β -diketone levels,

suggesting that the CER-U protein domain functions as a hydroxylase introducing the hydroxyl group into β -diketones (von Wettstein-Knowles, 1972).

Further studies using radioactively labelled precursors and tissue slices prepared from the *cer-c*, *cer-q* and *cer-u* mutants provided additional evidence that the β -ketoacyl-CoA compound generated by the β -ketoacyl elongase is the key intermediate in the synthesis of β -diketones and alkan-2-ols (Figure 5.2; Mikkelsen, 1984). This research demonstrated that the β -ketoacyl-CoA intermediate can be converted to a methylketone by a putative decarboxylase, and an alkan-2-ol by a putative methylketone reductase. Finally, the alkan-2-ol can give rise to an ester by reacting with an acyl-CoA (Figure 5.2). Alternatively, the β -ketoacyl-CoA intermediate can undergo successive condensations by the CER-Q β -ketoacyl synthase, followed by a number of chain elongations and decarboxylation or decarbonylation, resulting in the formation of a β -diketone. A hydroxyl group can then be introduced and oxidised to yield a hydroxy- β -diketone and a keto- β -diketone, respectively (von Wettstein-Knowles, 1993). The proposed biosynthetic pathway for the production of β -diketones and alkan-2-ol esters has not yet been verified by molecular and biochemical characterisation of the enzymes that catalyse the suggested reactions. This is clearly an essential next step in understanding the production of these wax constituents.

5.4 Triterpenoid biosynthesis

Triterpenoids exhibit a striking diversity of carbon structures and of derivatives with varying functional groups (Xu *et al.*, 2004). Based on rapidly accumulating chemical information on these structures, Ruzicka *et al.* in the 1950s proposed the concept of the biogenetic isoprene rule (Ruzicka, 1959). It provides the framework describing the biosynthetic pathways and mechanisms leading to the triterpenoid backbones (Eschenmoser *et al.*, 1955), from which the final products are derived by modification reactions. The predictions of the biogenetic isoprene rule have since been confirmed by numerous chemical, biochemical and molecular biological investigations. We thus have a fairly good knowledge of the biosynthetic processes leading to triterpenoid formation in general. Even though published reports on the biosynthesis of cuticular triterpenoids are lacking to date, it is very likely that much of the information available on triterpenoid biosynthesis in general can be applied to these plant surface specific compounds.

Most plant triterpenoids are, like steroids, derived from enzymatic cyclisation of 2,3-oxidosqualene, which is therefore the last common precursor in two separate pathways of primary and secondary metabolism (Abe *et al.*, 1993). Protonation leads to epoxide ring opening and creates a carbocation that can be attacked by π -electrons from one of the double bonds in the squalene molecule. A series of these intramolecular addition reactions leads to formation of four fused carbon cycles, the six-membered rings A, B and C in chair conformation and the five-membered D-ring. The dammarenyl cation thus formed is transformed by widening

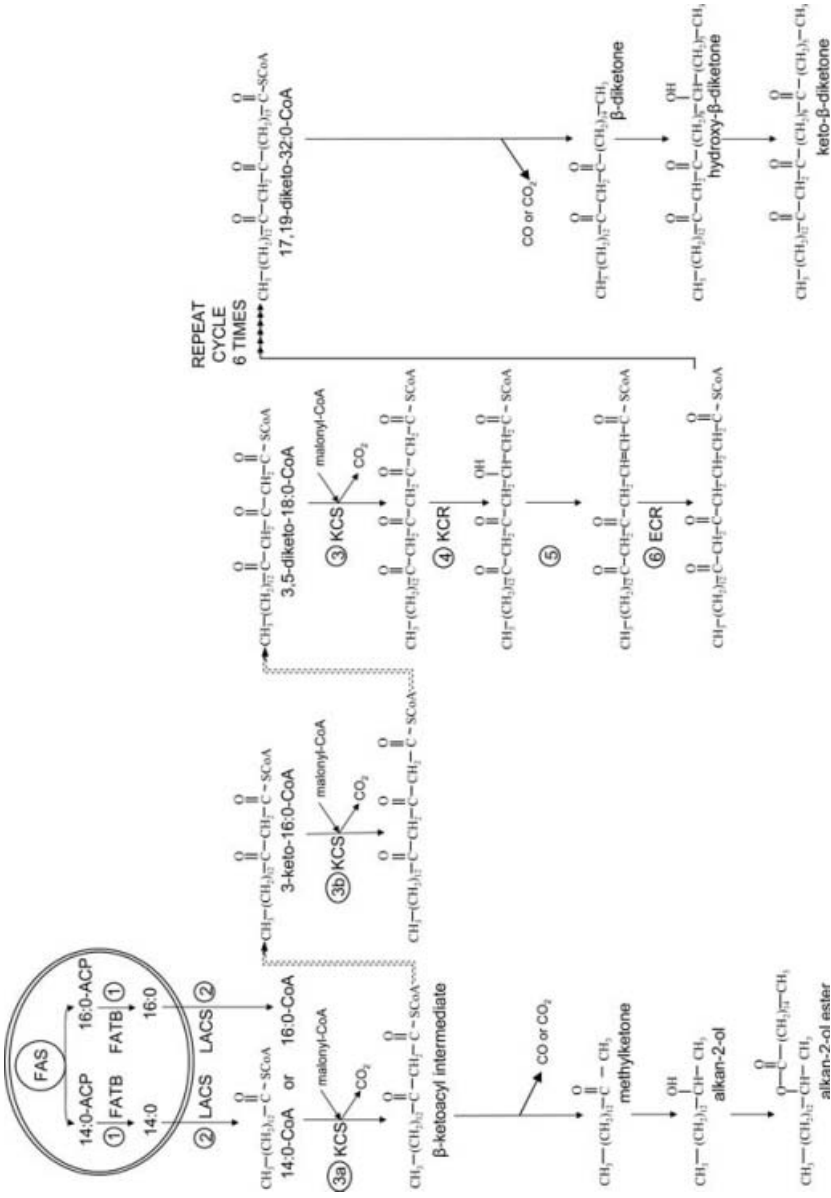


Figure 5.2 Biosynthetic pathways for the formation of β-diketones, alkan-2-ols and their derivatives. C₁₄ and C₁₆ fatty acyl-ACPs formed by the fatty acid synthase (FAS) in the plastid are hydrolyzed by an (1) acyl-ACP thioesterase (FATB), and during transport through the plastid envelope esterified to CoASH by a (2) long-chain acyl-CoA synthetase (LACS). C₁₄ and C₁₆ fatty acyl-CoAs undergo a (3a) condensation reaction with malonyl-CoA to yield β-ketoacyl intermediates, which can then be converted into methylketones, alkan-2-ols and alkan-2-ol esters. Alternatively, C₁₄ and C₁₆ acyl-CoAs can undergo two successive condensations carried out by one or two KCSs (3a and 3b), followed by several rounds of FAE to yield C₃₂ or C₃₄ β-diketoacyl intermediates used for the production of β-diketones, hydroxy- and keto-β-diketones. Enzymatic steps (1)–(6) shown in this figure are described in more detail in Table 5.1. Chemical formulas for one representative compound from each lipid class are shown.

of the D-ring, followed by formation of the E-ring. The product of this reaction sequence (the lupenyl cation) is deprotonated in many cases, leading to lupeol as one of the most widespread triterpenoids. Alternatively, it can be rearranged in a series of 1,2-hydride and/or 1,2-methyl shifts, and the resulting cations can be deprotonated to produce the various other triterpenoid structures. The products are isomeric structures $C_{30}H_{50}O$, either with a hydroxyl function on C-3 and a $C=C$ double bond in specific positions in the molecule, or with a carbonyl function at C-3 and a saturated carbon backbone.

It has been shown that all the reaction steps from oxidosqualene to the deprotonation products can be catalysed by a single enzyme – a triterpene synthase (Herrera *et al.*, 1998; Kushiro *et al.*, 1998). A number of triterpene synthases have been cloned and characterised from various plant species. In most cases, they form predominantly one product, for example β -amyrin or lupeol. The mechanism of the cyclisation reaction has been investigated in some detail in these two enzyme groups. Active site residues involved in the protonation of the epoxide substrate, and in the stabilisation of carbocation intermediates have been identified (Tanaka *et al.*, 2002). In addition, amino acids involved in determination of product specificity have been established, making it possible to switch a β -amyrin synthase into a lupeol synthase (and vice versa) by site directed mutagenesis of single residues (Kushiro *et al.*, 2000).

The only other product-specific triterpene synthase presently known is an isomultiflorenol synthase from *Luffa cylindrica* (Hayashi *et al.*, 2001). Two multifunctional triterpene synthases have been described and are the only enzymes currently shown to form α -amyrin (Husselstein-Muller *et al.*, 2001). Besides, they generate mixtures of either taraxasterol, Ψ -taraxasterol, multiflorenol, tirucalla-7,21-dienol, butyrospermol and baurenol, or of δ -amyrin, germanicol, taraxasterol, Ψ -taraxasterol and butyrospermol. The species-specific triterpenoid compositions may therefore be formed by multiple single-product enzymes in some cases, and by one (or a few) multi-product enzyme(s) in others. The triterpenoid compositions have not been reported for all the plant species from which relevant genes have been cloned; thus it is not possible to assess in all cases whether the set of *in vitro* characterised genes is sufficient to explain *in planta* product formation.

The same carbon structures, mostly with the hydroxyl function on C-3, have been reported for the cuticular triterpenoids of diverse species (Chapter 4, Section 4.2.3). For this reason it seems likely that very similar enzymes are involved in the biosynthesis of surface triterpenoids. Unfortunately, the spatial and temporal expression patterns of the triterpenoid synthase genes cloned to date have not been reported. The cuticular wax compositions of the species from which these genes were cloned have also not been investigated. Consequently, it is not clear whether the triterpenoid products of the enzymes characterised so far might be partially or completely exported to the cuticle. While the cuticle-relevance of the known genes/species is being tested, homologous genes from other species with reportedly high concentrations of cuticular triterpenoids should also be investigated.

5.5 Regulation of wax biosynthesis

Differences in plant resistance/susceptibility to environmental stresses, pathogens or insects have been linked to variations in wax accumulation (load) and wax composition among plant species (Eigenbrode and Espelie, 1995; Post-Beittenmiller, 1996; Chapters 11 and 12). The mechanisms by which plants control wax accumulation and composition are therefore of considerable interest. At present, however, the knowledge of these regulatory mechanisms is extremely limited.

Cer mutants of *A. thaliana* and *glossy* mutants of *Z. mays* have been valuable resources for the identification and isolation of genes associated with plant cuticular wax production. Four of the genes cloned from these mutant collections, *CER2*, *CER3*, *GL2* and *GL15*, have been proposed to encode regulatory proteins (Tacke *et al.*, 1995; Hannoufa *et al.*, 1996; Moose and Sisco, 1996; Negruk *et al.*, 1996; Xia *et al.*, 1996). Of those, only the *GL15* was suggested to encode a transcription factor that regulates leaf epidermal cell identity, whereas the identities of *CER2*, *CER3* and *GL2* gene products could not be deduced from their primary sequences, and their predicted functions in regulation of wax deposition remain to be confirmed.

Detailed expression analyses have been carried out for only two genes encoding wax biosynthetic enzymes, *CER2* and *CER6* (Xia *et al.*, 1997; Hooker *et al.*, 2002). Both studies documented that these genes were transcriptionally regulated in response to developmental cues. In addition, *CER6* transcription was induced by light and osmotic stress (Hooker *et al.*, 2002), environmental factors known to stimulate wax accumulation in a few species, for example *B. oleracea* (Baker, 1974). Somewhat unexpectedly, *CER2* expression was not light-, heat-, cold- or wound-inducible, and was unaffected by osmotic stress (Xia *et al.*, 1997).

Despite the available evidence that wax production is under transcriptional control, transcriptional activators upregulating the expression of wax biosynthetic genes during development and in response to environment have not yet been identified. Recently, however, two groups reported the isolation and characterisation of *WIN1/SHN1*, an *A. thaliana* transcriptional regulator of the *AP2/EREBP* family which, when overexpressed, dramatically enhances wax accumulation in *A. thaliana* leaves and stems, and results in a strikingly glossy leaf phenotype (Aharoni *et al.*, 2004; Broun *et al.*, 2004). A detailed examination of the molecular mechanisms underlying the wax hyperaccumulation in the leaves of 35S:*WIN1/SHN1* transgenic plants demonstrated that *WIN1/SHN1* overexpression resulted in the induction of several wax-related genes, including *CER1*, *CER2* and *KCS1* (Broun *et al.*, 2004), and consequently in a dramatic increase in leaf alkane, secondary alcohol and ketone levels (Aharoni *et al.*, 2004; Broun *et al.*, 2004). Extreme leaf glossiness and increased wax deposition on leaves and stems have also been detected in transgenic plants overexpressing the *WIN1/SHN1* paralogues *SHN2* and *SHN3* (Aharoni *et al.*, 2004; Broun *et al.*, 2004). In addition, RNA-blot and microarray analyses showed that other genes predicted to encode lipid biosynthetic enzymes, and proteins involved in cellular trafficking, including an ABC transporter were

up-regulated in the *35S:WIN1/SHN1* overexpressors (Broun, 2004). Similarly, *WXP1*, an *AP2/EREBP* domain transcription factor from *Medicago truncatula*, increases leaf cuticular wax accumulation and results in a glossy leaf phenotype when overexpressed under the control of the 35S promoter in alfalfa (*Medicago sativa*; Zhang *et al.*, 2005). Unlike in *A. thaliana*, however, where *SHN* transcriptional regulators activate the production of alkanes and their derivatives, the wax load increase in alfalfa is mainly due to a greater production of the C₃₀ primary alcohol triacontanol, the major component of the alfalfa leaf wax. Even though high levels of expression of *SHN* transcription factors under the control of the 35S promoter had pleiotropic effects on *A. thaliana* growth and development, and their exact *in planta* role could not be determined in the absence of loss-of-function phenotypes or fully silenced transgenic lines, it is tempting to speculate that the major function of the *SHN* clade of transcription factors in *A. thaliana*, and related *AP2/EREBP* domain transcription factors in other plants is the activation of the wax biosynthetic and export machinery in epidermal cells.

5.6 Wax biosynthesis and transport in the context of the epidermal cell

During formation and growth of aboveground plant organs, their surface area typically expands first due to division and later due to controlled anisotropic expansion of epidermal pavement cells. Cuticle formation and growth have to be synchronised with this expansion in order to completely coat the surface for protection at all times. Investigations into the development of cuticle structure and composition have in many cases found that the major cuticular components, for example, the epicuticular wax crystals on the tissue surface, are in place already at the earliest time points studied, as well as during ensuing surface expansion. During growth, large quantities of cutin and wax have to be deposited on the periclinal surface of the growing cells. It is generally accepted that the cuticular components originate in the epidermal pavement cells, starting from ubiquitous precursors such as acetyl-CoA and involving common fatty acyl intermediates. Consequently, the epidermal cells have to strike and maintain a delicate balance of biosynthetic activities, coordinating the large flux of lipophilic products towards the cuticle with other lipid generating pathways.

The large quantities of wax intermediates and products might also pose structural problems for the pavement cells, as they must handle saturated aliphatic molecules that, due to their lipophilic nature and very long-chain geometry, may interfere with the integrity of cell membranes (Ho *et al.*, 1995; Hamilton, 1998; Millar *et al.*, 1998). By analogy to numerous lipid transport processes in diverse eukaryotes, two fundamental mechanisms for handling the wax precursors and products may be distinguished. Both will likely be involved in channelling the molecules between biosynthetic enzymes and the cuticle. One possibility is that wax components are embedded in the membrane bilayers, spanning nearly their entire thickness. In that case special membrane microdomains or presently uncharacterised proteins might

help to accommodate them to prevent disruption of cellular membrane functions. Alternatively, special proteins might serve to solubilise wax compounds in the cytoplasm, possibly by binding them as single molecules at the site of synthesis, and shuttle them across aqueous compartments on their way to the cuticle.

En route to aliphatic wax compounds, 16:0-ACP and 18:0-ACP are synthesised in the plastids of pavement cells and hydrolysed by acyl-ACP thioesterases (see Section 5.2). During or after transfer through the plastid membranes, the free fatty acids are transformed into long-chain acyl-CoAs in the plastid envelope. Currently, nothing is known about the mechanism of acyl-CoA transport from the epidermal plastids to the ER, but at least two hypotheses may be considered. In many plant species and cell types, domains of the ER have been found located near the plastids, without apparent fusion or mixing of bilayers (Staehelein, 1997). This proximity may facilitate fatty acid transfer to the ER, involving non-vesicular mechanisms such as spontaneous desorption, diffusion and absorption. Alternatively, lipid transport from plastids to the ER could be facilitated by acyl-CoA binding proteins (ACBPs) (Johnson *et al.*, 2002). This class of proteins has been described in a wide variety of eukaryotes (Chye *et al.*, 2000), and a *S. cerevisiae* mutant lacking ACBP was found to have increased levels of C_{18:0} and reduced C_{26:0}. This result suggests that in yeast ACBP is involved in transport of acyl-CoAs towards the ER, where they are elongated from C_{18:0} to C_{26:0} for production of sphingolipids (Gaigg *et al.*, 2001). In plants, both a cytoplasmic ACBP from *A. thaliana* (Engeseth *et al.*, 1996; Leung *et al.*, 2005) and membrane associated ACBPs (Li and Chye, 2003) have been described. Although it is postulated that their role is to maintain an acyl-CoA pool in the cytosol (Engeseth *et al.*, 1996) or transport palmitoyl-CoA or oleoyl-CoA from the plastid to the ER (Leung *et al.*, 2005), their *in vivo* functions remain to be investigated. As the ACBP1 and ACBP2 were located in the plasma membrane (PM) rather than the cytoplasm (Li and Chye, 2003; Leung *et al.*, 2005), it is unlikely that they are trafficking lipids towards the ER. However, this does not preclude the possibility that other ACBP orthologues are involved in this transport process.

5.6.1 Intracellular sites of wax synthesis and trafficking of wax constituents

Multiple lines of evidence support the model that enzymes catalysing the elongation of long chain to VLCFAs are associated with the ER (Figure 5.3, scenario 1). In cell fractionation studies, fatty acid elongation activity was most enriched in microsomal fractions (Lessire *et al.*, 1985; Bessoule *et al.*, 1989), i.e. membrane preparations dominated by ER material. After the genes encoding VLCFA biosynthetic enzymes had been isolated, some of these enzymes have also been localised using fluorescent tags. For example, an *A. thaliana* β -ketoacyl-CoA synthetase (CER6) (Kunst and Samuels, 2003) and ECR (CER10) (Zheng *et al.*, 2005) were fused to the green fluorescent protein (GFP) and expressed in the corresponding mutants. Both enzymes complemented the mutant phenotypes, indicating that they were functional, and were localised throughout the ER of epidermal pavement cells (Figure 5.4).

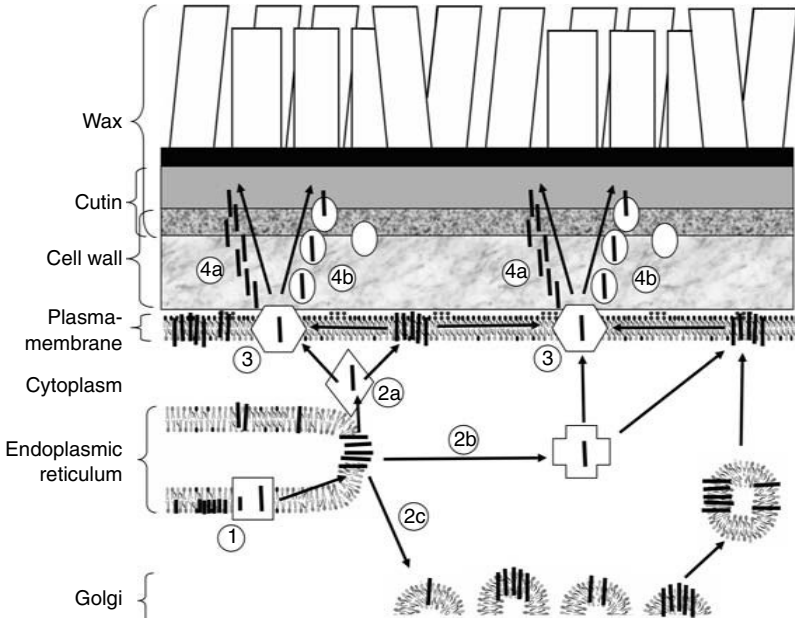


Figure 5.3 Hypothetical scenarios for export of aliphatic wax components to the cuticle. (1) C₁₆ and C₁₈ fatty acyl-CoAs are elongated by the fatty acid elongase (FAE, shown as a square) in the endoplasmic reticulum (ER). Subsequent modifications of very long-chain acyl precursors into alkanes, primary or secondary alcohols, or ketones are carried out by unknown enzymes and are, therefore, not shown. From the ER, the VLCFAs or their derivatives could be transported to the plasma membrane (PM) by three possible routes: (2a) direct ER to PM transport mediated by unknown proteins (shown as a diamond), delivering lipid either directly to an ABC transporter (shown as a hexagon) or into the PM bilayer; (2b) unknown lipid binding proteins could mediate transfer of VLCFAs to the ABC transporter or into the PM bilayer; (2c) cuticular lipids could move along the endomembrane system from ER to Golgi and on to the PM, either free in the lipid bilayer or in 'lipid rafts'. Once at the cell surface, wax components could be pulled out of the bilayer by ABC transporters (3) and either (4a) transferred directly through the cell wall or (4b) carried by non-specific lipid transfer proteins (LTPs) to the cuticle.

In contrast, when the *A. thaliana* CER10::GFP fusion was heterologously expressed in yeast, CER10 was localised only to one particular domain of the ER, the nuclear–vacuolar junction (Zheng *et al.*, 2005). This finding is in very good accordance with previous reports showing that the *S. cerevisiae* VLCFA ECR is also localised in this ER subdomain (Kohlwein *et al.*, 2001). In contrast, other components of the yeast FAE complex are located throughout the ER (and nuclear envelope). It may be speculated that the restriction of both native and foreign ECRs to one subdomain of the ER in yeast is required for the production of sphingolipid VLCFA precursors. To date, there is no evidence for a similar sub-compartmentation of lipid biosynthesis within the ER of plant epidermal cells, but the diverse pathways in this organelle might be spatially organised to a certain degree. The first committed steps of the pathways for the production of sphingolipids, cutin monomers and waxes presumably all co-occur in the epidermal ER. It remains to be determined

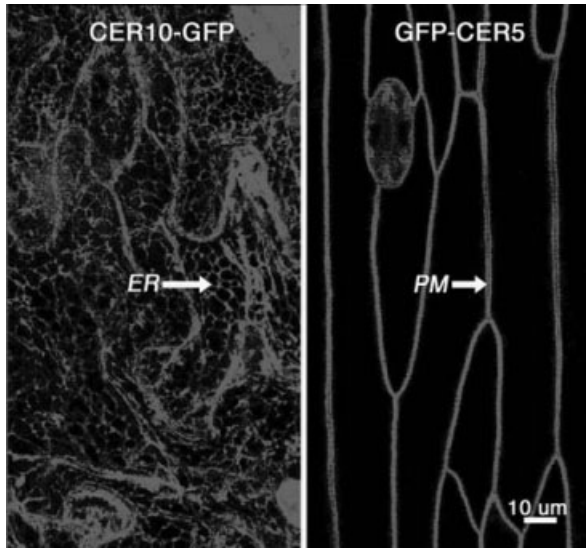


Figure 5.4 Green fluorescent protein (GFP) experiments demonstrate the location of the proteins involved in wax production. These images represent a projection of optic sections collected with the laser scanning confocal microscope. The enoyl-CoA reductase (CER10-GFP) component of the elongase was found in the endoplasmic reticulum of pavement cells in the leaf of *Arabidopsis thaliana*. ABC transporter CER5 was localised to the plasma membrane in *A. thaliana* stems (GFP-CER5). Magnification bar = 10 μm . This figure is produced in colour in the colour plate section, which follows page 249.

how the enzymes catalysing these branch point reactions compete for 16:0-CoA and 18:0-CoA as substrates, and how their activities are regulated in space and time.

Following elongation, VLCFAs are modified by various alternative pathways to form aliphatic wax components (see Section 5.3). Very little is known about the cellular localisation of the enzymes catalysing the corresponding reactions. Thus, it cannot be assessed whether compounds leave the ER as very long-chain acyl CoA intermediates, or as downstream wax constituents. In one extreme scenario, all the modifying enzymes might be located in the ER, and the entire mixture of wax constituents would be generated there. In this case the ensuing steps in the export towards the cuticle would have to accommodate a wide range of compounds, and the mechanisms should have little specificity. In the other extreme, the very long-chain acyl CoAs might be selectively trafficked from the ER to the PM for further modification and export.

The fact that conventional sample preparation techniques for electron microscopy (EM) extract lipophilic compounds (Reed, 1982) has made studies in lipid transport very difficult. For this reason, the mechanisms for transport of wax molecules (intermediates or products) from the ER to the PM are currently unknown. However, a number of alternative hypotheses for this aspect of cuticle formation

have been put forward (Figure 5.3), based either on circumstantial evidence or on analogies with other intracellular lipid transport processes (Kunst and Samuels, 2003; Schulz and Frommer, 2004). As one possibility, direct molecular transfer between both membranes could occur at sites where cortical ER is in close proximity to the PM (Figure 5.3, scenario 2a) (Staehelin, 1997). The second more speculative possibility is that small aggregates of wax components could also traffic from the ER to the PM, surrounded by oleosin-like proteins, similar to those surrounding oil bodies in oilseeds and the tapetum (Kim *et al.*, 2002; Jolivet *et al.*, 2004). Such small (>75 nm) lipid complexes might have eluded transmission electron microscopic investigations, because the structures are in the size range of one section thickness (70 nm typically) and also because the saturated components of the wax would not react with EM stains. As a third alternative, lipid-binding proteins similar to ACBPs have to be considered (Figure 5.3, scenario 2b), even though experimental evidence for their involvement is missing (see earlier). As a fourth possibility, saturated wax precursors could also be accommodated and transported by vesicles moving along the secretory pathway from ER to Golgi to the PM. As the VLCFA and their derivatives would influence the membrane fluidity and rigidity, they might be contained within specialised membrane microdomains, that is, 'lipid rafts'. Based on their detergent-resistance, characteristic microdomains of membranes have been isolated from plant cells growing in cultures. These 'lipid rafts' were especially sphingolipid- and sterol-rich (Mongrand *et al.*, 2004) and were associated with specific, often GPI-anchored, proteins (Borner *et al.*, 2005). Furthermore, those fractions of the rafts were enriched for PM proteins (Borner *et al.*, 2005). This finding was in good accordance with yeast and mammalian cells, where 'lipid rafts' have been postulated to function in sorting of membrane proteins and clustering of components involved in signalling (Brown and London, 2000; Mayor and Rao, 2004). It has been suggested that VLCFA-containing lipids, for example, sphingolipids, are transported in vesicles from the ER via the Golgi to the PM (Moreau *et al.*, 1988). Similarly, if wax precursors or components associate with sterols and/or sphingolipids in the 'lipid rafts', then they could be transported to the PM by vesicle traffic involving the Golgi (Figure 5.3, scenario 2c).

The epidermal cell structure of most plant species is not polarised, that is, the Golgi or the ER do not predominate under the periclinal surface of the cell in a way that would suggest a subcellular mechanism of handling and exporting the hydrophobic wax molecules. One exceptional case is the spectacular cork cells of *Sorghum bicolor*, which secrete long filaments of epicuticular wax. When studied with transmission EM, these cells have abundant ER on the periclinal side of the cytoplasm, facing the cuticle; however they contain few Golgi stacks (Jenks *et al.*, 1994). Correlated with wax secretion, these specialised cork cells have large vesicles with dark contents called 'osmiophilic globules'. Osmiophilic particles have also been observed in the cytoplasm of epidermal cells in deepwater rice during cuticle formation. They were observed fusing with the PM during cuticle synthesis and their production could be inhibited with monensin, indicating that a functional trans-Golgi structure is required for their synthesis (Hoffmann-Benning

and Kende, 1994; Hoffmann-Benning *et al.*, 1994). However, it is not clear what the 'osmiophilic' material represents. When probed with enzyme-gold particles and antibodies, the rice osmiophilic granules were found not to contain characteristic components of the epidermis surface, such as pectin or cutin. And while they tested positive with a proteinase-gold probe, proteins that might be involved in lipid export, such as lipid transfer proteins (LTPs), could not be detected (Hoffmann-Benning *et al.*, 1994). Thus, even though their presence correlated with cuticle synthesis, direct positive evidence for the role of these osmiophilic structures in cuticle formation could not be provided.

In summary, support for the hypothesis that wax components traffic through the ER–Golgi–PM route comes by analogy with sphingolipid transport and due to the presence of the apparently Golgi-derived but enigmatic osmiophilic vesicles. The argument against Golgi-mediated transfer is that there is no change in Golgi morphology such as increased number of vesicles associated with the stacks, as is typically observed during secretion.

5.6.2 Transport of wax through the PM

During the final step of intracellular trafficking, wax molecules (intermediates or products) have to enter into the PM. Again, there is currently no experimental evidence that would indicate the mechanisms involved in this process. Two fundamental possibilities can be distinguished – one involving direct loading of lipid molecules into specific PM proteins, and the other one involving initial partitioning of molecules into the membrane, followed by their association with proteins involved in further transport (Figure 5.3, scenario 3). These mechanisms might require energy for active detachment of lipid molecules from previous (protein or vesicle) complexes, and/or active loading onto PM proteins.

One recent advancement in understanding the mechanism of wax export came from the identification of an *A. thaliana* ATP binding cassette (ABC) transporter, CER5 (Pighin *et al.*, 2004). Evidence that this transporter is involved in wax export comes from the analysis of the *cer5* mutant in which wax components are reduced on the cuticle surface but instead accumulate inside the cells (Pighin *et al.*, 2004). Using a GFP tag, CER5 was localised to the PM of epidermal cells (Figure 5.4).

The CER5 gene encodes an ABC transporter related by sequence homology to the human ABCG subfamily, a group of proteins that transport lipids (Lorkowski and Cullen, 2002), and the *white–brown* complex (WBC) of *Drosophila melanogaster*, which transports eye pigment precursors (Ewart *et al.*, 1994). A survey of all genes containing ATP-binding cassettes in the *A. thaliana* genome identified the WBC-type subfamily as the largest single group with 29 members within this ABC transporter superfamily (Sanchez-Fernandez *et al.*, 2001). The CER5 protein (annotated as WBC12 in the *A. thaliana* ABC transporter survey) is the first ABC transporter of the WBC subfamily to have a biological role assigned to it in plants.

CER5 is a half-transporter, as its protein structure is predicted to contain one ATP binding cassette and transmembrane domain (TMD), rich in membrane spanning

α -helices. In all prokaryotic and eukaryotic ABC transporters known, the functional ABC transporter consists of two ABC domains and two TMDs (Higgins and Linton, 2004). The binding partner for CER5 could be either another CER5 molecule to form a homodimer, or another ABC half-transporter to form a heterodimer. If CER5 is directly exporting wax components out of the cell, judging by the *cer5* biochemical surface wax phenotype, it likely transports a variety of wax components. This is not unusual, as many ABC transporters have the ability to pump a wide variety of substrates. Previously, plant ABC transporters have been characterised primarily in heavy metal detoxification and secondary metabolite transport (Rea *et al.*, 1998; Jasinski *et al.*, 2001, 2003); however in mammals and bacteria, ABC transporters have been identified as lipid transporters in outer membrane lipid A export, in bile secretion, cholesterol homeostasis in macrophages, and exporters of β -sitosterol (Pohl *et al.*, 2005).

The ABC transporter CER5 is one candidate protein that could either directly extract lipids from cytosolic transport proteins, or indirectly accept them from within the PM. If the wax components are embedded in the PM, they could enter via a side port in the pore formed by the TMD, by analogy to lipid ABC transporters from bacteria and humans (Chang and Roth, 2001). For both the direct and the indirect mechanism, ATP hydrolysis can provide the energy required for loading molecules into the ABC transporter. Consequently, the next step in the process, that is, export into the cell wall, may be energetically promoted already at the stage of detaching molecules from the last intracellular transport unit, be it the PM or a cytosolic transport protein.

5.6.3 *Transport of wax through the cell wall to the cuticle*

Once wax components have been exported from the epidermal cells, they must traverse the cell wall. As the highly lipophilic wax molecules have very low solubility in the aqueous environment of the cell wall, this transport process has to be facilitated in some way. One possibility is that special structures exist in the periclinal cell wall of epidermal cells, characterised by relatively non-polar internal surfaces that help guide the wax molecules to the cuticle (Figure 5.3, scenario 4a). As such domains have not been characterised, wax transport through the cell wall has usually been attributed to lipid transfer proteins (Figure 5.3, scenario 4b) (Sterk *et al.*, 1991; Moreau *et al.*, 1998), although it must be stressed that there is also no direct experimental evidence confirming LTP participation in this process. In principle, LTPs are small enough to diffuse through the pectin matrix of the primary cell wall and the emerging data describing their properties make them interesting candidates.

Non-specific LTPs (nsLTPs) of plants are part of a larger superfamily of small, basic proteins that move phospholipids between lipid bilayers *in vitro* (Kader, 1996; Arondel *et al.*, 2000; Rogers and Bankaitis, 2000). A few representatives of the protein family have been localised in the epidermal cell wall (Thoma *et al.*, 1993) as well as in the cuticle (Pyee *et al.*, 1994). LTPs have been grouped into two classes, nsLTP1 (molecular weight, 9 kDa) and nsLTP2 (7 kDa) (Kader, 1996).

NMR spectroscopy and X-ray crystallography have been used to determine nsLTP1 protein structures from rice, barley and wheat. They contain four α -helices, stabilised by disulfide bonds between eight conserved cysteines, surrounding a flexible hydrophobic pocket (Douliez *et al.*, 2000a). Various techniques such as displacement of fluorescent analogues and intrinsic tyrosine fluorescence microscopy have been used to assess the interactions of LTPs with lipids. While nsLTP1 have been shown to bind to linear acyl lipids (Douliez *et al.*, 2000b), including cutin components such as ω -hydroxyhexadecanoic acid (Douliez *et al.*, 2000b, 2001), nsLTP2s showed lower affinity for fatty acids and phospholipids and higher affinity to sterols, and have been postulated to be important in plant defence (Blein *et al.*, 2002; Cheng *et al.*, 2004). None of these studies have tested the binding of VLCFAs or other wax components to LTPs. In summary, nsLTP1s are candidates for transport of cutin and wax monomers through the cell wall, but experiments directly testing their involvement have not been performed to date.

When wax molecules finally reach the cuticular layer, they can easily dissolve into the wax domains there. In case they arrive there as complexes bound to LTPs, the hydrophobic cargo could partition into the cuticle, freeing an LTP for another cycle of delivery. Within the hydrophobic environment of the cuticle, wax components self-arrange into intracuticular layers, epicuticular films and epicuticular crystals (see Chapter 4, section 4.6).

5.7 Concluding remarks

Formation of cuticular waxes requires two types of pathways: those involved in the biosynthesis of wax precursors and those for modifying them into diverse aliphatic lipid classes. Whereas cellular sites of production of VLCFA wax precursors and the biochemical reactions involved in this process have now been established, and at least one gene/enzyme for each step characterised, with the exception of the β -hydroxyacyl-CoA dehydratase, the majority of the proposed reactions leading to the formation of aliphatic wax components still await confirmation, and their subcellular location remains obscure. Information on the identity of the enzymes catalysing various steps of wax biosynthesis and their regulation, currently emerging from molecular and genomic studies in *A. thaliana* and maize, is essential to our overall understanding of cuticular wax deposition, and should provide a framework for rational modification of plant cuticles by genetic engineering to enhance the stress resistance of important agricultural commodities.

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6 Optical properties of plant surfaces

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6.1 Introduction

Plant organs are composed of many tissue types containing specialised cells differing in chemical composition and structure. This complexity results in complicated and variable interactions between radiation and plant matter. Among plant organs, the optical behaviour of leaves is probably the most studied because of its importance to the photosynthetic process (Terashima, 1989; Björn, 1992; Vogelmann, 1993; Smith *et al.*, 1997; Evans, 1999; Carter and Knapp, 2001; Ustin *et al.*, 2001; Vogelmann and Evans, 2002; Evans and Vogelmann, 2003), and for the interpretation of remote sensing data (Buschmann and Nagel, 1993; Zwiggelaar, 1998; Carter and Knapp, 2001; Ustin *et al.*, 2004).

This chapter reviews the optical characteristics of plant surfaces. As all three authors of this review are interested in various aspects of photosynthesis in higher plants, we primarily focus on surface optics of green leaves but also briefly discuss the surface optics of fruits and flower petals. In this review, we define 'plant surface' as any peripheral layer with the potential to influence radiation conditions inside the photosynthetic mesophyll of leaves; that is, the cuticle, epidermis and, in some cases, sub-epidermal layers. We consider natural ultraviolet (UV) radiation, which comprises of UV-B (280–315 nm) and UV-A spectral range (315–400 nm), because UV radiation can not only drive photosynthesis (McLeod and Kanwisher, 1962; Mantha *et al.*, 2001) but is also able to damage various components of the photosynthetic machinery (Day and Neale, 2002; Hollósy, 2002; Jordan, 2002; Kakani *et al.*, 2003). Naturally, we also discuss surface optics in the visible wavelength range (400–700 nm) which is the main energy source for photosynthesis: this spectral range can also damage components of the photosynthetic machinery if light energy is absorbed in excess of photosynthetic capacity (Osmond *et al.*, 1997; Huner *et al.*, 1998; Niyogi, 2000; Krieger-Liszkay, 2005). In this review, we restrict the use of the term 'light' to visible radiation only.

We briefly describe current methods for analysing surface optics and then discuss, in detail, not only optical surface properties arising from electronic absorption of radiation by pigments but also those resulting from non-absorptive interaction of radiation with matter: plant surface optics, of course, integrates both of these optical aspects.

Rather than provide complete coverage of the literature we have opted to concentrate on several especially interesting facets of surface optics in plants, and we apologise to those workers whose important works have not been cited. Consistent

with our restricted focus, we refer to relevant classical papers and recent papers but also include a number of reviews containing broader and more extensive literature compilations.

6.2 Methods to determine optical properties of plant surfaces

6.2.1 UV-visible absorbance spectrophotometry

Solutions of (epi)cuticular matter obtained by short-term extraction of plant surfaces with chloroform, or aqueous/methanolic extracts of epidermal strips peeled off plant organs are often measured spectrophotometrically to assess absorption properties of plant surfaces (see Section 6.3.1). These measurements are suitable for classifying the principal absorbing molecules present, and to reveal changes in their concentration, for example, during acclimation processes. Because optical conditions within plant surfaces often differ fundamentally from those in solutions, spectrophotometric data of extracts should not be used uncritically to evaluate the absorption of these compounds *in vivo*.

Stripping off the epidermis is not possible in many plant species; consequently, whole leaf extracts have been analysed spectrophotometrically to identify and classify epidermal UV-screening compounds (for a review see Searles *et al.*, 2001). The limitations of this method obviously include not only the different optical behaviour of pigments in solutions and *in vivo*, but also the difficulty of allocating variations in concentration of UV-absorbing molecules between upper and lower epidermis and leaf mesophyll (Kolb and Pfündel, 2005).

UV-Vis spectrophotometry has also been employed to analyse mechanically isolated epidermal strips or enzymatically prepared cuticles (see Section 6.3.1). Because isolation and subsequent handling can disrupt these layers, such spectroscopic studies should be accompanied by parallel microscopic investigations to confirm the intactness of the preparations. Furthermore, these layers scatter the measuring beam so that a part of the transmitted radiation, leaving the sample at oblique angles, misses the photodetector in standard spectrophotometers; therefore, spectrophotometric measurements of scattering samples tend to underestimate the sample transmittance. It is possible to reduce this error by placing the sample close to the photodetector, thereby increasing the angle of acceptance, and also by completely diffusing all radiation transmitted by the sample with an opal quartz plate (Butler, 1964). On the other hand, fluorescence excited by the measuring radiation might result in overestimation of transmittance particularly with highly absorbing samples.

6.2.2 Integrating sphere

Integrating spheres are hollow globes with highly reflective inner surfaces. In the so-called 'external' integrating sphere, the sample forms a defined area of the inner

surface. Also, an aperture through which photons can reach the detector is located on the inner surface. Orientation of the sample relative to the aperture is arranged to prevent photons originating in the sample reaching the photodetector directly, but photons at all angles of emergence can reach the detector after multiple reflection with comparable probabilities. Therefore, integrating spheres realise a very large acceptance angle and, hence, overcome the problem of photons missed by scattering in normal spectrophotometers.

In principle, transmittance of samples is calculated from the detector signal obtained with the interior illuminated through the sample, divided by the signal measured with direct illumination of the inner sphere. For reflection measurements, the sample is flipped over and illuminated from the inside of the sphere: by relating the signal obtained under these latter conditions with that recorded from a highly reflecting reference surface a value for reflectance is obtained. Absorbance of a sample is calculated according to:

$$\text{absorbance (\%)} = 100 (\%) - \text{reflectance (\%)} - \text{transmittance (\%)} \quad (6.1)$$

Equation 6.1 illustrates that reflectance, which determines the optical appearance of plant organs viewed from the illuminated side, is determined by absorption and transmission properties. We emphasise that absorbance is not to be confused with absorbance: in the absence of reflection, the logarithmic relationship between these two terms is given by:

$$\text{absorbance} = \log\{100/[100 - \text{absorbance (\%)}]\} \quad (6.2)$$

Integrating spheres are frequently employed to study intact leaves. At wavelengths of high electronic absorption by the leaf, information on surface reflectance can be derived from intact material (see Section 6.4.1). With plant species allowing the preparation of sufficiently large epidermal patches, optical properties of plant surfaces can be studied over the entire wavelength range of interest (Figure 6.1; Grammatikopoulos *et al.*, 1999; and references in Section 6.4.1).

6.2.3 Microfibre optics

Information on plant surface optics is also available from measurement of gradients of radiation intensities inside leaves using fibre microprobes (Vogelmann and Björn, 1984; Vogelmann and Haupt, 1985). These gradients are obtained with a fibre optics probe having a thickness of a few micrometres by advancing it stepwise through the tissue and recording the radiation accepted by the probe using a spectroradiometer. The steepness of intensity curves below the illuminated surface and the depth of penetration of the radiation are both key parameters from which absorption properties of surfaces can be derived. This technique has been useful in obtaining information on UV screening of leaf surfaces (Bornman and Vogelmann, 1988; Bornman and Vogelmann, 1991; Day *et al.*, 1992; Cen and Bornmann, 1993; Ålenius *et al.*, 1995; Turunen *et al.*, 1999; Olsson *et al.*, 2000; Liakoura *et al.*, 2003).

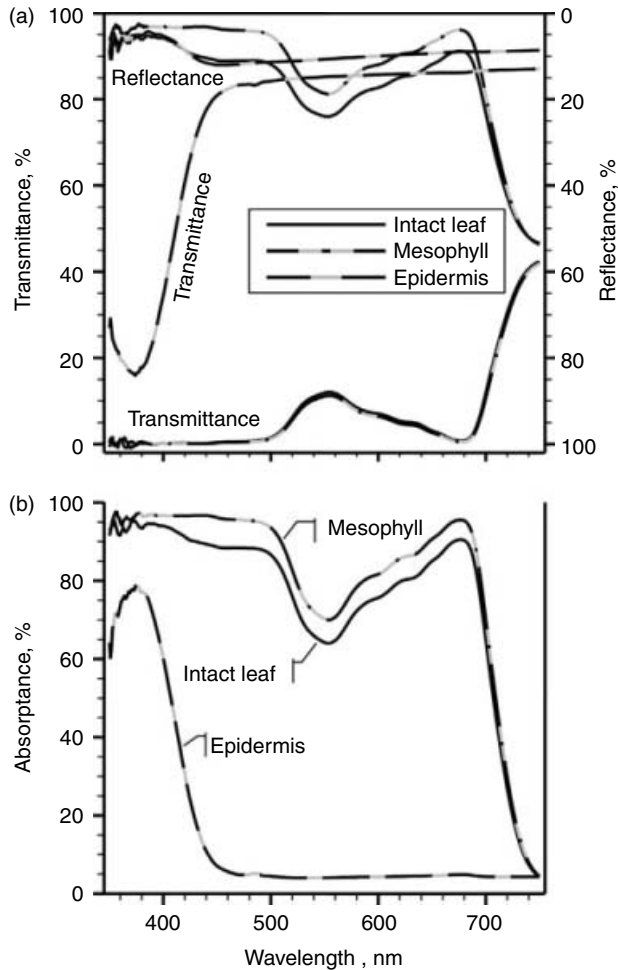


Figure 6.1 Some optical properties of leaf preparations from *Tulipa spec.* Reflectance and transmittance spectra (a), and absorbance spectra, calculated from the former according to Equation 6.1, (b) were recorded using whole leaves (labelled: intact leaf), leaves with the epidermis stripped off (mesophyll) and epidermal strips (epidermis). Each spectrum corresponds to the mean of four independent measurements of outdoor-grown material recorded with an external integrating sphere (1800-12 integrating sphere, Li-Cor, Lincoln, Nebraska) in combination with an LI-1800 portable spectrometer (Li-Cor). Panel (a) illustrates important aspects of surface optics: (1) removal of the epidermis decreases reflectance at wavelengths greater than 400 nm; (2) reflectance of intact leaves and epidermis is lowest below 400 nm; and (3) reflectance of the intact leaf and epidermis coincide when intact leaf or mesophyll transmittance is minimal between 350 and 480 nm and near 680 nm (see Section 6.4.1 for a discussion of these phenomena). Epidermal transmittance (a) is minimal below 400 nm, which is almost certainly due to the presence of colourless flavonoids located in epidermal cell vacuoles (see Section 6.3.1). The calculated absorbance spectra (b) show that the mesophyll absorbs radiation better than the intact leaf at wavelengths greater than 400 nm, and also, show efficient epidermal absorbance at wavelengths lesser than 400 nm (Jana Leide and E.E. Pfündel, unpublished data).

6.2.4 *UV-excited chlorophyll fluorescence*

UV fluorimetry allows the assessment of epidermal absorbance of intact leaves in the UV spectral range. The basis of the method relies on the efficient absorption of UV by chlorophyll (Cerovic *et al.*, 1999) and on the fact that a certain fraction of leaf chlorophylls, excited by absorption of radiation, emits red fluorescence when returning to the ground state (Dau, 1994). In intact leaves, UV excitation is reduced when epidermal UV screening is high and, under such conditions, relatively weak chlorophyll fluorescence is observed (Cerovic *et al.*, 1993; Sheahan, 1996; Mazza *et al.*, 2000).

Bilger *et al.* (1997) introduced the ratio of UV-excited fluorescence to that excited by blue–green light as an accurate measurement for epidermal UV screening with the rationale that visible radiation is not absorbed by the epidermis but fluorescence excitation in the visible and in the UV is subject to the same optical peculiarities of the individual sample. Consequently, this fluorescence excitation ratio (FER) method cancels out specific optical properties of the sample except that of epidermal UV screening. Recently, two portable devices have been developed: the Dualex leaf clip (Goulas *et al.*, 2004) and the UV-A-PAM fluorimeter (Bilger *et al.*, 2001, Krause *et al.*, 2003; Kolb *et al.*, 2003, 2005). Both permit repeated measurements of epidermal UV screening of the same sample under field conditions.

The Bilger approach has been strongly supported by the parallel relationship observed between UV transmittance data from spectrophotometric measurements of isolated epidermal strips and FER data (Markstädter *et al.*, 2001). The formation of anthocyanins, however, interferes with excitation by blue light and can, therefore, restrict the use of fluorimetry in red leaves (Barnes *et al.*, 2000). This problem can be overcome by choosing another reference excitation wavelength in the red (650 nm in Dualex), which is outside the anthocyanin absorption range (Goulas *et al.*, 2004). In addition, fluorescence excited at wavelengths within the anthocyanin absorption range when divided by fluorescence excited at wavelengths of high epidermal transparency can also be used to determine the epidermal screening due to anthocyanins *in vivo* (Agati *et al.*, 2005).

6.2.5 *Fluorescence microscopy*

Fluorescence microscopy can complement data obtained with the earlier techniques by obtaining two- and even three-dimensional information on the arrangement of structures and compounds of plant surfaces. This technique can localise absorbing substances by fluorescence: such data were useful in providing an understanding of the origin of fluorescence signals of entire leaves and, also, for the interpretation of remote fluoro-sensing data.

Epi-fluorescence microscopy is widely used to analyse leaf tissues and it has been much improved in the last decade due to progress in digital imaging, confocal laser scanning and multiphoton microscopy (Blancaflor and Gilroy, 2000). Increased performance of personal computers permitted the application of image restoration

by algorithms to remove out-of-focus signals in wide-field fluorescence microscopy (McNally *et al.*, 1999). Compared to wide-field fluorescence microscopy, which works well with relatively thin specimen ($<30\ \mu\text{m}$), both confocal and multi-photon mode microscopy yield crisp images with much thicker samples (Hutzler *et al.*, 1998; Feijo and Moreno, 2004). In addition to localisation of substances, fluorescence microscopy of leaves can yield information about the concentrations of substances as demonstrated in confocal fluorescence microscopic studies on photosystem distribution in leaves from C_4 photosynthesis plants (Pfundel and Neubohn, 1999).

Microscopic analysis of leaf optical properties can be performed by non-destructive observation of the adaxial and abaxial surfaces, as well as by imaging leaf sections: confocal laser scanning microscopy was employed to investigate, non-destructively, the blue–green fluorescence of UV-absorbing phenolics in barley leaves to a depth of $35\ \mu\text{m}$ (Hideg *et al.*, 2002); UV-induced fluorescence microscopic imaging of surfaces of intact wheat leaves was used to evaluate the contribution of epidermis cells and sclerenchyma bands to blue–green emission (Meyer *et al.*, 2003); and fluorescence microimage analysis of leaf surfaces was employed for *in situ* counting of stomata (Karabourniotis *et al.*, 2001).

Photobleaching and photodamage, however, are important limitations in the use of fluorescence microscopy to study living structures since the radiation, used to excite fluorescence, often forms free radicals that react with cellular constituents. Moreover, fluorophores can undergo molecular degeneration or rearrangement to form non-fluorescent species or may produce singlet oxygen, which will efficiently react with nearby bio-molecules. Multiphoton microscopes overcome most of these disadvantages because of their limitation of fluorophore excitation and photodamage to a relatively small focal volume (Diaspro and Sheppard, 2002).

When determining the intracellular location of compounds, non-ruptured cells are required for microscopy and, therefore, cross-sections of at least $50\text{--}100\ \mu\text{m}$ thickness are essential. Longitudinal sectioning is required to get intact epidermal cells of monocotyledonous species that can be $200\text{--}2000\ \mu\text{m}$ long (Wenzel *et al.*, 1997). Paradermal sections are particularly useful since they show intact cells from the epidermal and mesophyll compartments on the same image (Hutzler *et al.*, 1998).

Micro-spectrofluorimetry, which provides spectrally resolved information on microscopic areas, can identify individual compounds in the presence of several different fluorophores (Schnabl *et al.*, 1986; Opitz *et al.*, 2003) and provides the basis for selection of appropriate acquisition bands for multi-spectral imaging. Multi-spectral imaging, in which spectral information is added to each pixel, is a new and promising technology to identify auto-fluorescent plant molecules and to study their function (Berg, 2004). The introduction of highly sensitive CCD (charge coupled device) detectors permits significant reduction of acquisition times (Agati *et al.*, 2002; see Figure 6.2) and, therefore, ensures wider use of multispectral imaging in the future. Recently, multispectral imaging was successfully used in a study of the intracellular location of polyphenols in species of the Mediterranean basin

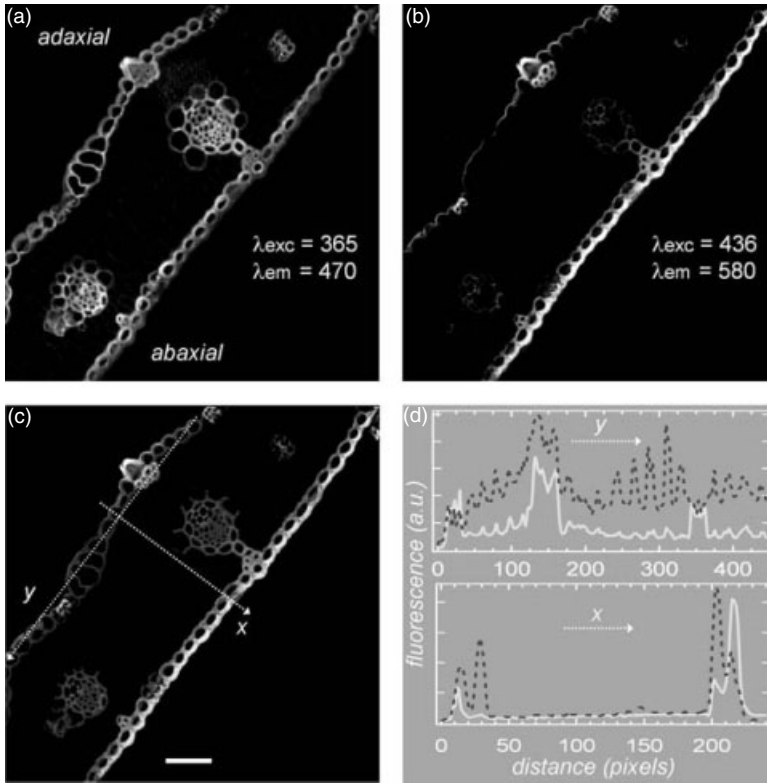


Figure 6.2 Multispectral autofluorescence microimaging of a 100- μm -thick cross-section from a *Triticum aestivum* L. leaf in phosphate buffer. The autofluorescence images in panel (a) was excited at 365 nm and detected in the blue range at 470 nm, and that in panel (b) was excited at 436 nm and detected in the yellow range at 580 nm. Combination of monochrome images, (a) and (b), with blue and yellow colours assigned to the 470 and 580 emission bands, respectively, is shown in panel C (bar = 50 μm). Panel (d) depicts fluorescence intensity profiles for the 470 and 580 nm bands along the two, x and y, directions indicated by dotted arrows in (c). Panels (c) and (d) clearly reveal inhomogeneous fluorescence characteristics and, thus, quite large variations in spatial localisation of different fluorescing compounds: the UV-induced blue fluorescence emanates from cell walls, while the blue-induced yellow signal appears to be confined to cuticles, guard cells and sclerenchyma bands (G. Agati, Corrado Tani and Z.G. Cerovic, unpublished data). This figure is produced in colour in the colour plate section, which follows page 249.

(Tattini *et al.*, 2004, 2005) and also for the characterisation of leaf damage induced by ozone stress (Bussotti *et al.*, 2005).

6.2.6 Auto-fluorescence and reagent-induced fluorescence

The early microscopic studies of Harris and Hartley (1976) suggested that the main contributors to blue–green fluorescence of leaf surfaces included hydroxycinnamic

acids esterified to epidermal cell-wall polysaccharides, lignin in sclerenchyma tissue and cutin in cuticles. Cuticles of many monocot and dicot species fluoresced when excited with UV-A radiation (Harris and Hartley, 1980; Hartley and Harris 1981). It is uncertain, however, if UV-A-excited fluorescence also arises in part from flavonoids which have been reported to occur in cuticular waxes (Wollenweber and Dietz, 1981). Trichomes, both glandular and non-glandular, can also contribute to superficial auto-fluorescence (Karabourniotis and Fasseas, 1996; Meyer *et al.*, 2003; Agati *et al.*, unpublished data).

Tissue localisation of compounds exhibiting weak or undetectable auto-fluorescence, including many polyphenolics, can be obtained by using specific fluorescence intensifiers (Hutzler *et al.*, 1998). A widely used staining procedure for leaf sections, to visualise flavonoids, employs solutions of Naturstoff reagent (NR) which is diphenylboric acid 2-aminoethylester (Dai *et al.*, 1995; Schnitzler *et al.*, 1996; Hutzler *et al.*, 1998). This reagent was previously used to detect flavonoids in thin layer chromatography by inducing secondary fluorescence (Markham, 1989). However, the products formed by reaction of specific flavonoids with NR have not yet been identified, and we must also be aware that NR is not totally specific for flavonoids. Indeed, it reacts also with hydroxycinnamic acids and their derivatives by forming fluorescent products (Agati *et al.*, 2002). Separation of fluorescence from different classes of compounds can be obtained only by using different excitation wavelengths (Tattini *et al.*, 2004).

Alkaline conditions also induce strong fluorescence in both flavonoids (Schnabl *et al.*, 1986; Markham, 1989) and hydroxycinnamic acids (Harris and Hartley, 1976; Meyer *et al.*, 2003). Because of this lack of specificity, this alkaline test cannot be easily applied to localise specific compounds in leaf sections. However, the combination of the alkali treatment with appropriate excitation wavelengths can help to discriminate between different phenolics.

6.3 Electronic absorption of radiation

Electronic absorption of radiation is a key factor determining plant surface properties. Substantial absorption at the short-wavelength edge of natural radiation, that is the UV-B region, depends on the presence of linear or cyclic compounds exhibiting four or more conjugated double bonds (Cockell and Knowland, 1999). The normal components of plant waxes lack such conjugated double bond systems (see Chapter 4). Accordingly, dissolved cuticular wax from needles of two conifer species did not absorb significantly above 300 nm (Bornman and Vogelmann, 1988) and penetration of UV radiation into needles of the conifer *Picea pungens* was not affected by wax removal (Day *et al.*, 1992); similarly, wax extraction had little effect on the UV absorption of isolated cuticles (Baur *et al.*, 1998). Epicuticular matter from *Olea chrysophylla* also absorbed very little natural UV (Karabourniotis *et al.*, 1992) and epicuticular material from *Pinguicula vulgaris* showed only a single peak at 277 nm which is outside the UV-B range (Mendez *et al.*, 1999).

Cutin monomers (see Chapter 3) and the carbohydrates of cell walls also lack the extended double bond systems and, hence, cannot effectively absorb natural UV radiation. However, isolated cuticles of some plant species exhibit conspicuously high UV-B absorption (Krauss *et al.*, 1997; Baur *et al.*, 1998), suggesting the presence of UV-absorbing substances. Similarly, UV-absorbing compounds have been located in wax layers and cell walls of some species but are also present in vacuoles (see later). Furthermore, substances affecting optical characteristics and absorbing in the visible range occur in plant surfaces. In this chapter, we divide all compounds which contribute to surface optics by absorption of UV and visible radiation into three groups: (1) phenolics, including hydroxycinnamic acids, colourless flavonoids and anthocyanins, (2) betalains and (3) carotenoids.

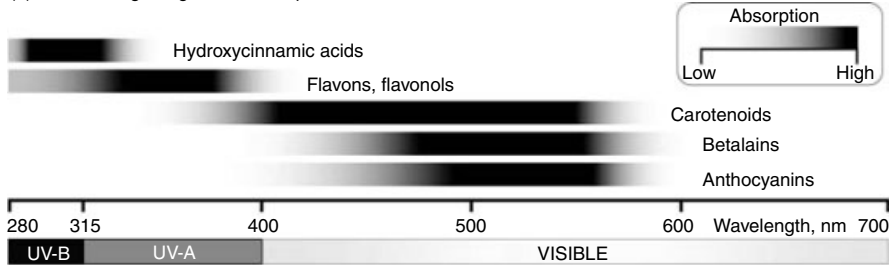
Compounds of all groups are not only involved in absorption of radiation but also exhibit free-radical scavenging functions which is outside the scope of this chapter. Information on the latter properties have been summarised for hydroxycinnamic acids (Chen and Ho, 1997; Foley *et al.*, 1999; Son and Lewis, 2002), for colourless flavonoids (Pietta 2000; Cotellet 2001; Amić *et al.*, 2003; Williams *et al.*, 2004), for anthocyanins (Wang *et al.*, 1997; Neill and Gould, 2003), for betalains (Pedreño and Escribano, 2000; Kanner *et al.*, 2001; Cai *et al.*, 2003), and for carotenoids (Di Mascio *et al.*, 1991; Pfündel and Bilger, 1994; Young and Lowe, 2001). Also, consistent with the title and focus of this chapter, we will not discuss the occurrence and function of absorbing compounds located in the interior of plant organs.

6.3.1 Phenolics

Plant phenolics include hydroxycinnamic acids and flavonols and are products of general phenylpropanoid metabolism, in which the initial step is the elimination of ammonia from the aromatic amino acid phenylalanine to form cinnamic acid. This acid can be hydroxylated in the presence of O₂ by a mono-oxygenase to 4-hydroxycinnamic acid (i.e. *p*-coumaric acid) which has a C₆-C₃ structure. Coumaric acid may also be formed directly by elimination of ammonia from tyrosine (Hahlbrock and Scheel, 1989; Rosler *et al.*, 1997). There are a multitude of flavonoids but the first committed step in their synthesis, catalysed by chalcone synthase, is the condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA forming three molecules of CO₂ and the simplest flavonoid, chalcone, with a C₆-C₃-C₆ skeleton (Forkmann and Heller, 1999).

4-Hydroxycinnamic acids. Hydroxycinnamic acids (see Figure 6.3) and their simple derivatives, absorb mainly in the UV-B and the short-wavelength UV-A range (Lichtenthaler and Schweiger, 1998; Kolb *et al.*, 2001; Meyer *et al.*, 2003; Kolb and Pfündel 2005). Consequently, high concentrations of peripherally located hydroxycinnamic acids can efficiently protect plants from the known deleterious effects of UV-B radiation (Hollósy, 2002). In fact, hydroxycinnamic acids have been shown to screen the leaf mesophyll in *Arabidopsis thaliana* from UV-B radiation (Li *et al.*, 1993; Landry *et al.*, 1995; Sheahan, 1996).

(a) Wavelength regions of absorption of radiation



(b) Chemical structures

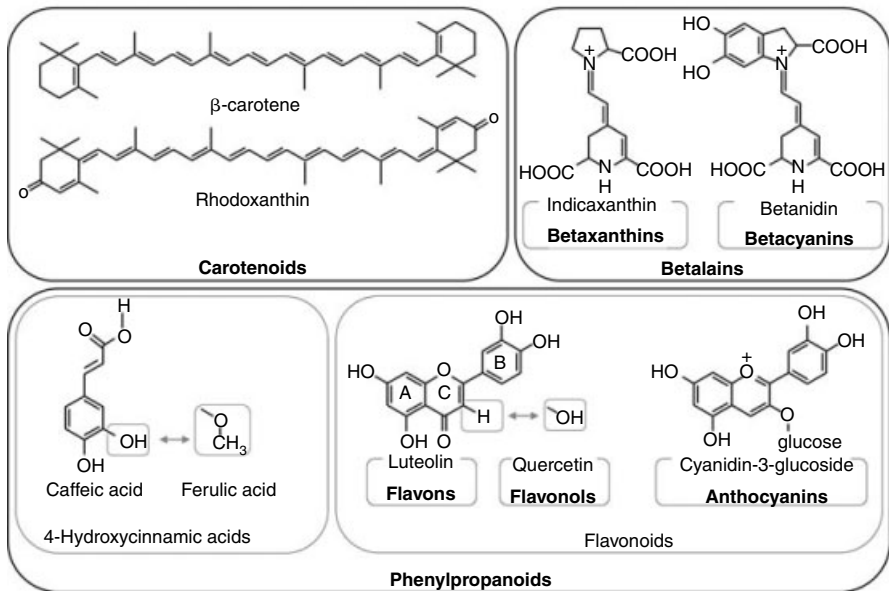


Figure 6.3 Schematic representation of absorption regions of the major groups of compounds involved in electronic absorption properties of plant surfaces (a) and chemical structures of representative compounds from these groups (b). For detailed information on surface absorption see Section 6.3 (structures from Bate-Smith, 1962; Isler, 1971; Borriss and Libbert, 1985; Harborne, 1988; Strack and Wray, 1989).

Hydroxycinnamic acids occur covalently bound to epicuticular wax constituents of leaves (Markstädter, 1994; Schmutz *et al.*, 1994; Ruhland and Day, 2000; Liakopoulos *et al.*, 2001), isolated cuticles (Baur *et al.*, 1998) and cell walls (Harris and Hartley, 1976; Strack *et al.*, 1988; Akin, 1995; Lichtenthaler and Schweiger, 1998; MacAdam and Grabber, 2002), but water-soluble hydroxycinnamic acids are also found in vacuoles of epidermal cells (Strack and Sharma, 1985; Schnabl *et al.*, 1989). It has been suggested that insoluble bound hydroxycinnamic acids in

cuticles of leaves from various woody species (Krauss *et al.*, 1997) or in cell walls of conifer needles (Schnitzler *et al.*, 1996; Fischbach *et al.*, 1999) provide screening against UV-B radiation; however, a general screening function for bound hydroxycinnamic acids is questionable because extraction of soluble phenolics from isolated epidermal peels often results in highly UV-transmitting samples (Lautenschlager-Fleury, 1955; Caldwell, 1971; Robberecht and Caldwell, 1978; Markstädter *et al.*, 2001).

The importance of soluble hydroxycinnamic acids for UV-B screening in leaves varies significantly between species and it has been suggested that they are of only minor significance for *Secale cereale* (Burchard *et al.*, 2000), *Vicia faba* (Markstädter *et al.*, 2001), *Ligustrum vulgare* (Tattini *et al.*, 2004) and *Hordeum vulgare* (Kolb and Pfündel, 2005); however, increased epidermal UV-B screening of greenhouse-grown grapevine leaves (*Vitis vinifera*), during acclimation to natural radiation, arises mainly from synthesis of soluble hydroxycinnamic acids (Kolb *et al.*, 2001; Kolb and Pfündel, 2005).

In fruits, data on optical properties of hydroxycinnamic acids are sparse. The presence of hydroxycinnamic acids has been demonstrated in the vacuoles of *V. vinifera* berries (Moskowitz and Hrazdina, 1981) but they contribute little to UV screening in the white *V. vinifera* cultivar 'Bacchus' (Kolb *et al.*, 2003). Significant amounts of the hydroxycinnamic acid derivative verbascoside have been demonstrated in the skin of olive fruits (Servili *et al.*, 1999) which might play a role in UV-B screening. It is uncertain if simple hydroxycinnamic acids contribute to the optical properties of flower surfaces but they can affect petal colouration by forming complexes with flavonoids (Harborne and Williams, 2000).

Colourless flavonoids. The so-called colourless flavonoids, flavones and flavonols (Harborne, 1988), exhibit an absorbance peak located in the UV-A spectral region (about 350 nm), with another peak near 260 nm, that is, outside the natural UV range (Cerovic *et al.*, 2002). Colourless flavonoids, accumulated in the leaf epidermis could, therefore, screen the mesophyll against the damaging effects of UV-A radiation on photosystem II (Turecsányi and Vass, 2000; Nayak *et al.*, 2003; Pfündel, 2003). Flavonoids can also be covalently linked to hydroxycinnamic acids as in the needles of *Pinus sylvestris* (Schnitzler *et al.*, 1996) and in the leaves of *Pisum sativum* (Weissenböck *et al.*, 1986): these molecules combine the absorption properties of both hydroxycinnamic acids and colourless flavonoids.

The absorbance trough in the UV-B spectrum of simple colourless flavonoid derivatives does not necessarily signify inefficient UV-B screening because variations in absorption by these flavonoids correlated with variations in epidermal absorption determined fluorometrically in both the UV-A and in the UV-B spectral range (Burchard *et al.*, 2000; Mazza *et al.*, 2000; Bilger *et al.*, 2001; Markstädter *et al.*, 2001; Kolb and Pfündel, 2005).

Soluble glycosylated flavonoids have been demonstrated in the vacuoles of leaves from many plant species (Tissut and Ravel, 1980; Hrazdina *et al.*, 1982; Weissenböck *et al.*, 1984, 1986; Hutzler *et al.*, 1998; Kolb *et al.*, 2001) but

flavonoids are also bound to cell walls of conifer needles (Strack *et al.*, 1989; Schnitzler *et al.*, 1996; Hoque and Remus, 1999) and their non-polar derivatives are also constituents of wax layers on leaf surfaces (Wollenweber and Dietz, 1981; Harborne and Williams, 2000; Onyilagha and Grotewold, 2004).

Recent data, from leaves of *Phillyrea latifolia*, support the view that cuticular flavonoids may play some role in UV screening of leaves (Tattini *et al.*, 2005); however, there is no general consensus on the importance of non-vacuolar flavonoids for UV screening of leaves. Indeed, the increase in UV-A screening in leaves, induced by UV radiation, can be attributed to vacuolar flavonoids (Schnitzler *et al.*, 1996; Burchard *et al.*, 2000; Markstädter *et al.*, 2001; Kolb *et al.*, 2001; Kolb and Pfündel, 2005) while non-vacuolar flavonoids seem to play a negligible role in variable UV screening.

Flavonol glycosides have been demonstrated in the vacuoles of white *V. vinifera* berries (Moskowitz and Hradzina, 1981). Fluorescence microscopy on the berries showed that the flavonols are accumulated in the multi-layered skin of the fruit suggesting that they function as UV-screening compounds. This was confirmed by the correlation between absorption of isolated flavonoids and skin absorption in both the UV-A and UV-B regions (Kolb *et al.*, 2003). Flavonols have been suggested as UV-screening compounds in apple (*Malus pumila*) fruits (Merzlyak *et al.*, 2005). It was estimated that cuticular flavonoids accounted for approximately one third (at most) of the total flavonoid UV screening in the *M. pumila* skin (Solovchenko and Merzlyak, 2003). Further, flavonoids were located in the skin layer of olives (*Olea europaea*) (Servili *et al.*, 1999) and flavonoid precursors were found in tomato (*Lycopersicon esculentum*) fruit cuticle (Baker *et al.*, 1982) which could be consistent with a UV-screening function for flavonoids.

Substantial amounts of colourless flavonoids have been found in the epidermal vacuoles of petals from a number of plant species (Kay *et al.*, 1981). The confinement of the UV-absorbing flavonoids to only certain areas of the petal can result in patterns of UV reflection which might increase their attraction to pollinating insects with UV vision (see Section 6.4.1 and Chapter 13). Colourless flavonoids can also affect the visible colour of flowers by acting as co-pigments to anthocyanins (Harborne, 1988).

Anthocyanins. Anthocyanidins, the chromophores of anthocyanins, are formed from colourless flavan-3,4,-diols (Harborne, 1988; Koes *et al.*, 1994; Turnbull *et al.*, 2000). Formation of anthocyanin, by glycosidation of anthocyanidin, is important for stabilising the chromophore *in vivo*; consequently, the failure to glucosylate flavonoids in grape-skin tissue results in an anthocyanin-free, white *V. vinifera* berry (Boss *et al.*, 1996). At acidic pH values, anthocyanins exist as flavylum cations which absorb maximally in the green wavelengths between 500 and 550 nm with negligible absorption in the red region (Harborne, 1988; Strack and Wray, 1989; Stintzing and Carle, 2004). The absorption characteristics of anthocyanins are affected not only by the pH but also by substitution patterns, presence of co-pigments and by metal ions chelated to the anthocyanins (Harborne, 1988; Strack

and Wray, 1989). Anthocyanins also have an absorbance maximum between 267 and 275 nm (Harborne, 1989) and, when hydroxycinnamic acids are covalently linked to anthocyanins, another maximum occurs in the UV-B and short wavelength UV-A regions (310–330 nm; Strack and Wray, 1989).

Anthocyanins are located in vacuoles (Strack and Wray, 1989) and accumulate especially in the epidermis of leaves of several plant species including *P. sativum* (Hrazdina *et al.*, 1982), *Pinus banksiana* (Huner *et al.*, 1998), *Zea mays* (Pietrini *et al.*, 2002) and *Lactuca sativa* (Neill and Gould, 2003). These epidermally located anthocyanins can reduce the intensity of light reaching the photosynthetic mesophyll tissue. The light-screening function of anthocyanins in *L. sativa* cv. 'Dark Lollo Rosso' was recently investigated by Neill and Gould (2003). The anthocyanins were synthesised only in a part of the leaf lamina where they are confined to the adaxial epidermis. The presence of anthocyanins increases total leaf absorbance from 35 to 75% at 550 nm and from 79 to 88% at 440 nm. If we disregard leaf reflectance, these absorbance data correspond to a reduction of transmittance by approximately 2.5-fold in the green region and 1.8-fold in the blue region; thus, epidermally located anthocyanins are competent to screen out blue and green radiation.

Anthocyanin-dependent screening of the photosynthetic mesophyll from excessive light intensities has, indeed, been suggested to prevent deleterious photoinhibitory processes (Chalker-Scott, 1999; Steyn *et al.*, 2002; Gould, 2004) which arise from inefficient use of absorbed light quanta under unfavourable conditions (Osmond *et al.*, 1997; Huner *et al.*, 1998; Niyogi, 2000; Krieger-Liszskay, 2005). It has also been suggested that light screening by anthocyanins assists to retrieve nutrients from the light-sensitive, senescing leaves by preventing high light intensities damaging the resorption system (Hoch *et al.*, 2001, 2003). Anthocyanins, however, have also been found to be confined to the lower epidermis of some aquatic plants (Lee, 1986) which is incompatible with a light screening function. Anthocyanins appear to be unimportant in UV screening of leaves which agrees with the relatively low absorption of simple anthocyanins in the natural UV range (Close and Beadle, 2003; Gould, 2004).

Anthocyanins might also assume some light-screening functions in the skin of *M. pumila* fruits (Merzlyak and Chivkunova, 2000) and in the exocarp of pods from the Leguminous tree *Bauhinia variegata* (Smillie and Hetherington, 1999). The peripheral localisation of anthocyanins suggests a possible light screening function in *O. europaea* fruits (Agati *et al.*, 2005). The anthocyanins in the sub-epidermal tissues of red-grape berries (Moskowitz and Hrazdina, 1981) could also provide screening against high-light intensities.

In the petals of many flowers, anthocyanins are confined to the vacuoles of epidermal cells (Kay *et al.*, 1981). These anthocyanins produce red to magenta flower petals, but co-pigmentation with flavonoids or chelation of metal cations can shift the absorption peak in the visible region to produce blue colours that are the preferred attractant to bee pollinators (Harborne and Williams, 2000).

6.3.2 Betalains

Betalains (Figure 6.3) are immonium conjugates of betalamic acid comprised of yellow betaxanthins, with amino acids or amines as the conjugating moiety and red-violet betacyanins, which are conjugated to various substituted *cyclo*-Dopa (i.e. *cyclo*-dihydroxy-phenylalanine) derivatives (Strack *et al.*, 2003). Betaxanthins have an absorption peak near 480 nm and the betacyanins have a corresponding peak between 530 and 550 nm with a further peak between 270 and 280 nm due to the Dopa moiety (Pedreño and Escribano, 2000; Cai *et al.*, 2001a,b). Acylation of some betacyanins with hydroxycinnamic acids gives rise to a third absorbance maximum between 300 and 330 nm (Strack *et al.*, 2003; Stintzing and Carle, 2004).

Betalains occur only in some families of the order of *Caryophyllales* which includes other families that synthesise anthocyanins. Because the betalain and anthocyanin pathways appear to be mutually exclusive (Kimler *et al.*, 1970; Stafford, 1994; Mabry, 2001), it was suggested that betalains assume the same functions as anthocyanins (Steyn *et al.*, 2002; Stintzing and Carle, 2004). Betalains, like anthocyanins, are located in vacuoles (Stintzing and Carle, 2004) and they also accumulate in the upper leaf epidermis of ice-plant leaves (*Mesembryanthemum crystallinum*) in response to high radiation intensities (Vogt *et al.*, 1999; Ibdah *et al.*, 2002) suggesting that betalains can protect against high light intensities. Betalains also replace anthocyanins as flower and fruit pigments, presumably, to attract not only insect pollinators but also fruit-eating animals as a seed dispersal mechanism (Kay *et al.*, 1981; Kobayashi *et al.*, 2000; Cai *et al.*, 2001a,b; Butera *et al.*, 2002; Christinet *et al.*, 2004). It is unclear, however, if betalains function as light screens in fruits as has been proposed for anthocyanins.

6.3.3 Carotenoids

Most, but not all, plant carotenoids are made up of a C₄₀ carbon skeleton including a central polyene chain and two end rings (Figure 6.3). The number of conjugated double bonds ranges between 9 and 14 giving rise to absorption of blue and blue-green light, respectively (Vetter *et al.*, 1971; Britton, 1985, 1995; van den Berg *et al.*, 2000). If the central polyene chain is in the all-*trans* configuration, the UV absorption of the carotenoid is low; with *cis* configurations, however, the UV absorption peak is higher and can reach roughly one-third of the maximum absorption in the visible range (Molnár and Szabolcs, 1980; Bialek-Bylka *et al.*, 1998; Phillip *et al.*, 1999). It is unclear, however, if accumulation of *cis*-carotenoids is sufficient to affect plant surface optics in the UV.

Carotenoids are synthesised in plastids (Lichtenthaler, 1999; van den Berg *et al.*, 2000; Hirschberg, 2001) where they are normally located. In chromoplasts, the yellow, orange or red carotenoid colours are apparent, but in the green chloroplast, these colours are masked by the green chlorophylls (Bartley and Scolnik, 1995). In green leaves, plastids are absent from epidermal cells, with the exception of stomatal guard cells, although some extreme-shade plants do possess chloroplasts in normal

epidermal cells (Héban and Lee, 1984; Lee 1986); hence, carotenoid effects on the optical surface properties of green leaves can only arise from sub-epidermal layers.

During acclimation to cold seasons, the leaves of some gymnosperms develop red–brown colours due to synthesis of large amounts of rhodoxanthin (Ida, 1981; Weger *et al.*, 1993). This colouration by carotenoids was confined to the upper leaf side (Weger *et al.*, 1993) which is consistent with carotenoid-dependent screening of visible radiation. In fact, Han *et al.* (2003) reported less light stress during winter in rhodoxanthin-synthesising foliage of wild-type *Cryptomeria japonica* than in a non-rhodoxanthin-accumulating mutant. More spatial distribution studies of rhodoxanthin within conifer leaves are needed to determine if rhodoxanthin is accumulated in the upper mesophyll layers to form an adequate shield against light stress in the bulk mesophyll beneath. Screening of light by carotenoids in senescing leaves of Norway maple (*Acer platanoides*) was also proposed by Merzlyak and Gitelson (1995) on the basis of reflectance and transmittance spectra. Furthermore, carotenoids, located in *M. pumila* peel, have been suggested to protect the fruit against deleterious light intensities together with anthocyanins (Merzlyak *et al.*, 2002).

Carotenoid-containing chromoplasts of the yellow–red coloured petals of wallflower (*Erysimum cheiri*), are derived from chloroplasts situated in the epidermal cells of the petal (Weston and Pyke, 1999). In a survey of flowering plants, Kay *et al.* (1981) found that carotenoids are often confined to the petal epidermis. A combination of epidermally located chromoplasts with unpigmented mesophyll, which provides back-scattering of non-absorbed light (see Section 6.4.1), could intensify colour to efficiently attract pollinators.

6.4 Non-absorptive optical properties

6.4.1 Reflectance

Radiation can not only be absorbed at surfaces, but redirection of photons at transitions of phases with different refractive indices also affects the optical behaviour of plant surfaces. This subject has been reviewed by Grant (1987), Vogelmann (1993) and Barnes and Cardoso-Vilhena (1996). In brief, radiation can be reflected at the border between air and a smooth cuticle surface resulting in specular reflectance rather than diffuse reflectance which arises from a perfect matte surface (i.e. a so-called Lambertian surface). Specular reflectance exhibits polarisation and directional intensity which are both affected by the angle of incident radiation and the viewing angle (Woolley, 1971).

Natural surfaces which are composed of structures that are larger than the wavelength of the incident light can reflect radiation specularly. Surface structures which are much smaller than the wavelength of the incident radiation can, however, be much more effective in reflecting radiation of shorter rather than longer wavelengths. Radiation from this so-called Rayleigh scattering may be polarised

depending on particle frequency (Grant, 1987). The large variability of plant surface structures, for instance, brought about by epicuticular wax crystals or leaf hairs (trichomes), result in manifold types of surface reflection to which both specular reflectance and Rayleigh scattering can contribute to various degrees. A further complication arises when photons which are transmitted by the outer surface of a plant organ are redirected back to this surface by multiple reflection and scattering in deeper tissue. This is known as diffuse reflection; that is, the reflected radiation intensity in any direction varies according to Lambert's cosine law and it is not polarised. A study of light scattering by leaves led Gates *et al.* (1965) to conclude that whole leaf diffusion was more of the Mie rather than of the Rayleigh type, because radiation at all wavelengths was scattered more or less equally.

Leaf specular reflectance has been analysed using instruments that allow measurements of direction and polarisation of radiation (Woolley, 1971; Liang *et al.*, 1997; Jacquemoud and Ustin, 2001; Raven *et al.*, 2002). Results obtained with leaves and individual plants are taken into account in radiative transfer models for vegetation canopies in which the contribution of specular reflectance to radiation fluxes has been considered (Gastellu-Etchegorry *et al.*, 1996; Andrieu *et al.*, 1997). Because the fraction of polarised radiation to total leaf reflectance may vary markedly between species (Grant *et al.*, 1993), it was also suggested that discrimination between specular and diffuse radiation in remote sensing could help to distinguish between different types of vegetation (Barnes and Cardoso-Vilhena, 1996).

Many studies on leaf optics, however, do not differentiate between specular and diffuse radiation but they measure total reflectance from perpendicularly illuminated leaves using integrating spheres. To understand reflectance characteristics, it is important to realise that, at wavelengths of efficient absorption inside a leaf, the probability of multiple scattering events of a single photon is reduced and, hence, reflection from internal structures is low (Grant, 1987). This explains why whole reflectance is low in the blue and red spectral range where photosynthetic pigments absorb strongly (Figure 6.1). Reduction of diffuse reflection by chlorophyll absorption also accounts for the close association between reflectance in the far-red spectral range and chlorophyll concentration (Buschmann and Nagel, 1993; Carter and Knapp, 2001; Gitelson *et al.*, 2003).

In the UV spectral region, absorption by UV-screening phenolics in the epidermis is additional to chlorophyll absorption in the leaf mesophyll (Cerovic *et al.*, 1999, 2002). Hence, UV reflectance of leaves is assumed to originate at or near the leaf surface (Grant *et al.*, 2003) and, therefore, non-absorptive attenuation of radiation by the plant surface can be assessed from whole-leaf reflection measurements in the UV.

Glabrous surfaces. Upper surfaces of many glabrous (or smooth) leaves from mountain and alpine plants exhibit reflectance values in the UV-A below 7% (Caldwell, 1968). These data have been supported by others: leaves of *Z. mays*

reflected 5% of UV radiation (Woolley, 1971); glabrous *Eucalyptus* leaves exhibited UV-B reflectance of 5% (Robberecht *et al.*, 1980); UV-reflectance values ranging between 5 and 10% were measured for *P. sativum* leaves (Donkin and Price, 1990; Gonzalez *et al.*, 1996); leaflets from bean plants (*Phaseolus vulgaris*) reflected 5% of radiation at 360 nm (Bullas-Appleton *et al.*, 2004); green leaves of *A. platanoides* exhibited UV reflectance of 5% (Merzlyak and Gitelson, 1995); and, pecan leaves (*Carya illinoensis*) reflected between 4 and 8% of UV radiation (Qi *et al.*, 2003). Grant *et al.* (2003) determined UV reflectance values of approximately 5% for leaves of 20 different deciduous tree species.

The above data from intact leaves agree well with UV-reflectance measurements using isolated epidermal strips from six different species which exhibited reflectance in the UV between 4 and 9% (Gausmann *et al.*, 1975). With the reservation that specular reflectance at oblique angles of incident radiation is probably higher than that observed under the more frequently used conditions of perpendicular illumination, published data suggest that UV reflectance originating at the leaf periphery is negligible in glabrous leaves. In the visible region, the absence of efficient absorption in the epidermis might result in somewhat higher surface reflectance (Figure 6.1). Nonetheless, we believe that reflection by the glabrous leaf surface does not significantly intercept penetration of visible radiation into the leaf and, therefore, does not normally restrict availability of visible radiation for photosynthesis.

In flowers of a number of plant species, UV reflection can vary markedly within a single petal so that in radial flowers peripheral high UV reflectance contrasts with low UV reflectance near the centre, and this contrast might help to attract pollinating insects with UV vision. In *Brassica rapa* flowers, Ômura *et al.* (1999) determined that flower reflectance at 350 nm at the outer edge of the petal was greater than 20% but lesser than 10% in the central part. Extrapolating from the situation in green leaves, it is likely that in petals the presence of UV-absorbing compounds modulates UV reflectance originating in deeper layers (cf. Harborne, 1988); indeed, the highly UV-reflecting zones of *Hypericum calycinum* petals exhibited much smaller concentrations of UV-absorbing compounds when compared with zones of low UV reflectivity (Gronquist *et al.*, 2001). Further, petals of a *Petunia hybrida* mutant, in which accumulation of all flavonoids was blocked, appeared much brighter in UV photography compared with wild-type flavonoid-containing petals (Mol *et al.*, 1998).

Glaucous surfaces. In some leaves, specific waxes on the surface of the epidermis produce a glaucous (or blue-green) appearance and some of these glaucous leaves reflect radiation better than glabrous ones. For instance, glaucous needles of blue spruce (*P. pungens*) reflected 25% of UV radiation but non-glaucous needles from the same species reflected only 10% (Reicosky and Hanover, 1978) confirming an earlier report (Clark and Lister, 1975) that mechanical removal of the wax bloom from glaucous blue spruce needles reduced the high UV reflection values of glaucous needles to those of glabrous needles. In both studies, reflection by

the needle surface increased with decreasing wavelengths which is consistent with Rayleigh-type scattering (Day *et al.*, 1992).

High short-wavelength reflection with lesser reflection in the longer visible wavelengths permits specific screening of UV without reducing the availability of visible light for photosynthesis. A 25% reduction of UV radiation by reflection as demonstrated in *P. pungens*, however, would screen the UV rather inefficiently and appears unimportant compared to the highly efficient UV-screening capacity of many conifer needles (Day *et al.*, 1992; Fischbach *et al.*, 1999). Similarly, data from other species suggests that high UV reflection in glaucous leaves is relatively insignificant in UV screening. Glaucous leaves of *Echeveria* sp. reflected 20% of UV radiation (Robberecht *et al.*, 1980), and UV reflectance of 25 and 35% was determined for glaucous leaves of two *Eucalyptus* species (Holmes and Keiller, 2002). In leaves of *Triticum turgidum*, reflectance in the visible range increased with the amount of epicuticular wax but always remained below 30% at 400 nm which was the shortest wavelength used in this study (Johnson *et al.*, 1983). *Sorghum bicolor* leaves, with a dense filamentous wax surface, also reflected 25% of UV-B radiation (Grant *et al.*, 2003).

On the other hand, powdery epicuticular waxes on leaves of the succulent rosette-plant *Dudleya brittonii* provided appreciable protection against UV but also against visible radiation with reported reflectances of 80% in the UV and 60–70% in the visible range (Mulroy, 1979). Wax removal reduced reflectance to that of naturally occurring, non-glaucous leaves of *D. brittonii*. *Kalanchoe pumila*, like *D. brittonii*, is a Crassulaceae species and also has the powdery wax surface associated with relatively high reflectance values. Young leaves reflected 40% of blue radiation between 400 and 500 nm but only 5% after removal of epicuticular waxes (Eller and Willi, 1977). Reflection of visible radiation by developed leaves of *Cotyledon orbiculata*, also a Crassulaceae, was 60% and was reduced to 10% following wax removal (Barker *et al.*, 1997). Wax layers which reflect visible radiation efficiently, however, do not always exhibit high UV reflectance. In *K. pumila*, reflectance dropped from 33% at 500 nm to below 10% in the UV-B (Holmes and Keiller, 2002). The physical basis for the different reflectance characteristics is unresolved. In any case, high reflection of UV or of visible radiation by waxes forms a long-lasting shield against photo-stress which might confer advantages to plants growing under continuous excessive light intensities.

On the lower side of leaves of some species, epicuticular waxes have remarkably high reflectance of visible radiation (cf. Feldhake, 2002). It has been proposed that the lower surface of such leaves minimises loss of non-absorbed radiation by reflecting it back to the mesophyll (Smith *et al.*, 1997); fluorescence and fibre optics measurements, however, indicate that intensities of non-absorbed radiation at the lower mesophyll border are relatively small (Cui *et al.*, 1991; Bornman *et al.*, 1991; Vogelmann and Evans, 2002) so that back reflection would rescue little light. Indeed, when the lower epidermis was removed from leaves of four herbaceous species, only minor effects on leaf absorbance spectra in the visible range were observed (Lin and Ehleringer, 1983).

It is noteworthy that high reflectance of radiation does not always accompany special wax layers but can also arise from discontinuity of refractive index at sub-epidermal air spaces which are present along the entire leaf lamina of the *argenteum* mutant of *P. sativum* (Hoch *et al.*, 1980), and also as patches in leaves from blessed milk-thistle (*Silybum marianum*) and zucchini (*Cucurbita pepo* ssp. *pepo* convar. *giromontina*).

In many fruits, UV photography revealed significant reflection of UV radiation which is considered to be a foraging signal for animals perceiving UV radiation and thereby assisting in seed dispersal (Burkhard, 1982; Willson and Whelan, 1989). In the latter work, a survey of fruits from 53 species showed that high UV reflectance values were frequently associated with glaucous surfaces. These high UV reflectance values, ranging between 10 and 30%, were reduced to 10% or less when the wax layer was removed. Similarly, recent studies with bilberries (*Vaccinium myrtillus*) by Honkavaara *et al.* (2002, 2004) revealed UV reflectance values of about 10%, when the berries were covered with a waxy bloom, decreasing to 5% after wax removal. High UV reflection by fruits is not always associated with a glaucous surface: Altshuler (2001) found that some tropical fruits, not covered by wax bloom, still exhibited UV reflectance values higher than 20%.

Trichomes. Trichomes are cellular protuberances on plant surfaces of relatively high height to width ratios and show various degrees of complexity (Wagner *et al.*, 2004). In some plant species, leaf trichomes reflect radiation efficiently and, thus, reduce radiation intensities inside the leaf. Reflection of visible radiation by trichomes was thoroughly studied in the drought-deciduous brittlebush (*Encelia farinosa*). With trichome layers isolated from thick pubescent leaves, Ehleringer and Björkman (1978) measured reflectance values of around 70% in the green and red wavelength regions. At shorter wavelengths, reflectance dropped to 50% but absorptance increased in parallel; hence, trichomes of *E. farinosa* are highly reflective but their absorption may contribute to light screening in the blue region. Removal of leaf hairs increased leaf absorptance in the visible region from about 40 to 80% and produced an absorptance spectrum similar to non-pubescent leaves of the closely related *Encelica californica*. Trichomes may also be involved in UV screening as the densely pubescent leaves of *Argyroxiphium sandwicense* reflected about 40% of UV radiation (Robberecht *et al.*, 1980).

Efficient reflection of radiation, however, is not a universal feature of trichomes. Isolated trichome layers from two *Olea* species showed reflectance values below 5% in the UV and below 20% in the visible (Karabourniotis *et al.*, 1992). The pubescent *Espeltia schulzii* leaves reflected less than 5% of UV-B radiation (Robberecht *et al.*, 1980). Leaves of 23 trichome-covered plant species always reflected less than 15% of radiation at 330 nm and less than 25% at 680 nm (Holmes and Keiller, 2002). Reflectance at 680 nm did not exceed 20% in pubescent upper leaf surfaces of some Bromeliaceae and removal of trichomes caused only minor reductions in reflectance (Pierce *et al.*, 2001).

In essence, reflection by trichome layers of incident radiation is quite variable. The factors determining this variability include the density and thickness of the trichome layer (Robberecht *et al.*, 1980), trichome shape (Baldini *et al.*, 1997) or the presence of air-filled, highly light-scattering trichomes (Ehleringer and Björkman, 1978).

Water and salt. Radiation can also be reflected by water droplets, from rain or hydathodes, residing on the top of plant surfaces. Reflection properties of water droplets on leaves have received little attention compared with their function as condensing lenses. Brewer *et al.* (1991) calculated that, in focal spots of water lenses, incident radiation is intensified 20-fold or more and, because some plant species retard water droplets on their leaf surfaces (Brewer *et al.*, 1991; Brewer and Smith, 1997), spots of deleteriously high radiation intensities can develop inside leaves when exposed to the sun. Both focusing and reflection of radiation are affected by the angle of incident radiation and by the ratio of collimated to diffuse radiation. More detailed information on the radiation microenvironment of wet surfaces is required to better comprehend the optical effects of surface water.

Salt deposits on leaves of salt-secreting mangrove species or of halophytic species of the genus *Atriplex* are responsible for another remarkable surface-optics phenomenon: salt deposits on such leaves increase their reflectance of radiation (Björkman and Demmig-Adams, 1995). In the desert shrub *Atriplex hymenelytra*, the relation between surface salt and reflectance was characterised in detail by Mooney *et al.* (1977). Leaves of *A. hymenelytra* are covered with salt-containing bladders which are hydrated when plants are well supplied with water; under drought stress, however, these bladders dry out and the salt crystallises. In the presence of salt crystals, reflectance at 550 nm approached values close to 60% but this value dropped below 30% in well-hydrated leaves. The changes in reflectance were related to dilution of surface salt which occurred without variations in the total amount of salt. Remarkably, the decrease of reflectance from highest to lowest values can occur within a couple of days after irrigation of drought-stressed plants.

The efficiency of surface reflection by salt crystals is approximately comparable to the extreme reflectance values of waxes and trichomes (described above); hence, salt deposits can shield the leaves from intense radiation. Compared with the more permanent and stable reflection of radiation by waxes and trichomes, reflective screening by salt deposits on leaves of *A. hymenelytra* varies rapidly in response to water availability, thus, permitting more flexible acclimation of light-harvesting to ambient conditions.

6.4.2 *Optical effects of surface architecture*

Lens effect. Many leaf epidermal cells have convexly shaped surfaces, which result in a pavement-like appearance of the plant surface. Because cell interiors exhibit higher refractive indices than air, these cells can act as condensing lenses (Vogelmann, 1993). The potential advantage of epidermal lens-type focusing in

tropical shade plants is the enhancement of light harvesting for photosynthesis (Bone *et al.*, 1985; Lee, 1986). This was achieved in *Selaginella uncinata* by directing radiation, incident on the epidermis at off-normal angles, towards chloroplasts situated within the epidermal cell or, in *Anthurium warocqueanum*, by focusing radiation towards the chloroplast-rich regions beneath the epidermis. The uneven surface in such light-focusing leaves probably improves light-harvesting by decreasing specular reflection, as suggested by Bone *et al.* (1985), who observed lower reflectance values in light-focusing shade leaves compared with non-focusing sun leaves.

Focusing of radiation, however, is not confined to shade plants (Vogelmann *et al.*, 1996). Using image analysis, these authors demonstrated that collimated light, transmitted by isolated epidermal strips from sun plants, was highly concentrated within focal spots below the epidermis. Clearly, chloroplasts located in focal spots may be photo-damaged in exposed leaves so that epidermal focusing could be detrimental under certain conditions. Similarly, as in the case of focusing effects of water droplets, it is not yet clear how plants cope with the potential photo-damage at spots of extreme radiation intensities; therefore, more information on surface optics is needed to better understand the role of epidermal focusing in leaves.

Many flower petals possess lens-like epidermal cells, which protrude above the adaxial surface as conical structures (Kay *et al.*, 1981). By comparing flowers of wild-type *Antirrhinum majus* with a mutant, which shows only slightly domed epidermal cells (Noda *et al.*, 1994), Gorton and Vogelmann (1996) elucidated the optical properties of the wild-type petal surface. They suggested that absorption of light is optimised in wild-type petals because of efficient focusing of radiation into anthocyanin-containing epidermal vacuoles; mutant cells, however, were less efficient in concentrating radiation and the focal plane was in the unpigmented mesophyll. In agreement with Bone *et al.* (1985), who suggested that the presence of convex epidermal cells reduces specular reflection, the wild-type petals exhibited lower reflectance values than the mutant; different flavonoid concentrations in the wild-type and mutant lines might also have contributed to the differences in reflectance properties.

Conical epidermal cells, besides intensifying floral colour by optimising absorption, also scatter light reflected back from the petal interior resulting in a velvety appearance of the petal surface (Kay *et al.*, 1981; Mol *et al.*, 1998). Both enhanced light absorption and scattering of reflected light could be important signals for attraction of pollinating insects (Glover and Martin, 1998).

The sieve effect. The optical sieve effect is a peculiarity of the epidermal layer which arises when epidermal vacuoles accumulate highly absorbing concentrations of pigments while the interstitial spaces, anticlinal cell walls and intervacuolar protoplasm are relatively transparent (Butler and Norris, 1962; Fukshansky, 1981; McClendon and Fukshansky, 1990; Evans *et al.*, 2004). A distinct property of the sieve effect is that these transparent spots generally increase transmittance values

of the entire epidermis but the significance of these spots becomes greater with increasing difference between vacuolar and intervacuolar absorption.

The sieve effect for UV radiation was visualised using epidermal peels placed on a film which fluoresces in the blue when excited with UV radiation (Day *et al.*, 1993). Microscopic examination of the film revealed high transmission of UV radiation between epidermal cells and also through stomatal pores. The optical heterogeneity of these epidermal layers was confirmed by measurements using a fibre-optic micro-probe which revealed large spatial intensity variations below the epidermis. The sieve effect was not detected in needles of *P. pungens* and this was attributed to the presence of UV-absorbing phenolics in the cell walls of conifers but not in the herbaceous species investigated. Similarly, a quantitative relationship between epidermal UV absorbance and UV absorbance of extracted phenolics was successfully established for both barley and grapevine leaves when the sieve effect was taken into account (Kolb and Pfündel, 2005). Therefore, transparent areas between completely non-transparent vacuoles might determine the lower limit of epidermal transmittance in the UV range of many plants.

Structural colour. In addition to absorption and wavelength-dependent scattering of radiation, the optical behaviour of nanometric structures can produce a coloured appearance of plant surfaces. Leaves of *S. uncinata* exhibit blue-iridescence when grown under extreme-shade conditions but are green in response to more direct sunlight. The different appearance corresponded to higher reflectance in the blue but lower reflectance in both the green and red spectral ranges of the shaded compared with the sun-exposed leaves (Héban and Lee, 1984). The blue colour was attributed to constructive interference caused by thin layers which are comparable in thickness to the wavelengths involved and also differ in their refractive index from the surrounding media.

Considering the wavelength of maximum reflection, and that the refractive index of the layer is always higher than that of its surroundings, Héban and Lee (1984) predicted a thickness of about 70 nm for the optical layer in *S. uncinata* and confirmed this calculation by electron microscopy which detected two opaque layers of the appropriate thickness in the outer cell wall of the upper epidermis. Destructive interference at longer wavelengths explains why blue leaves show decreased reflectance within the green and red spectral range.

We argued (see Section 6.4.1) that peripheral reflectance does not significantly obstruct penetration of light into leaves. However, absorption of dense canopies results in extremely low light intensities and a relative enrichment of far red photons. In extreme-shade plants growing under such conditions, photosynthesis depends literally on each single photon and, hence, decreased reflection of red wavelengths by thin-film interference might play a significant role in optimising light-harvesting (Lee, 1986, 1997). Interference phenomena, comparable to *S. uncinata*, have been observed in other species of *Selaginella* and also in two remotely related flowering plant species, *Begonia pavonina* and *Phyllagathis rotundifolia*, and in the fern *Trichomanes elegans*. In *T. elegans*, however, interference is caused by

special chloroplasts (called iridoplasts) located in epidermal cells, which contain periodically arranged thylakoid stacks spanning the entire chloroplast (Lee, 1986, 1997).

Structural colouration due to interference phenomena was further reported for fruits of species of the genus *Elaeocarpus* (Lee, 1991). In these fruits, interference originates from a three-dimensional lattice formed by extracellular polysaccharide strands of about 80 nm thickness but are still located inside the upper epidermal cell wall. More recently, Lee *et al.* (2000) discovered structural colouration in fruits of *Delarbreia michieana* (Araliaceae). Generally, fruit colouration is thought to attract animal ingestion and consequent seed dispersal. A specific advantage of structural colouration may be greater stability and longevity resulting in longer-lasting appeal to animals compared with normal pigment colouration, which fades faster with fruit decomposition.

6.5 Concluding remarks

We have summarised the many factors contributing to the optical behaviour of plant surfaces. Most of the individual aspects of surface optics have been characterised for specific plant species or plant groups in which they appear of particular importance. A general pattern of the relative significance of the various optical features, however, is not yet apparent. Moreover, published data sets are often limited to a certain aspect of surface optics; thus, not only the relative significance of electronic absorption of radiation and of non-absorptive optical properties is often not defined but also the relative contributions of surface domains (e.g. epicuticular wax, cuticle or cell vacuole) to the total surface optical characteristics are not evaluated.

Obviously, despite the immense success of research during recent decades, our general understanding of plant surface optics is still fragmentary. To obtain a more holistic comprehension, future research on surface optics requires investigations at different levels of complexity by combining analytical methods to identify optically active molecules, spectrometric characterisation of surface optics and microscopic evaluation of structures and distribution of absorbing compounds. Little is known about the forces that have driven the evolution of so many different kinds of optical features. Analyses of energy costs for syntheses of various optical elements in different species in comparison with the advantages conferred by specific optical features, together with studies clarifying the emergence of optical traits during evolution, will help to further advance our understanding of the role and function of the many forms of plant surface optics. Understanding the role of plant surface optics also requires consideration of other possible functions, e.g. their potential signalling role which can influence plant–animal interactions.

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7 Transport of lipophilic non-electrolytes across the cuticle

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7.1 Introduction

Plants come into contact with their environment at two interfaces: the roots and the aboveground parts. Primary parts not having undergone secondary growth are covered by the cuticle which forms the most extended interface between the interior of the plant and its atmospheric environment. Across this interface, the exchange of solutes, vapours and gases takes place.

This chapter is devoted to the transport of organic compounds across the cuticle. The scope will be restricted to lipophilic organics without charge. These can be non-dissociable compounds or the non-dissociated species of weak bases or acids. They may be either derived from external anthropogenic or biological sources (e.g. air pollutants, active ingredients of plant protection formulations) or from the interior of the plant (primary or secondary metabolites). Transport of polar compounds like highly water-soluble organics or ions is discussed in Chapter 8.

In the present chapter we will address the question of how lipophilic non-electrolytes (LNE) can enter the cuticle, especially which sources and points of entry exist. Partitioning will be described as the process which governs the uptake of LNE into the interior of the cuticle. The major part of this chapter will be given to various questions related to the transport of LNE across the cuticle. After introducing the laws of mass transfer (as appropriate to this system) methods for measuring cuticular transport parameters will be presented. The meaning of these parameters in physical terms and what they can tell us about the molecular structure of the transport-limiting barrier of the cuticle will be delineated. Tools for predicting cuticular permeability from easily available properties of the chemicals will also be discussed. In the final section of the chapter we will treat the chemical nature, mode of action and practical application of transport-enhancing chemicals which play a considerable role in the agrochemical industry.

7.2 How do LNE enter the cuticle?

The transport of LNE across the plant cuticle is a dissolution–diffusion process. This means that the movement of an organic molecule from an adjacent solution into the cuticle precedes the diffusion of the molecule within the cuticle itself (Riederer, 2004). In order to analyse the processes and limits of LNE transport

across the cuticle, we need a clear understanding of the sources and points of entry of the molecules and the relevant partitioning processes.

7.2.1 Sources

For entering the cuticle, LNE have to come close to its surface. This may be either the external surface bordering the atmospheric environment or the internal surface adjacent to the outer epidermal cell wall of the plant tissue (Chapter 2). There is an extremely large variability of the types of sources and the physical states of chemicals coming into contact with the outer cuticular surface while the inner surface of the cuticle solely borders on aqueous solutions present in the cell walls of the interior plant tissue.

With regard to the extreme diversity of external sources and the large spectrum of exogenous compounds, we will consider the consequences of this variability on the initial steps of LNE transport across the cuticle. One of the most straightforward cases of initial steps is the LNE uptake from aqueous solutions. These solutions may be derived from rain, fog, clouds, dew, exuded by plant hydathodes or released from arthropods or fungi on the plant surface. In plant protection, in most cases, complex formulations instead of pure aqueous solutions will be applied to the plant surface.

Solutions may contain LNE of anthropogenic and natural origin (Levsen *et al.*, 1991; Valsaraj *et al.*, 1993; Fingler *et al.*, 1994). Anthropogenic compounds originate from incineration processes in industry, traffic and heating as well as from volatilisation of adjuvants from plastics and coatings. Natural sources of LNE in deposited solutions are organisms, chemical processes in the atmosphere and wildfires.

Besides wet deposition of LNE, atmospheric particulate matter is also deposited onto the plant surface (Davidson and Wu, 1990; Rosell *et al.*, 1991; Moser *et al.*, 1992; Wesely and Hicks, 2000). The size of particulate matter ranges from microscopic aerosols to visible particles. The origins of the particles are either anthropogenic, atmospheric or biological. Part of the carbon fraction of particulate matter in the atmosphere above forested areas is derived from plant cuticular waxes eroded by the action of wind (Simoneit, 1986; Rogge *et al.*, 1993). The nature of the particles can be either completely organic or may contain an inorganic nucleus with adsorbed organic material. In addition, small parts of organisms coming into contact with plant surfaces like bacteria and yeasts (Chapter 11), the feet, antennae or eggs of arthropods (Chapter 13) or the hyphae and spores of fungi (Chapter 12) may be also considered as particles. They may also contribute LNE delivered to the cuticular surface.

There is a continuum between aqueous solutions on the one side and particulate deposits on the other as water may evaporate from solutions leading to solid deposits (see later). Alternatively, LNE associated with particulate matter may be (re-) dissolved when the plant surface is wetted. The degree to which droplets dry up obviously depends on relative humidity but also on the (additional) presence of

inorganic or organic compounds of low points of deliquescence. In the presence of such compounds, aqueous solutions are reduced only to more or less concentrated mixtures of LNE solutes, water and hygroscopic salts (Tang, 1980; Burkhardt *et al.*, 2001; Schönherr, 2001).

In addition to pure aqueous solutions we may find on plant surfaces a wide spectrum of colloidal systems like suspensions (oil or water based) or emulsions. They are all widely used in pesticidal formulations and exhibit very complex behaviour when water and organic solvents gradually evaporate and mesomorphic residues evolve on the cuticular surface (Tadros, 1989, 1990; Stock and Briggs, 2000). One of the most important traits of pesticidal formulations is that oils and surface-active compounds contained within them can lead to enhanced spreading of the solution on the leaf surface.

Dry deposition onto cuticular surfaces may also occur in the form of gases or as vapours from volatile or semi-volatile organic compounds. As in the case of solids, the sources of these air-borne compounds may be either atmospheric, anthropogenic or biological. The volatiles of biological origin either derive from plants (Arey *et al.*, 1991; Winer *et al.*, 1992) or from arthropods as pheromones or allelochemicals (Cardé and Bell, 1995). Re-volatiliation (Ince and Inel, 1991; Bedos *et al.*, 2002) from solid or liquid deposits on plant surfaces is another source of vapours in the direct vicinity of the cuticle. In this case, vapours even of compounds having very low vapour pressures may be present in the unstirred air layer covering the leaf surface (Nobel, 1991; Schuepp, 1993) at concentrations much higher than expected from the solid nature of the chemical (Figure 7.1). Obviously, the concentrations and amounts of vaporous compounds in the vicinity of the cuticle depends on the physico-chemical properties of the chemical and wind conditions (Riederer, 1995; Riederer *et al.*, 2002).

While the so far listed sources of exogenous chemicals reaching the cuticular surface are diverse and sometimes dependent on environmental conditions and time, the situation at the inner surface of the cuticle is comparably simple. The compounds occurring *de novo* in the apoplastic solution are exclusively of biological origin. They are derivatives of the primary or secondary metabolism of the plant.

7.2.2 *Points of entry*

In principle, uptake into the cuticle will proceed for a LNE reaching the plant surface in every physical state. However, the points of entry into the plant are accessible to the deposits to variable degrees (Figure 7.2). The simplest situation is the entry of gases and vapours which have easy access to the total leaf surface irrespective of its fine structure and topography.

However, the uptake of compounds contained in deposits in the liquid or solid state strongly depends on a variety of factors whereby the roughness of the cuticular surface is the most important. The deposited solutions may appear in a variety of geometric forms from droplets sitting only on the tips of epicuticular wax crystals, sessile droplets being in direct contact with smooth cuticular surfaces to thin films

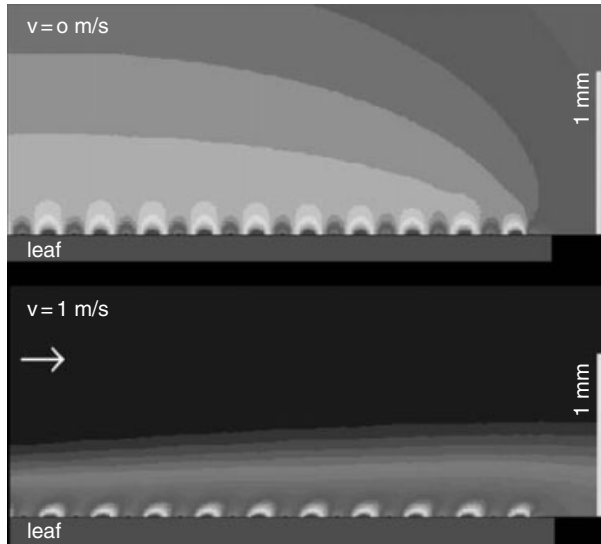


Figure 7.1 Isolines of the relative concentration of a semi-volatile compound in the unstirred layer in the vicinity of the leaf surface. Relative concentrations are colour-coded from red (highest) to dark blue (lowest). In the finite-element model leading to this picture a regular distribution on the leaf surface of solid deposits of the organic compound (red spots) and stomata was assumed. The concentration distributions at two wind speeds (0 and 1 m s^{-1}) are shown. Figure from Riederer *et al.* (2002) by kind permission by Oxford University Press. This figure is produced in colour in the colour plate section, which follows page 249.

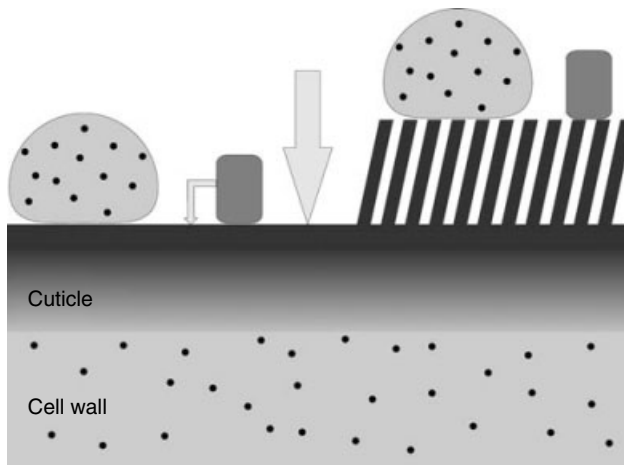


Figure 7.2 Schematic representation of the points of entry of lipophilic neutral compounds into the plant cuticle. Entry from sessile droplets, solid deposits, the vapour phase and the cell-wall solution is shown. The figure is not to scale.

covering large contiguous areas of the plant surface. Only when the solution gets into immediate contact with the film of epicuticular wax lining the outer surface of the cuticle will direct uptake occur.

Essentially the same is applicable to particles as long as they are larger than the structures contributing to the roughness of the plant surface. Again, a particle has to get in touch with the outer lining of the cuticle. Only then contact transfer of molecules from the solid or adsorbed state to the cuticular surface will be possible. With semi-volatile compounds, this barrier may be overcome by vapour emitted to the unstirred layer.

7.2.3 Partitioning

When a compound either in solution or in the solid or vapour state comes into direct contact with the outer surface of the cuticle it starts to move into it. The concentration in the cuticle phase increases but this increase is not indefinite: the thermodynamics governing this process limit the concentration in the cuticular phase to a degree relative to the concentration in the outer phase. In the simplest case, equilibrium is reached between both phases. This equilibrium can be described and analysed by the partition coefficient which is characteristic both to the chemical in question and to the nature of the phases involved. The partition coefficient is the ratio between the equilibrium concentrations C (in molar, i.e. volume-based units) in any two compartments y and z according to:

$$K_{yz} = \frac{C_y}{C_z} \quad (7.1)$$

For a deposit of an aqueous solution on the cuticular surface the partitioning occurs between the cuticular phase (C_C) and the water phase (C_W) leading to the cuticle/water partition coefficient

$$K_{CW} = \frac{C_C}{C_W} \quad (7.2)$$

When the chemical on the outside is in the vapour phase the cuticle/air partition coefficient applies

$$K_{CA} = \frac{C_C}{C_A} \quad (7.3)$$

with C_A being the equilibrium concentration in the vapour phase. The cuticle/air partition coefficient can also be derived from K_{CW} and the air/water partition coefficient K_{AW} . The latter is related to the Henry's law constant H according to

$$K_{CA} = \frac{H}{RT} \quad (7.4)$$

For further detailed treatments of partitioning processes and how to analyse them quantitatively see Riederer (1990, 1995).

The partitioning of weak organic acids or bases between an aqueous and the cuticle phase is complicated by the fact that these compounds dissociate depending on the pH of the aqueous phase. During equilibration between the cuticular

and the aqueous phase the non-dissociated species of weak electrolytes is highly preferred. When equilibrium concentrations are measured, only the analytical concentration in the aqueous phase can be obtained. This concentration covers both the non-dissociated and the dissociated species of the weak electrolyte. The resulting apparent cuticle/water partition coefficient K_{CW}^{pH} at a given pH is related to the K_{CW} of the non-dissociated species by

$$K_{CW}^{pH} = \frac{K_{CW}}{1 + 10^{pH - pK'_a}} \tag{7.5}$$

where pK'_a stands for the ionic-strength corrected acid dissociation constant of the weak electrolyte. When the partitioning of weak bases is studied the pK'_a can be obtained from the basic dissociation constant pK'_b according to $pK'_a = 14 - pK'_b$.

Under field conditions, temperatures of the leaf surfaces may vary from below 0 to approximately 45°C under temperate climatic conditions (Larcher, 2003). As partition coefficients are equilibrium constants from a thermodynamic point of view they also change with temperature. For LNE the enthalpy of the phase-transfer process is negative indicating that the transfer of a molecule from its aqueous solution into the lipid cuticle is an exothermic process (Riederer and Schönherr, 1986). As a consequence, the equilibrium will shift towards the aqueous phase when the temperature increases and thus the partition coefficient will exponentially decrease (Figure 7.3).

Cuticle/water partition coefficients of LNE can be experimentally determined. The results obtained become unreliable when K_{CW} is close to 1 and larger than 10^7 .

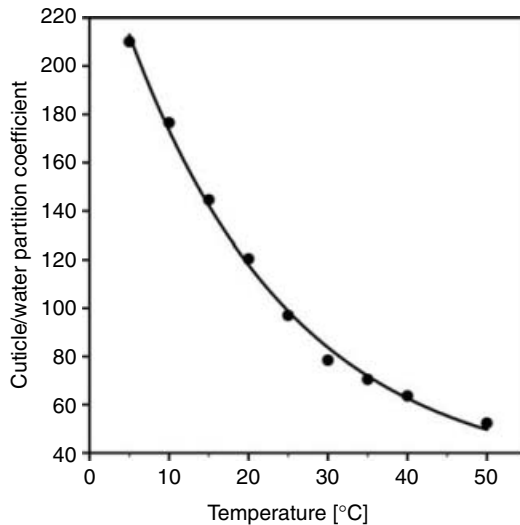


Figure 7.3 Temperature dependence of the cuticle/water partition coefficient of 4-nitrophenol in the dewaxed cuticle of tomato (*Lycopersicon esculentum*) fruits. The internal concentration of 4-nitrophenol at all data points is 0.001 mol kg⁻¹. Data from Riederer and Schönherr (1986).

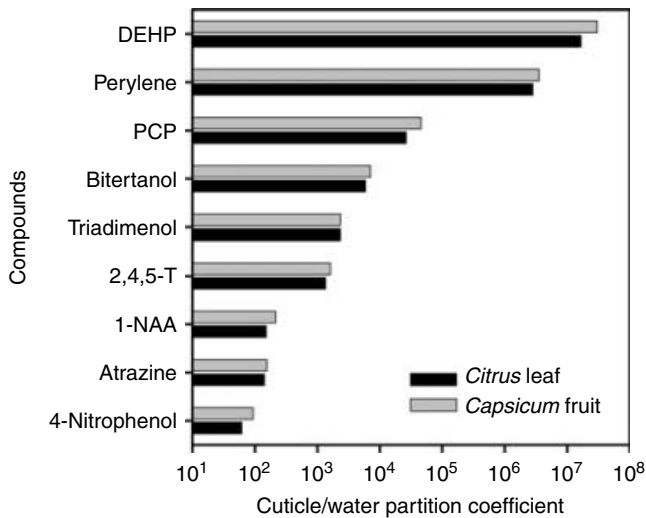


Figure 7.4 Variability of the cuticle/water partition coefficient of different lipophilic non-electrolytes in the cuticle of *Citrus aurantium* leaves and *Capsicum annuum* fruits (DEHP, diethylhexylphthalate; PCP, pentachlorophenol; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; 1-NAA, 1-naphtylacetic acid). Data from Riederer (1995).

For many LNE, values have been published with leaf cuticular membranes from *Citrus aurantium* and fruit cuticles from *Lycopersicon esculentum*, being the most widely used types of cuticles (Riederer and Schönherr, 1984; Shafer and Schönherr, 1985; Kerler and Schönherr, 1988a,b; Bauer and Schönherr, 1992; Baur *et al.*, 1997c). A compilation of values can be found in Riederer (1995). On account of their importance for predicting LNE permeability (see later) it can be assumed that K_{CW} of much more compounds have been measured so far which, for reasons of confidentiality, do not appear in the open literature. The data available show, however, that K_{CW} values vary drastically when they are determined for different LNE. This variability is due to the widely varying lipophilicity of LNE. A further source of variability is the plant species, the cuticle of which is used for the experiments. When comparing K_{CW} of 9 compounds of very different lipophilicities, the partition coefficients for the *C. aurantium* leaf cuticle are consistently smaller than that of *L. esculentum* fruits (Figure 7.4). In an extended study with 2,4-dichlorophenoxyacetic acid it has been shown that the variability of cuticle/water partition coefficients was larger within a single species than among 11 different species (Riederer and Schönherr, 1984).

The direct experimental determination of cuticle/water partition coefficients is impossible or impractical in many cases and for many reasons. The most important reason for this becomes evident when we consider the plethora of LNE in the environment and within the plant. For this reason, it is plainly impossible to obtain experimental values for each of them. Consequently, an indirect method has

been established by using fundamental physico-chemical properties of the chemical as predictors for K_{CW} . Quantitative property–property relationships (QPPR) were established between the cuticle/water partition coefficient and the corresponding 1-octanol/water partition coefficient K_{OW} (Schönherr and Riederer, 1989; Riederer, 1995):

$$\log K_{CW} = 0.057 + 0.970 \log K_{OW} \quad (r = 0.987) \quad (7.6)$$

Similarly, the aqueous solubility (S_W in mol l^{-1}) of LNE which is also related to lipophilicity can be used for predicting K_{CW} of a given compound (Schönherr and Riederer, 1989; Riederer, 1995):

$$\log K_{CW} = 1.118 - 0.569 \log S_W \quad (r = 0.978) \quad (7.7)$$

Values of K_{OW} and S_W may be obtained from the literature (Leo *et al.*, 1971; Hansch and Leo, 1979; Banerjee *et al.*, 1980; Valvani *et al.*, 1981) or databases. An alternative way for obtaining basic physico-chemical properties for input into QPPR is by using estimation programmes like the US Environmental Agency's 'Estimation Program Interface (EPI) Suite' which is available for free from <http://www.epa.gov/oppt/exposure/docs/episuite.htm>.

In addition to property–property relationships for estimating cuticle/water partition coefficients, topological descriptors of molecular structure can be used (Sabljic *et al.*, 1990; Riederer *et al.*, 1995). The equations derived are called quantitative structure–property relationships (QSPR) and are based on non-empirical descriptors of molecular structure. One type of descriptors successfully used is the connectivity index calculated from the topology of the molecule. For further information on the concept consult the book by Kier and Hall (1976). Another possibility for correlating physico-chemical to structural properties is employing ad-hoc properties of the molecule by counting defined structural fragments. This fragment approach has been successfully applied (Riederer *et al.*, 1995) to the prediction of the de-waxed cuticle (MX)/water partition coefficient of monodisperse alcohol ethoxylate surfactants. The prediction equation derived is

$$\log K_{MXW} = -1.78 + 0.52C - 0.17E \quad (r = 0.998) \quad (7.8)$$

with C being the number of carbon atoms in the alcohol moiety and E the number of ethoxy groups of the alcohol ethoxylate.

Plant cuticles are highly asymmetric having a large inner sorption compartment consisting mainly of cutin and a comparably small ($\leq 10\%$ of total volume) outer compartment where waxes predominate the properties (Schönherr and Riederer, 1988). This has profound implications for the actual partition coefficients valid at the points of entry into the cuticle which consist of cuticular waxes. Partition coefficients have been measured in the system reconstituted cuticular waxes/water (wax/water partition coefficient, K_{WAXW}). Schönherr and Baur (1994) and Burghardt *et al.* (1998) showed that they are directly proportional to the whole-cuticle/water partition coefficient but, on the average, are one order of magnitude lower according to

$$\log K_{WAXW} = -1.108 + 1.010 \log K_{CW} \quad (r = 0.994) \quad (7.9)$$

Thus, there is a concentration step by a factor of ten within the cuticle at the boundary between the cuticular wax and the cutin-dominated inner volume.

7.3 Transport through the cuticle

7.3.1 Laws of mass transfer

Diffusion is the process by which molecules (including LNE) mix when regions of higher concentration (or more rigorously: higher chemical potential) come into contact with regions of lower concentration (chemical potential). There are two ways for analysing this process. First, it can be described by the First Fick's law and a diffusion coefficient, a fundamental and scientific description necessitating that all properties of the system are known. Second, when this condition cannot be met, the process can be characterised by a mass transfer coefficient which is often used in technical applications and gives a simpler description of the whole process (Cussler, 1997).

When we assume that the transfer of mass occurs across an interface between two well-mixed solutions we expect that the amount transferred is proportional to the concentration difference and the interfacial area:

$$\text{amount transferred} = k (\text{interfacial area}) (\text{concentration difference}) \quad (7.10)$$

where the proportionality coefficient k summarises the properties of the interface and the physico-chemical properties of the molecule. This overall entity is called the mass transfer coefficient. When the transport across a membrane is considered – as it is the case with the cuticle – the mass transfer coefficient is called the permeance P [m s^{-1}] of the membrane. Thus, the flux N [$\text{mol m}^{-2} \text{s}^{-1}$] of LNE across the cuticle can be described by

$$N = P(C_i - C_o) \quad (7.11)$$

where C_i and C_o are the LNE concentrations at the inner and the outer sides of the cuticle. This equation is valid if both the inner and outer compartments are in the liquid state.

The permeance P is a coefficient combining several properties which, in this special case, cannot easily be measured separately:

$$P = \frac{DK}{l} \quad (7.12)$$

D [$\text{m}^2 \text{s}^{-1}$] is the diffusion coefficient in the limiting layer of the cuticle, K the partition coefficient between the limiting layer and the adjacent reservoirs and l [m] is the length of the diffusive path. It is justified to assume that the wax-encrusted outer portion of the cuticle acts as the layer limiting the transcuticular transport of LNE. It will be shown in the following paragraph how the permeance P and, under special experimental conditions, the mobility of molecules in the cuticle can be measured.

7.3.2 Measuring transport in cuticular fractions

Transport in the plant cuticle is much more complex than predicted by the First Fick's law. This fact becomes important when we try to analyse the mass transfer coefficient for transport across the cuticles quantitatively in terms of D , K and l according to Equation 7.12. Fick's laws assume homogeneity of the membrane in the direction of diffusive transport which evidently is not valid in the case of the cuticle. Electron (Chapter 2) and polarised light microscopic (Sitte and Rennie, 1963) evidence makes us suppose that plant cuticles are asymmetric with a gradient of fine-structure and the presence of waxes from the physiological outer to the inner side. The first experimental evidence for functional cuticular asymmetry was provided by Schönherr and Riederer (1988) using a method called simultaneous bilateral desorption. In this experimental setup a radiolabelled compound is simultaneously desorbed from both the outer and the inner surface of a cuticular membrane. Previous to the experiment the cuticle is loaded with the compound and, after drying, it is mounted between two aqueous compartments. This setup allows recording simultaneously the amounts desorbed from both sides. The results showed that within 6 h nearly the total quantity of the radiolabel sorbed in the cuticle was desorbed towards the inner surface while only 2–5% was lost over the outer surface. Further evidence for asymmetry of cuticles was derived from the study of the transcuticular diffusion of ions (Tyree *et al.*, 1990).

From these facts we can conclude that the plant cuticle is laminar consisting of an outer transport-limiting skin making up approximately 10% of total cuticular thickness and an inner layer which may act primarily as a sorption compartment for LNE (Bauer and Schönherr, 1992; Schönherr and Bauer, 1992). The solubility and mobility of permeating LNE in the limiting skin is much lower than in the sorption compartment implying that the former is in fact the transport-limiting barrier. The barrier properties of the skin can be attributed to cuticular waxes which are preferentially deposited in the outer fractions of the cuticle as shown by polarised light microscopy (Sitte and Rennie, 1963). This assumption as to the identity of the transport-limiting barrier is corroborated by experiments comparing the permeance of native cuticular membranes (CM) and dewaxed cuticles called polymer matrix membranes (MX). For 2,4-dichlorophenoxy acetic acid (2,4-D) it was found that the permeance of MX membranes was up to four orders of magnitude higher than that of CM (Riederer and Schönherr, 1985).

When it comes to determining cuticular permeability experimentally there are a number of methods available which almost exclusively were developed in the laboratory of J. Schönherr. The simplest and most intuitive method is the measurement of trans-cuticular transport across a cuticle mounted as a septum between two compartments (Figure 7.5a). One compartment contains a solution of the LNE in question (donor compartment) while samples are taken at regular intervals from the solution in the receiver compartment (Kerler *et al.*, 1984). In the steady state (i.e. when rates of diffusion are independent of time), the flux of the molecules across the membrane can be derived from the slope of a plot of the total amount diffused (M_t) versus time.

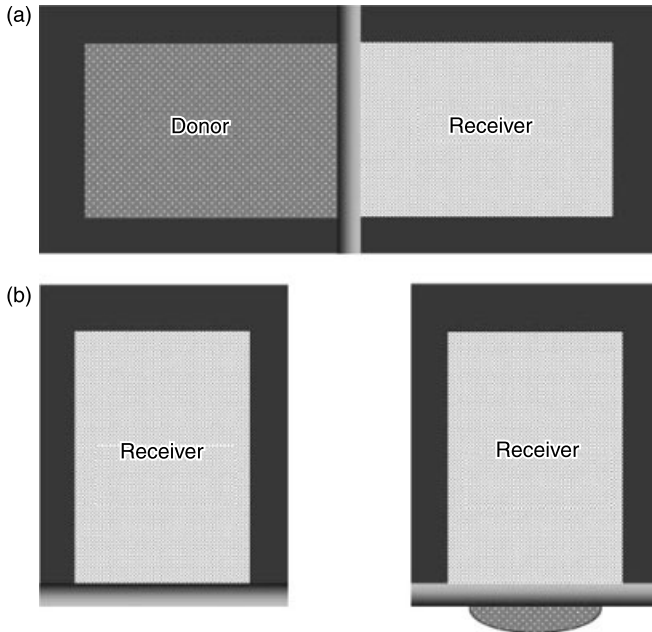


Figure 7.5 Schematic representation of the different setups for the determination of transport properties of the plant cuticle. (a) Setup for studying permeation across the cuticle separating two liquid-filled compartments; (b) unilateral desorption from the outer side (UDOS); (c) simulation of foliar uptake (SOFU).

When the concentration of the LNE in the receiver chamber is kept at or close to zero, the permeance P can be derived according to Equation 7.11 from the flux N and the LNE concentration in the donor compartment C_i . Procedures for obtaining mass transfer coefficients for trans-cuticular diffusion of LNE under non-steady state conditions are outlined by Hartley and Graham-Bryce (1980). The permeance P is an overall property specific for a given cuticular membrane and a given LNE having the disadvantage that it cannot be dissected into D , K and l according to Equation 7.12 (Riederer and Schönherr, 1985).

A method which allows to analyse cuticular transport properties in a more mechanistic way is a procedure called ‘unilateral desorption from the outer surface’ (UDOS) (Bauer and Schönherr, 1992; Schönherr and Bauer, 1992). This method is similar to simultaneous bilateral desorption as outlined earlier with the distinction that the desorption medium is added only to the chamber facing the physiologically outer surface of the cuticle (Figure 7.5b). Prior to the experiment the cuticle is again loaded with LNE which is preferentially sorbed in the large inner sorption compartment. Thus, diffusion occurs from the cuticular sorption compartment across the limiting skin (transport-limiting barrier) to the receiver chamber of the apparatus used. The major benefit of this setup is that the mobility of a LNE molecule in the transport-limiting barrier can be measured independent of its partition coefficient.

Desorption in UDOS experiments proceeds as a first-order process which theoretically would continue until equilibrium of LNE activity is attained between the sorption compartment of the cuticle and the receiver chamber. Using the terminology established by Bauer and Schönherr (1992) the first order rate constant for the desorption process is designated by k^* . This quantity is obtained from the slope of a plot of $-\ln(1 - M_t/M_0)$ versus time. When the thickness of the cuticle as a whole (Δx) is known and a good estimate for the thickness of the outer limiting skin (Δx_{skin}) is available, the thickness of the inner sorption compartment (Δx_{soco}) can be estimated. From k^* and the appropriate thicknesses the diffusion coefficient D of the LNE molecule in the transport-limiting skin can be deduced semi-quantitatively from

$$D = k^* \cdot \Delta x_{\text{skin}} \cdot \Delta x_{\text{soco}} \quad (7.13)$$

Thus, the development of the UDOS method made experimentally available good estimates of the diffusion coefficient. This was a major advance in cuticular transport studies. It should be noted, however, that the absolute values of D depend on the quality of the estimate for Δx_{skin} (Riederer and Schreiber, 1995). Under this premise, the mobility of LNE molecules in the limiting skin can be mechanistically analysed in terms of molecular size and in the presence of substances acting on wax properties (see later).

Another method for measuring LNE mobility has been developed by Schreiber and Schönherr (1993). In this case, the diffusion coefficients of LNE molecules in reconstituted cuticular waxes are obtained. For this purpose waxes solvent-extracted from cuticular membranes are deposited on suitable supports (aluminium disks) and loaded with a radiolabelled compound. Subsequently, the wax-covered material is bathed in a suitable desorption medium where the radiolabel starts to diffuse into the bathing medium. When the LNE concentration in the bathing medium is kept essentially zero, rates of desorption are solely the function of the solute concentration in the wax.

On plotting the amount desorbed until a given time t (M_t) versus the square root of time, a linear desorption graph is obtained which indicates that the kinetics obey Fick's second law and that it is independent of concentration. The appropriate solution of Fick's second law for the geometry of the experimental system is

$$\frac{M_t}{M_0} = \frac{4}{\Delta x} \cdot \sqrt{\frac{D}{\pi}} \cdot \sqrt{t} \quad (7.14)$$

with M_0 and Δx being the total amount of LNE loaded into the wax and the thickness of the wax layer, respectively. From the slope of the linear portions of plots of M_t/M_0 versus the square root of time the diffusion coefficient D can be calculated according to Equation 7.14. There is a limitation of this approach: cuticular waxes must be assumed to be reconstituted on the supports in a manner equivalent to their native physical structure. In addition, the actual thickness of the wax layer is difficult to assess as the wax often is not evenly distributed on the supports.

For some purposes, mimicking as closely as possible the processes occurring during the uptake of LNE from a droplet on the leaf surface is the relevant question. Then, another method is the approach of choice. This method has been called 'simulation of foliar uptake' (SOFU) (Schönherr and Baur, 1994,1996; Baur and Schönherr, 1997). Small droplets of a solution containing LNE are deposited on the outer surface of the cuticle (Figure 7.5c) and under controlled air humidity the diffusion from the droplet into a receiver chamber is monitored. In this case, the molecules cross both the limiting skin and the inner sorption compartment as it happens in the steady-state setup described earlier. On the contrary, in SOFU diffusion proceeds under non-steady state conditions. It is a valuable method for studying the effects of evaporation, concentration changes in the deposit and of relative humidity on cuticular penetration rates. Quantitative evaluation of the experiments is equivalent to that described for UDOS. Recently, SOFU has been modified by employing a piece of agar instead of the aqueous desorption chamber (Popp and Riederer, unpublished results). This approach has been modified from that of Santier and Chamel (1996). It allows for performing SOFU experiments also with very delicate cuticular membranes.

7.3.3 Analysis of transport properties

The transport properties outlined in the previous section can not only be used for quantitatively estimating the uptake and loss of LNE across the plant cuticle but they also provide insight into mechanisms of the transport process. Thus, they provide indirect information on the physical structure of the transport-limiting barrier. This information is accessible when cuticular transport parameters are measured as functions of solute size and temperature.

Bauer and Schönherr (1992) investigated for the first time the effect of solute size on mobilities (k^*) of LNE in plant cuticles. They found good correlation between $\log k^*$ and the molar volume (V_x) estimated according to Abraham and McGowan (1987), when using eleven cyclic test compounds. Subsequently, the diffusion coefficients of both cyclic and linear (aliphatic) LNE were determined in reconstituted cuticular waxes and correlated to their respective V_x (Schreiber and Schönherr, 1993). Good correlation between the diffusion coefficients and molecular size was obtained but relationships with different slopes were found for cyclic and linear LNE. Very low diffusion coefficients for linear LNE were obtained when aliphatics were included in the wax mixture used for covering the aluminium supports. This can be interpreted as being due to incorporation of the linear LNE into the crystalline domains of the wax.

Later, the physical meaning of the slope and the y-intercept of plots of $\ln k^*$ versus V_x (Figure 7.6) was recognised (Schönherr and Baur, 1994). The y-intercept $k^{*\circ}$ is the hypothetical mobility of a molecule of zero molecular size. It was suggested to be indicative of the tortuosity of the diffusive pathway across the cuticular transport-limiting barrier. The slope of the graph β gives the size-selectivity of the transport limiting barrier. Its reciprocal value is proportional to the free volume of

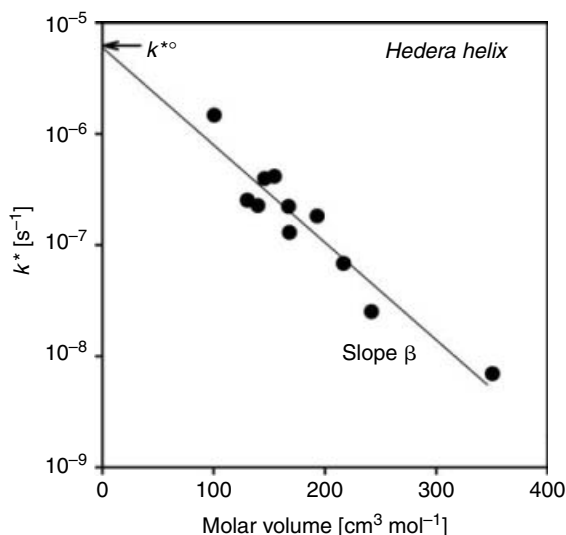


Figure 7.6 Size-dependence of the rate constants k^* from unilateral desorption from the outer side (UDOS) experiments with English ivy (*Hedera helix*) leaf cuticles. Data from Buchholz *et al.* (1998).

diffusion. It was shown in an extended study that k^{*o} for aliphatic LNE was lower by one order of magnitude than that for cyclic compounds while values of β were not significantly different (Baur *et al.*, 1996). The size-selectivity parameter β is independent of plant species (six species tested) while k^{*o} differed by three orders of magnitude (Buchholz *et al.*, 1998). From this, it can be concluded that the mobility of a hypothetical zero-volume LNE (and thus the tortuosity of their diffusive pathway) is the sole factor causing the differences of solute mobilities in the cuticle and permeabilities across the cuticles among plant species.

Another physical factor influencing permeability and mobility parameters is temperature. There are numerous reports in the applied literature that uptake of LNE into leaves increase with temperature. The first study demonstrating this temperature effect for the permeability of isolated leaf cuticles was done by Norris and Bukovac (1969). When we want to understand the temperature effects mechanistically, measuring cuticular permeability as a function of temperature would not provide all the necessary information. Permeance P is a composite quantity containing both the partition coefficient K and the diffusion coefficient D (Equation 7.12). It was shown earlier (Riederer and Schönherr, 1986) that sorption into the cuticle and thus K_{CW} decreases with increasing temperature. When the temperature dependence of the diffusion coefficient in reconstituted cuticular wax is studied (Krauß and Riederer, unpublished), D for six LNE compounds were found to increase steeply (Figure 7.7).

The majority of the studies on temperature effects on mobility have been performed in the UDOS system measuring k^* in a temperature-dependent way (Baur

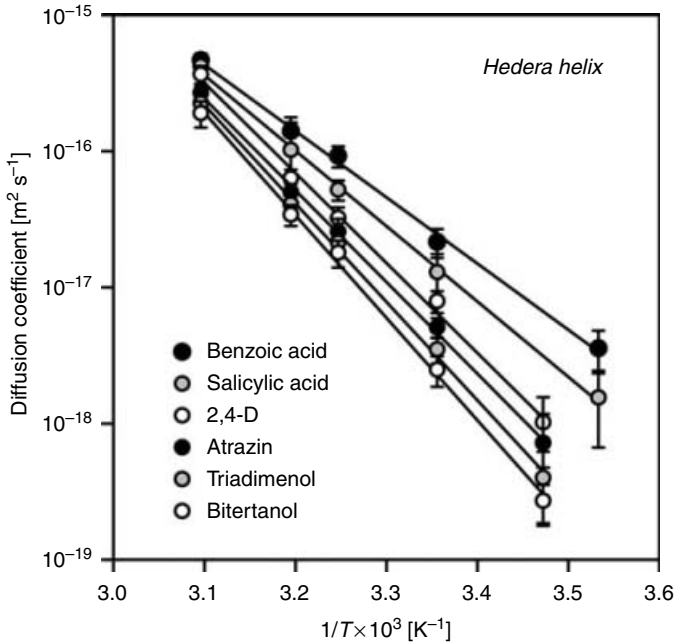


Figure 7.7 Temperature dependence of the diffusion coefficients of lipophilic non-electrolytes in the reconstituted leaf cuticular wax of English ivy (*Hedera helix*). 2,4, 2,4-dichlorophenoxyacetic acid (unpublished results from Krauß and Riederer).

and Schönherr, 1995; Schönherr and Baur, 1996; Baur *et al.*, 1997a; Schönherr *et al.*, 2000, 2001). Values of k^* (and thus mobilities) determined for the isolated cuticles of 12 plant species and 8 LNE compounds over a temperature range from 15 to 78°C varied up to 1000-fold (Baur *et al.*, 1997a). Arrhenius plots ($\ln k^*$ versus $1/T$) were linear in most cases and were interpreted according to

$$\ln k^* = \ln k_0^* - \frac{E_D}{RT} \quad (7.15)$$

where the pre-exponential factor k_0^* is proportional to entropy while E_D is the enthalpy (energy) of activation of diffusion (Vieth, 1991). Activation energies of diffusion in the range from 75 to 189 kJ mol^{-1} depending on plant species and solute size were deduced. A further analysis of the thermodynamics of the diffusion process revealed valuable insight into the mechanisms and the environment within which the LNE molecules diffuse across the cuticular transport barrier. Both the size selectivity and variability between individual cuticles decreased with increasing temperature. When the pre-exponential factor k_0^* of the Arrhenius equation was plotted against the activation energy of diffusion E_D , an excellent linear fit was obtained. This indicates that the free energy in the system is the same for all plant species and all LNE. This finding gives good evidence that diffusion of different

chemicals occurs in the same micro-environment (Baur *et al.*, 1997a). It further leads to the conclusion that LNE molecules of different size ($130\text{--}346\text{ cm}^3\text{ mol}^{-1}$) and lipophilicities (K_{CW} from 25 to 10^8) use similar diffusion paths in the limiting skin of all plant species tested and implies that the regions in the cuticular wax open for diffusion of LNE molecules have very similar physico-chemical properties.

7.3.4 Prediction of transport properties

Transport properties of plant cuticles can be measured experimentally using the methods and formalisms outlined earlier. This is useful as only then mechanisms of transport and the dependence on physico-chemical properties of LNE can be inferred. However, from a practical point of view, there are two major limitations: (1) only a small number of plant species have leaves with an astomatous surface and/or cuticular membranes that can be isolated enzymatically (Schönherr and Riederer, 1986), and (2) the diversity of LNE is far too large so as to allow measuring transport properties for all of them (Schönherr and Riederer, 1989). Therefore, efforts were undertaken to predict either the cuticular permeance or the mobility in the limiting skin of plant cuticles from basic physico-chemical properties.

A fairly simple method for predicting permeance P is based on the observation that values of P for 2,4-dichlorophenoxy acetic acid and the cuticles of eleven plant species ranged over two orders of magnitude (Riederer and Schönherr, 1985). In other words, the variation between the species with the lowest and the one with the highest P was a factor of approximately 100. As a first approximation, values of P were found to vary with the lipophilicity (K_{CW} or K_{OW}) of the LNE molecules leading to the predictive equations

$$\log P = 0.704 \log K_{OW} - 11.2 \quad (r = 0.91) \tag{7.16}$$

and

$$\log P = 0.734 \log K_{CW} - 11.3 \quad (r = 0.95) \tag{7.17}$$

These equations for the prediction of permeance were derived from data for *C. aurantium* leaf cuticles. The data for this plant species are at the lower limit of the range of P observed.

However, lipophilicity cannot account for the total variation observed in the P values of plant cuticles. This is predicted by Equation 7.12 from which can be deduced that the diffusion coefficient characterising the mobility of LNE molecules in the limiting skin is another determinant of permeance. As previously shown, mobility varies with molecular size and, therefore, the inclusion of a parameter characterising this property should improve the predictive power. Introducing the molar volumes (V_M) of the LNE molecules calculated according to LeBas (Reid *et al.*, 1977) the following prediction equation was obtained (Kerler and Schönherr, 1988b; Schönherr and Riederer, 1989)

$$\log P = 238 \frac{\log K_{CW}}{V_M} - 12.5 \tag{7.18}$$

with $r = 0.98$. Thus, by including a size-related parameter the predictive power further increases as compared to Equation 7.17. A more sophisticated predictive model based on experimental data from UDOS and using a formalism proposed by Potts and Guy (1992) is outlined elsewhere (Schönherr and Baur, 1994).

7.3.5 *Cuticular permeability in perspective*

Up to now, we have seen how cuticular permeability can be determined experimentally, what it means in terms of the physical processes involved and how it can be predicted from fundamental physico-chemical properties. For practical purposes, however, when the uptake of LNE into the interior of the leaf is to be assessed (e.g. in the application of plant protection agents or in ecotoxicology) cuticular permeability is only one quantity among several (Trapp, 1995).

It should be remembered that, according to Equation 7.11, the flux of LNE across the plant cuticle is the function of two independent parameters – the permeance and the concentration difference, that is, the driving force. In the majority of cases the concentration of any LNE in the aqueous cell wall solution adjacent to the physiologically inner side of the cuticle is close to zero because the cuticle/water partition coefficients K_{CW} are greater than 1. In addition, there will be no accumulation of material in the vicinity of the inner boundary of the cuticle because transport in the water-filled cell wall is by orders of magnitude faster than in the cuticle (Schönherr and Baur, 1994).

In contrast to this, the situation on the outer surface of the cuticle is much more complex. When the donor is a dilute solution of LNE in water, the driving force is only a function of LNE concentration and the partitioning between the aqueous solution and the waxes of the limiting skin. Note that the wax/water partition coefficient is approximately ten-fold lower than the cuticle/water partition coefficient (see earlier). Assessing the driving force for transport across the cuticle becomes more intricate when other than dilute aqueous solutions are considered. This is especially true when LNE serving as plant protection agents are applied as a complex formulation of water, surfactant, oils, solvents and humectants. These organic additives increase the overall solubility of LNE in the formulation on the leaf surface. After deposition of a droplet of formulation onto the cuticular surface, the concentrations of all components start to change because volatile formulation components begin to evaporate.

The driving force of a LNE across the cuticle will be at the maximum when evaporation proceeds until a saturated solution of the LNE in the formulation residue is attained (Schönherr and Baur, 1994). This situation has been investigated by Baur *et al.* (1997c) by using pure long-chain polyethylene glycol (PEG) as a model for neat formulation residues without any free water. They found that the cuticle/PEG partition coefficients for seven compounds with K_{CW} ranging from 10^{-1} to 10^7 were essentially identical and close to unity. This has an important implication because under such circumstances the partitioning term in Equation 7.11 is one and

therefore the flux of LNE across the cuticle depends exclusively on the mobility of the molecules in the limiting skin.

Another aspect becomes relevant when we consider the uptake of a LNE into the mesophyll of a leaf: the resistances contributed by both the cuticle and the underlying tissues. Theoretically, it is conceivable that the further diffusion of some chemicals across the thick mesophyll tissue underneath the cuticle proceeds more slowly than the transport across the cuticle and consequently may control the rate of the whole process. Riederer (1995) has analysed semi-quantitatively this situation. Under conservative assumptions the contribution of the mesophyll is between 2 and 0.0001% (for LNE with $K_{OW} = 10^7$ and $K_{OW} = 1$, respectively) of the total resistance of the uptake process. Thus, under all realistic circumstances penetration across the cuticle definitely is the rate-limiting step for LNE uptake into plant leaves. This clearly demonstrates how important it is to quantitatively measure and analyse cuticular permeability parameters in all cases where non-volatile LNE crossing the leaf/atmosphere interface of a plant are to be studied.

7.4 Enhancing the transport properties of the cuticle

Cuticular permeability for LNE is not a static property but may change under the influence of temperature and ontogenetic state. Cuticular properties also change in the presence of certain chemicals. While little is known about such chemicals produced by the plant itself or by other organisms a vast literature deals with chemicals which are technically used in pesticidal formulations. Thus, having discussed the properties of the cuticular transport-limiting barrier for LNE and the methods currently available to study them, in this section we will turn our attention to the possibilities that exist to modify these transport properties. In a first instance we will present a conceptual model for the transport barrier and how we can measure the influence that transport-modifying molecules can have on this barrier. The pharmaceutical and the polymer industry have a long tradition of trying to understand how barrier materials like the mammalian skin or polymers can be made more permeable to small organic molecules. We will review which lessons can be learned from their experience. Finally, we will discuss the molecules which in the agrochemical industry have to date been most successful in practical applications.

7.4.1 Modes of action of uptake enhancers

The currently accepted conceptual model of the cuticular transport barrier states that for LNE the transport-limiting barrier is formed by the cuticular wax. The waxy surface film and the embedded cuticular waxes form this barrier. There is a wealth of experimental data showing that by extraction of the waxes, the mobility of LNE in the cuticle and the permeability of the cuticular membrane are increased dramatically (Riederer *et al.*, 1989; Baur *et al.*, 1996; Knoche and Bukovac, 2000).

It has also been demonstrated that the epicuticular wax layer influences surface wetting solely and thus the contact area of the penetrating chemical with the leaves. The removal of the epicuticular layer has no influence on water permeability (Baur and Schönherr, 1998). Chapters 1 and 4 of this book deal in detail with this layer and its chemical composition, which is different from that of the underlying surface and embedded waxes.

After entering the surface waxes, the LNE encounter an environment which has been described by a brick-and-mortar model where crystalline fractions of the wax act as bricks and amorphous fractions are the mortar. The diffusion of LNE is postulated to take place only in the amorphous volume elements (Riederer and Schreiber, 1995). The crystalline regions are not accessible to the diffusing molecules and thus act as impermeable barriers which increase the tortuosity and thus the length of the diffusion path (Buchholz *et al.*, 1998; Baur *et al.*, 1999a). As discussed earlier, it has been found that the differences in permeabilities are not due to different size selectivities between species, but to differences in tortuosity and thus path-length of diffusion (Buchholz *et al.*, 1998). This was also suggested as an explanation for the observation that the thickness of the plant cuticle does not correlate with barrier properties (Baur *et al.*, 1999a).

This structural model is in agreement with the experimental evidence found for a number of molecules in various plant species (see earlier). It also agrees with the evidence for the structure of waxes (Basson and Reynhardt, 1988; Dorset, 1995; Gerson *et al.*, 1999). The studies done on plant cuticular waxes are limited though (Reynhardt and Riederer, 1991, 1994). While it seems clear that crystalline components are present, it has not been possible to date to characterise the amorphous fraction, nor has it been possible to define the relative ratio of the two fractions. Studies done with polymers loaded with ceramic flakes have given indications that solid loading and aspect ratio of the solid particles affect tortuosity and consequently the apparent diffusion coefficient (Falla *et al.*, 1996; Cussler, 1997).

It is only relatively recently, that the term 'mode of action' has come into use to describe how certain molecules change the envisaged molecular structure of the cuticular wax and lead to an increase in the transport of LNE across the plant cuticle and further into the interior tissues and in particular into the vascular system of plants (Baur and Schönherr, 1998). The bulk of the mechanistic work on transport enhancement has concentrated on alcohol ethoxylate type surfactants and some work has been done on oils.

The term accelerator has been coined for a chemical which increases the permeation of itself and/or other molecules through the cuticle. This can be achieved either through an increase in the diffusion coefficient of the LNE or through a modification of its partitioning into the wax or both. The underlying premise is that the accelerator itself partitions into the wax and modifies its physical structure in order to facilitate the subsequent penetration of LNE. It is clear from this statement that accelerators must be LNE themselves.

Experimental evidence for this hypothesis is available from electron spin resonance (ESR) experiments (Schreiber *et al.*, 1996) and NMR measurements

(Reynhardt and Riederer, 1991, 1994; Schreiber *et al.*, 1997). These experiments have shown that the degree of disorder increases significantly in reconstituted cuticular wax if it contains monodisperse alcohol ethoxylate surfactants of the type also used as accelerators. This is interpreted as an increase in fluidity of the amorphous fraction of the cuticular wax which in consequence leads to an increased mobility of the diffusion of LNE and thereby to enhanced permeation.

Temperature-dependent studies on LNE mobility in isolated cuticles done in the presence and absence of accelerators further confirm this (Schönherr and Baur, 1996; Baur *et al.*, 1997a; Baur and Schönherr, 1998; Buchholz and Schönherr, 2000; Schönherr *et al.*, 2001). Activation energies for diffusion decrease in the presence of accelerators showing that the energy requirements for the displacement of the diffusing molecules are reduced.

7.4.2 *Transport enhancing compounds*

There is a multitude of chemicals with diverse chemical structures which, up to now, have been identified as accelerators of LNE mobility in plant cuticles. There is a growing number of reviews dealing with this subject (Riederer and Schönherr, 1990; Kirkwood, 1993; Gauvrit and Cabanne, 1993; Foy, 1996; Baur, 1998; Zabkiewicz, 2000). In recent years a trend became visible away from a conventional descriptive compilation of effects towards a search for evidence-based structure/activity relationships which are more widely applicable. Transport enhancing chemicals can be roughly grouped into non-ionic amphiphilic molecules (non-ionic surfactants) and oils or solvents. Non-ionic surfactants of the alcohol ethoxylate type are probably the most encountered type of accelerating molecules in plant protection formulations. In addition to the acceleration, they may also have a positive influence on spreading of the droplet on the leaf surface and other formulation properties not directly related to cuticular penetration. Significant work has been done to characterise their influence on transport properties (Schönherr, 1993; Stock and Holloway, 1993b; Riederer *et al.*, 1995; Baur *et al.*, 1997d, 1999b; Burghardt *et al.*, 1998).

The work done in the laboratories of Baker (Stevens and Baker, 1987; Stevens *et al.*, 1988; Baker *et al.*, 1992), and Holloway (Stock and Holloway, 1993a,b) have given us important rules about how alkyl ethoxylate type surfactants influence the foliar penetrations of LNE (active ingredients) into intact leaves of different plants. Now well-known rules have been derived stating that low degrees of ethoxylation preferably enhance the transport of lipophilic compounds, while a high degree of ethoxylation better works for hydrophilic compounds.

An often-overlooked aspect of the work by Holloway and co-workers is the interaction between the surfactants and the model LNE under study. In a series of double label studies, it was shown that while the accelerating surfactants enhance the mobility of the model compounds, their own rate of uptake is reduced (Stock *et al.*, 1992; Stock and Davies, 1994). It was suggested that this is mainly due to a saturation of the sorption compartment.

It may be an 'accident of history' (Briggs and Bromilow, 1994) that oil or solvent additives have been largely neglected in systematic studies in the past. This is even more surprising as it is relatively easy to obtain chemically pure oils while non-ionic surfactants in practice are never pure compounds but are technical mixtures having a more or less broad distribution of homologues. Oils can be divided into triglycerides of plant or animal origin, modified natural oils (e.g. alkylated fatty acids), terpene oils, aromatic oils, naphthenic oils and branched or linear paraffinic oils (Hamilton, 1993; Gauvrit, 1994b). Most work with chemically well-defined oil additives has been done with triglycerides and their esterified derivatives. Important information on activity and overall uptake into leaves has been obtained (Nalewaja *et al.*, 1986; Gauvrit, 1994a). The work done by Hall *et al.* (1998) is particularly interesting in this context despite not having been done on cuticles. Their work demonstrates that the volatility of certain oils like methyl oleate can lead to substantial losses of the accelerators within the time relevant for biological activity.

The bulk of the systematic work with well-characterised natural oils and their derivatives has been reported by the groups of Gauvrit, Chamel and Cabanne. Again, the majority of the studies relate to LNE penetration into intact leaves. Some studies by the group of Cabanne have been done on isolated cuticles though, giving the possibility to compare the results of both methods. Studies with radiolabelled oils showed how different oils behave when they are applied to a cuticle (Santier and Chamel, 1996). This study also highlighted how critical it is to do a detailed analysis of the fate of the chemical diffusing across the cuticle into the plant. While triglycerides hardly penetrated into the cuticle at all, more than 50% of the applied methyl oleate disappeared into the waxes and the cuticle. On the other hand, only a small percentage emerged on the inner side of the cuticle into the receiver compartment.

Methyl oleate led to a strong acceleration of active ingredient diffusion in the cuticle, while triglycerides hardly had an effect at all. In a different study, monoglycerides were studied as accelerators and were found to have a similar but slightly weaker effect than methyl oleate (Langlois *et al.*, 1997). This was true for the longer-chain unsaturated derivatives (oleyl, linoleyl, etc.). Shorter chain or unsaturated monoglycerides (C7 or C11 with no or one double bond) had no accelerating effect at all. The same was true for isopropyl amides of oleic acid. The lack of effect of these latter molecules was attributed to the fact that they were solids and so could not easily be taken up by the cuticular waxes.

The pronounced uptake of methyl oleate into cuticles is in agreement with observations made in the polymer industry where a swelling of PVC and polypropylene was observed when exposed to methyl oleate (Hayashi *et al.*, 1994; Ducruet *et al.*, 1996). For oleic acid it has been suggested that continuous domains of liquid accelerator establish in the transport-limiting barrier of human skin. The observed apparent diffusion coefficient then can be envisaged as a composite of diffusion through the barrier itself and through those liquid domains (Golden *et al.*, 1987).

Another plant-derived type of oil which has been described as an accelerator known as (mono)terpenes. Commercially available products are derivatives of pine oil. In particular terpineol has been investigated by Cabanne and co-workers

(Cabanne, 2000; Pullen, 2004). In whole-plant studies terpineol was seen to have a stronger effect than butylated fatty acids and to enhance the potency of the latter. Wax diffusion studies have confirmed the strong accelerating properties (A. Friedmann, unpublished data). A potential drawback of terpenes is their volatility (Hall *et al.*, 1998) which can become quite relevant, since the vapour pressure of terpineol is higher than that of methyl oleate.

As mentioned earlier, the distinction between mineral and naphthenic oils has been made in the literature (Hamilton, 1993; Gauvrit, 1994b). The two terms describe linear (branched and straight chain) and cyclic aliphatic oils derived from petroleum, respectively. These materials form one of the most commonly used adjuvant types for agricultural use. Nevertheless, there is little systematic work done in this area and to our knowledge nothing on isolated cuticles or reconstituted waxes. The most thorough systematic work was done by Hall *et al.* (1998). Other studies have looked at the effects of commercially available materials on intact leaves (Nalewaja *et al.*, 1986; Manthey *et al.*, 1989). Given the importance of these compounds due to their widespread use as adjuvants, but also as pollutants from jet and car fuel, it is important that more systematic work be done in the future.

Derivatives of phosphoric acid have also been described as accelerators. Despite having been first mentioned at the same time as some of the oil adjuvants, it is only very recently that they received appropriate attention. Phosphates, in particular tri-butyl-phosphate (TBU) have been described as accelerators (Schönherr, 1994; Buchholz and Schönherr, 2000; Schönherr *et al.*, 2001; Shi *et al.*, 2005). In their experiments these authors measured diffusion data and activation energies for TBU, and compared them to a number of other known accelerators. It was suggested, that the accelerating effects were due to a plasticisation of the barrier-forming waxes.

Phosphonates with various substitution patterns have been used in a number of technical applications. The use to increase the penetration and activity of some fungicides (Stock *et al.*, 1998; Kleiner *et al.*, 1998a,b) or other plant protection actives (Stock *et al.*, 2002; Piper *et al.*, 2003) was published. Phosphinates have also been mentioned (Kleiner *et al.*, 1998a,b; Piper *et al.*, 2003) but there is little experimental data available in the published literature. Unfortunately, published data for any cuticle or wax mobility studies with any of the phosphoric acid derivatives are lacking.

From the comparative work with radiolabelled accelerators it is clear that the accelerator has to penetrate into the wax to have an effect on the permeation. There has been some debate as to whether the accelerator has to match the diffusion properties of the molecules it is accelerating. In the case of alcohol ethoxylate surfactants this is likely to be the case since they can partition out of the cuticle into the living tissue of the plant where they are metabolised. In order to maintain a reasonable concentration in the cuticle, the surfactant should not move significantly faster or slower than the molecule it accelerates. For oils this is slightly different. They have to move into the cuticle faster than other LNE, but due to their high lipophilicity, they hardly partition into the plant tissue. In other words, once a concentration is

built up in the cuticle, it is sustained over a longer period than for more hydrophilic accelerators.

The work on intact leaves has been paralleled with mechanistic studies on isolated cuticles. The first work was reported in the late 1980s (Geyer and Schönherr, 1988; Shafer and Bukovac, 1989). Mechanistic studies were performed with the different experimental approaches described earlier including UDOS, SOFU and desorption from reconstituted waxes (Burghardt *et al.*, 1998). These studies have shown that a wide range of organic compounds act as accelerators for the mobility of LNE in plant cuticles. Transport-enhancing activity was demonstrated for mono- and polydisperse alcohol ethoxylates (Schönherr, 1993; Baur and Schönherr, 1997; Schönherr and Baur, 1997; Baur *et al.*, 1997b, 1999a; Schönherr *et al.*, 2000, 2001), primary alcohols (Schönherr, 1993), glycols (Schönherr, 1993), diethyl sebacates (Schönherr *et al.*, 2001) and tributyl phosphates (Schönherr *et al.*, 2001). Using radiolabelled octaethylene glycol dodecyl ether it was shown that alcohol ethoxylate-type surfactants are taken up into and diffuse across the cuticle. When the reservoir of accelerator is exhausted its concentration in the transport-limiting barrier decreases. Thereby its effect on LNE mobility in the barrier is also decreased and finally disappears completely (Schönherr and Baur, 1997).

The effects of polydisperse alcohol ethoxylate surfactants on the penetration of six organic compounds varying in size and lipophilicity were also investigated (Baur *et al.*, 1997b). The accelerator effect depended on the chain-length of the alcohol ethoxylates and diminished with increasing length of the polyoxyethylene chain. Surfactant effects on solute mobility increase with the size of the solutes and lead to almost identical mobilities for all compounds irrespective of molecular size. Equivalent effects were found both for mono- and polydisperse alcohol ethoxylates. When the dose–effect relationships (log mobility versus accelerator concentration) were studied, linear graphs were obtained for monodisperse alcohol ethoxylates but curves convex to the x -axis were found for diethyl sebacate and tributyl phosphate (Schönherr *et al.*, 2001).

An interesting insight into the structural changes underlying the accelerating effect of certain molecules is provided by the work of Baur *et al.* (1999b). They studied the temperature dependence of LNE mobilities (in an UDOS setup) in the presence and absence of alcohol ethoxylates and tributyl phosphate. These accelerators considerably decreased the activation energy of diffusion of the LNE tested. The decrease in activation energies corresponded to 50- to 275-fold increases of the solute mobilities at 15°C. These data suggest that the decrease in activation energies with the concomitant accelerating effects on mobility contributes considerably to the effects of accelerating compounds.

7.5 Conclusions

Plant cuticles are permeable to lipophilic non-dissociated organic chemicals either of anthropogenic or biogenic origin. These compounds may either enter the cuticle

from the atmospheric environment (e.g. pollutants, plant protection agents) or from the internal tissue of the plant (primary and secondary metabolites). Permeability depends on the lipophilicity of the compound (described by the cuticle/water or 1-octanol/water partition coefficient) and its size (molar volume). A variety of experimental setups has been established in order to determine whole-cuticle permeability or the mobility of LNE in the transport-limiting barrier as well as in reconstituted cuticular waxes. Certain compounds primarily used in plant protection formulations increase the mobility of LNE in the transport-limiting barrier and thus the rates of transport across the cuticle. These compounds which are of diverse chemical structure are thought to plasticise the cuticular waxes.

Even though a consistent picture of cuticular LNE transport has evolved during the last two decades some questions remain open. So, in the future the molecular structure, localisation and actual thickness of the transport limiting waxes need to be further evaluated. There is much uncertainty on how the interaction between the cutin matrix and waxes influences the transport properties of plant cuticles. Further, the relationship between the pathways for LNE and polar ionised and non-ionic compounds across the cuticle must be studied in order to obtain a full picture of cuticular permeability. Finally, the effect of mobility enhancing accelerator molecules on the transport-limiting barrier and the appropriate structure/activity relationships need to be further elucidated in order to make possible the rational use of these compounds in pesticide formulations.

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8 Characterisation of polar paths of transport in plant cuticles

Lukas Schreiber

8.1 Introduction

As presented in the introduction and discussed in Chapter 9 of this book, one of the main functions of the plant cuticle is the protection of land-living plants against uncontrolled water loss (Schönherr, 1982; Riederer and Schreiber, 2001). Since water is a polar molecule, an efficient transport barrier for water should have a hydrophobic nature. Plant cell walls composed of carbohydrates are polar, attract water and thus cannot form a barrier for water diffusion. In technical applications cellulose membranes are covered with a thin layer of paraffin wax which renders them lipophilic and water impermeable (Fox, 1958). A similar solution has already been involved in plants about 500 Mio years ago. As described in detail in Chapters 2, 3 and 4 of this book, outer epidermal cell walls composed of carbohydrates are covered with a thin lipophilic polymer membrane – the plant cuticle – which is composed of the polymer cutin (Kolattukudy, 2001) and solid, partially crystalline cuticular waxes deposited onto and within the cutin polymer (Walton, 1990). Cuticular waxes form the main transport barrier for water since cuticular permeability increases by factors from 10 to 100 upon wax extraction (see also Chapter 9).

8.1.1 Penetration of non-ionic, lipophilic molecules across cuticles

Within the last 30 years permeability of water and non-ionic, lipophilic compounds across isolated plant cuticles has been studied intensively (for details see Chapters 7 and 9). These studies resulted in a simple solubility/mobility model for the cuticular transport barrier (Schönherr and Riederer, 1989; Riederer and Schreiber, 1995). As a measure for solubility partition coefficients (e.g. cuticle/water partition coefficients) are used (Riederer and Schönherr, 1984). Mobility is characterised either by diffusion coefficients (Schreiber and Schönherr, 1993) or by rate constants of desorption (Bauer and Schönherr, 1992). Higher values of partition coefficients indicate a higher solubility and higher values of diffusion coefficients or rate constants stand for a higher mobility of a respective compound in the lipophilic cuticular transport barrier. Thus, with increasing solubility and/or mobility overall permeability increases. Water permeability was found to be correlated with the permeability of lipophilic molecules (Niederl *et al.*, 1998; Schreiber, 2002). It was therefore concluded that the cuticular transport of non-ionic, lipophilic molecules as well as

that of polar water molecules can be described on the basis of two parameters – solubility and mobility.

8.1.2 *Penetration of polar and charged molecules across cuticles*

According to the solubility/mobility model of cuticular penetration, diffusion of charged substances like organic ionic compounds (e.g. glyphosate) and inorganic ions (e.g. K^+ or Ca^{2+}) across the lipophilic transport barrier of cuticles should not be possible. Ions are characterised by a hydration shell (Stein, 1967) which does not allow them to dissolve in a lipophilic phase. Nevertheless, permeation of polar and ionic compounds across cutinised leaf surfaces has already been reported since the first half of the last century. Qualitative approaches either investigating foliar uptake of ionic fluorescent dyes by microscopy (Strugger, 1939) or uptake of radiolabelled sucrose by autoradiography (Franke, 1964) indicated that these polar compounds are taken up by leaf surfaces. These results were confirmed by further studies analysing the foliar uptake of inorganic ions or dyes (Lord *et al.*, 1979; Eichert and Burkhardt, 2001; Schlegel *et al.*, 2005). All of these qualitative studies had in common the fact that highest rates of uptake were observed in the vicinity of stomata, in and at the bases of trichomes and across the cuticle over anticlinal epidermal cell walls. This indicates a pronounced lateral heterogeneity of the cuticular transport barrier covering the leaf surface.

8.1.3 *Effect of air humidity on cuticular barrier properties*

Furthermore, it was shown that the cuticular transport barrier is sensitive to humidity (Schönherr and Mérida, 1981; Schreiber *et al.*, 2001). In isolated cuticles, an increase of humidity from 2 to 100% resulted in an increase of the cuticular water flow by a factor between 2 and 3 (Figure 8.1). This observation leads to the conclusion that water can be absorbed by polar moieties, which are present in the lipophilic cuticle (Wattendorf and Holloway, 1984; Chamel, 1991). As a result barrier properties of the cuticle decrease and in turn permeability for water increases with increasing cuticular water content. Based on these partially contradicting results describing cuticular penetration of non-ionic, lipophilic molecules as well as polar, charged molecules, it was postulated that the plant cuticle should be composed of two parallel paths of diffusion (Schreiber *et al.*, 2001; Schönherr and Schreiber, 2004; Schlegel *et al.*, 2005): (1) the lipophilic transport path composed of lipophilic cutin and wax domains and (2) a polar transport path presumably composed of polar aqueous pores (Figure 8.2).

8.2 Results providing evidence for polar paths of transport

With few exceptions ion permeability of cuticles has rarely been investigated in the past. In one interesting and very detailed approach KCl diffusion across isolated

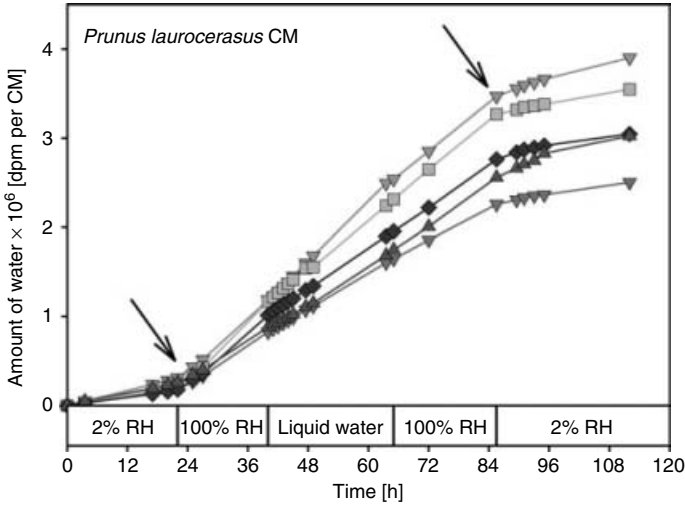


Figure 8.1 Cuticular fluxes of $^3\text{H}_2\text{O}$ across isolated *Prunus laurocerasus* L. cuticles at different external receiver conditions, varying between 2 and 100% air humidity and pure liquid water. Transpiration kinetics of five individual isolated cuticular membranes are shown. Black arrows indicate changes in cuticular barrier properties. Data from Schreiber *et al.* (2001).

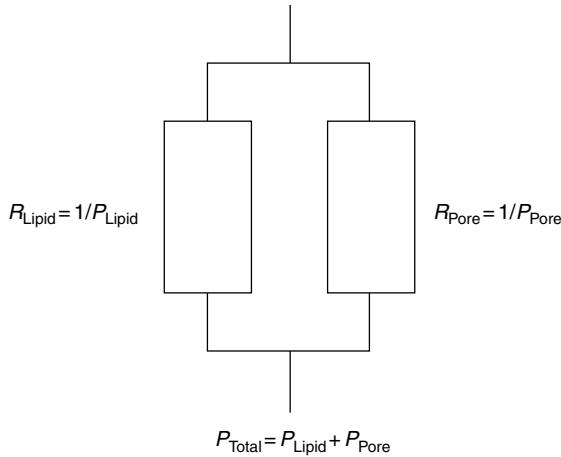


Figure 8.2 Model for the two paths of cuticular transport. The resistances $R_{\text{Lipid}} = 1/P_{\text{Lipid}}$ and $R_{\text{Pore}} = 1/P_{\text{Pore}}$ are arranged in parallel. P_{Total} is the overall permeability of the cuticle calculated from the sum of P_{Lipid} and P_{Pore} . Data from Schreiber *et al.* (2001).

cuticles of *Citrus aurantium* was investigated (Tyree *et al.*, 1990, 1991, 1992). From this data the authors have concluded that ion diffusion across cuticles must occur along polar pores traversing the cutin polymer and the waxy transport barrier. In a series of recent papers investigating inorganic ions (Schönherr, 2000, 2001; Schönherr and Luber, 2001; Schönherr and Schreiber, 2004; Schlegel *et al.*,

2005) as well as charged organic molecules (Schönherr, 2002) it was shown convincingly that polar and charged compounds in fact can penetrate isolated astomatous cuticles as well as intact stomatous leaf surfaces. These studies included different inorganic salts (potassium and calcium salts) and glyphosate, different experimental factors (humidity, temperature, presence or absence of cuticular waxes, plasticisers, molecular size of the solute and light) and isolated cuticles or intact leaves from seven plant species (*C. aurantium*, *Malus domestica*, *Populus canescens*, *Pyrus communis*, *Schefflera actinophylla*, *Stephanotis floribunda* and *Vicia faba*). The results obtained in these experiments strongly support the conclusion that polar paths of transport across cuticles are present.

8.2.1 Factors influencing permeability of inorganic ions and charged molecules across plant cuticles

Rates of penetration of calcium salts (Schönherr, 2000, 2001) and glyphosate (Schönherr, 2002) increase by a factor between 2 and 3 in a similar way as water permeability (Schreiber *et al.*, 2001) when relative humidity is increased up to 100%. Sorption of water to polar domains in the plant cuticles obviously leads to a swelling of the cutin polymer, which in turn increases the penetration of ionic compounds. In contrast, temperature had no (calcium chloride), or only a very small, effect on cuticular penetration, and activation energies calculated from Arrhenius plots were close to zero. This indicates that diffusion of these polar and charged compounds takes place in an aqueous environment and not in a solid, partially crystalline lipophilic phase composed of wax and cutin. Furthermore, extraction of cuticular wax from isolated cuticles, normally thought to form the main transport barrier of the cuticle, rarely had any effect on the rates of calcium chloride penetration (Schönherr, 2000). In accordance with this, plasticisers effectively increasing cuticular penetration of lipophilic molecules (Buchholz and Schönherr, 2000) rarely affected cuticular penetration of calcium chloride or glyphosate.

A dependence of the rates of cuticular penetration on the size of different calcium salts was observed when investigating isolated astomatous *P. canescens* cuticles (Schönherr and Schreiber, 2004) and intact stomatous *V. faba* leaf surfaces (Schlegel *et al.*, 2005). Since calcium salts are hydrated in aqueous solution as a result of which their exact molar volume cannot be estimated easily, molecular weights of the water-free salts were used as a measure of molecular size. It has been shown that the rates of cuticular penetration across *P. canescens* cuticles decrease by a factor of about 10 when the molecular weights of the calcium salts increase from 100 to 500 g mol⁻¹ (Figure 8.3). A weaker, however still significant, dependence on molecular size can be observed with *V. faba* leaf surfaces, where rates of penetration decrease by a factor of 3 when molecular weights of the calcium salts increase from 100 to 500 g mol⁻¹ (Figure 8.4). In addition a pronounced effect of light on calcium salt penetration across the stomatous *V. faba* leaf surfaces was observed (Schlegel *et al.*, 2005). In the dark, overall rates of salt penetration are nearly two-fold lower while the size selectivity is not significantly different from light (Figure 8.4). This observation

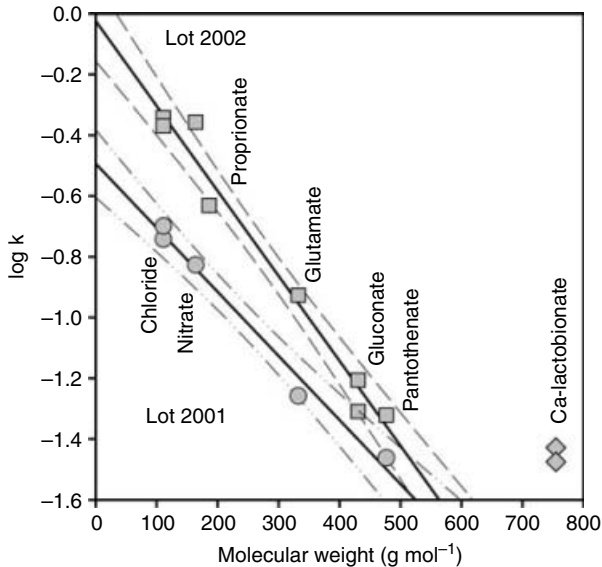


Figure 8.3 The effect of anhydrous molecular weight (MW) on the logarithms of the rate constants ($\log k$) of penetration of calcium salts across isolated *P. canescens* cuticles. Linear regression lines with 95% confidence intervals for the regression lines are shown. Data from Schönherr and Schreiber (2004).

provides a further piece of evidence for the involvement of the cuticle covering stomatal cells in foliar penetration of polar and charged molecules. In the dark when stomatal closure can be assumed to be at its maximum, preferential sites of uptake in the vicinity of stomata are obviously less accessible than in the light. Infiltration of stomata in the light is unlikely to account for the different rates of uptake in the light and the dark because size selectivity is the same in both situations (Figure 8.4).

8.2.2 Ion permeability in comparison to permeability of uncharged molecules

On comparing the penetration of polar and charged molecules, across cuticles with the penetration of non-ionic lipophilic molecules, obvious differences become evident. For the effects of humidity and light on rates of cuticular penetration comparable data for lipophilic molecules are not available, but for the other factors comparisons are possible. In contrast to ionic molecules, pronounced effects of temperature on cuticular penetration of water (Riederer and Schreiber, 2001; Schreiber, 2001) and lipophilic non-electrolytes (Baur *et al.*, 1997) have been described. Activation energies calculated from Arrhenius plots are significantly higher than zero and range from about 50 kJ mol^{-1} for water (Schönherr *et al.*, 1979; Schreiber, 2001) to nearly 200 kJ mol^{-1} for lipophilic molecules (Baur *et al.*, 1997; Buchholz and Schönherr, 2000). These fairly high activation energies for cuticular transport are a good indication that lipophilic molecules in fact diffuse

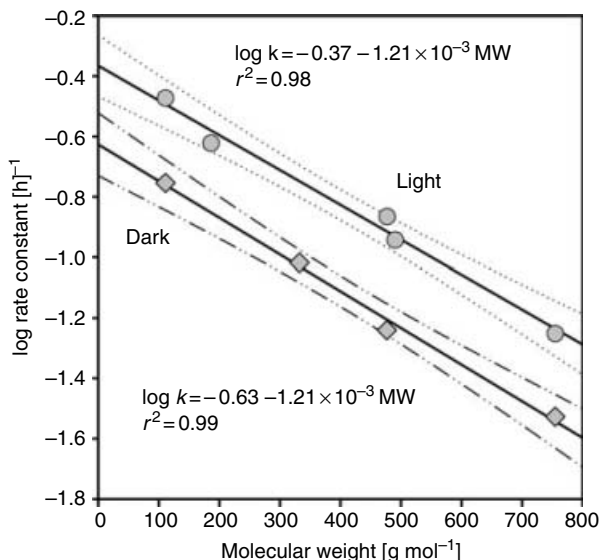


Figure 8.4 The effect of molecular weight on rate constants of penetration of calcium salts into *V. faba* leaf discs in the light and the dark. Linear regression lines with 95% confidence intervals for the regression lines are shown. Data from Schlegel *et al.* (2005).

in lipophilic domains of cutin and wax, which are solid and partially crystalline aggregates (Reynhardt and Riederer, 1991).

In a similar way as temperature, lipophilic plasticisers have been shown to increase rates of cuticular penetration not only of non-ionic lipophilic molecules (Schönherr and Baur, 1994; Buchholz and Schönherr, 2000) but also of water (Riederer and Schönherr, 1990) by factors up to 20-fold and even more. The same plasticisers were nearly ineffective when applied in experiments with inorganic ions and charged organic molecules (Schönherr, 2000, 2002). This is further evidence for the occurrence of two parallel paths of transport across plant cuticles. Obviously, lipophilic plasticisers sorb to lipophilic cutin and wax domains which are the regions where non-ionic lipophilic molecules diffuse across the cuticle. Thus, in this case plasticisers can accelerate the diffusion of lipophilic molecules, whereas they are excluded from the polar paths of transport where the diffusion of polar and charged molecules across the cuticle takes place. Furthermore, wax extraction has been described to strongly increase cuticular penetration of water (Schönherr, 1976) as well as that of lipophilic molecules (Schönherr and Riederer, 1989), whereas there was rarely an effect on cuticular penetration of inorganic ions and charged organic molecules (Schönherr 2000, 2002).

Finally, it is obvious that the dependence of cuticular penetration of lipophilic molecules on the size of the molecules is much stronger (Buchholz *et al.*, 1998) compared to that of inorganic ions (Schönherr and Schreiber, 2004). Whereas the

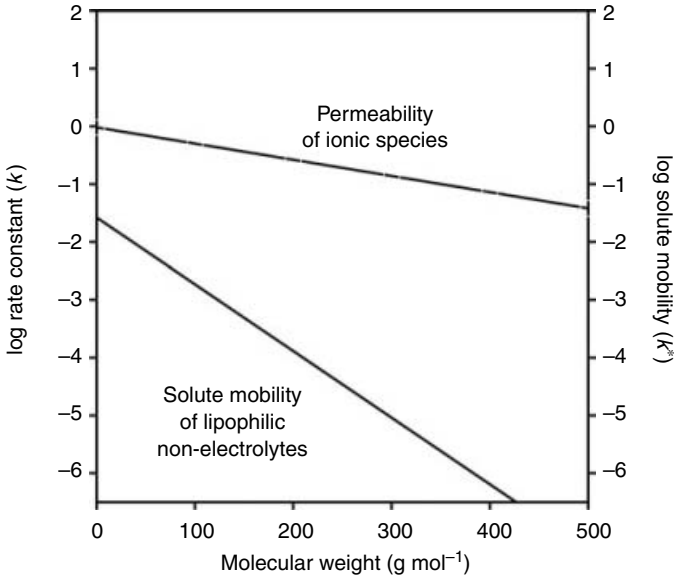


Figure 8.5 Correlation between molecular weights of solutes and rate constants of penetration of ionic species k across isolated poplar (*P. canescens*) cuticles [experimental data from Schönherr and Schreiber (2004)] or mobilities of lipophilic species in poplar cuticles [experimental data from Buchholz *et al.* (1998)]. Data from Schönherr and Schreiber (2004).

rates of cuticular penetration of inorganic ions decreases by about one order of magnitude when molecular weight increases from 100 to 500 g mol⁻¹, rates of cuticular penetration of lipophilic molecules decrease in the same range of molecular weight by more than three orders of magnitude (Figure 8.5). This discrepancy between lipophilic and ionic compounds again indicates that the two different groups of compounds diffuse in physico-chemically different phases in the plant cuticle.

8.3 Significance of polar paths of transport

All results described so far can best be unified in a model postulating two parallel and independent paths of diffusion across the plant cuticle (Figure 8.2): (1) a lipophilic path of transport accessible for non-ionic lipophilic molecules and (2) a polar path of transport available for inorganic ions and charged organic molecules (see Section 8.1.3). For water, being a small and uncharged polar molecule, both pathways of transport are probably accessible. Water solubility in the lipophilic cutin and wax domains will be low, but its mobility can be expected to be high. Significant increases in rates of cuticular water flow upon wax extraction, increases in temperature and the application of lipophilic plasticisers are very good evidence that water can diffuse along the lipophilic transport path. Furthermore, water permeability is highly correlated with the permeability of lipophilic molecules (Niederl *et al.*, 1998). However,

the pronounced effect of humidity on cuticular water permeability indicates at the same time that water can also diffuse to a certain extent along polar paths of transport. Future experimental approaches will have to estimate to what extent both separate paths of transport across plant cuticles contribute to the measured overall transport of water, although it can be speculated that most of the water will probably diffuse along the lipophilic path because it should amount to the largest fraction of the total leaf surface area (for a further discussion of this presently controversial issue see Chapter 9, Section 9.2)

In the past, the existence of these polar paths of diffusion across plant cuticles has largely been overseen or even neglected in cuticular transport physiology. Some exceptions are studies which showed qualitatively the occurrence of preferential sites of foliar penetration for polar and charged molecules (Strugger, 1939; Franke, 1964; Eichert and Burkhardt, 2001). Several reasons are responsible for this phenomenon. First of all, most experimental approaches in the past investigated the penetration of lipophilic molecules across isolated cuticular membranes from a few selected model species (e.g. Schönherr and Riederer, 1989; Kirsch *et al.*, 1997; Niederl *et al.*, 1998). Lipophilic molecules exclusively pass along the lipophilic transport pathway and thus the presence or absence of polar paths cannot be detected.

Second, preferential sites for the foliar penetration of polar compounds are not evenly distributed over the leaf surface, but they are often correlated with the occurrence of special differentiations of the epidermis. Highest rates of uptake of polar compounds are observed in the vicinity of stomata (Franke, 1964; Eichert and Burkhardt, 2001; Schlegel and Schönherr, 2002) and in trichomes (Strugger, 1939; Schlegel and Schönherr, 2002). This can nicely be demonstrated by analysing the precipitation of silver in the apoplastic cell wall after application of silver nitrate to the leaf surface (Schlegel *et al.*, 2005). Highest amounts of silver precipitations in *V. faba* leaves have been detected in the vicinity of stomata and at the base of and in trichomes (Figure 8.6). However, in nearly all experimental approaches of the past, cuticles were isolated from astomatous sides of trichome-free leaves (Riederer and Schreiber, 2001; Schreiber *et al.*, 2001). In addition, permeability of water as a small polar molecule showed an excellent correlation with that of lipophilic molecules and thus in the past there was no need to assume the existence of a polar transport pathway. Polar paths of transport in cuticles can only be detected when charged organic molecules or inorganic ions are investigated, because these substances can penetrate cuticles exclusively *via* polar paths whereas the lipophilic domains are not accessible to them.

8.3.1 *Chemical nature of polar domains in cuticles*

One major still unresolved question is related to the nature of this polar path of transport. Neither the size nor the chemical nature of these postulated polar paths of transport is known at the moment and future experimental approaches will have to solve these problems (for a theoretical approach see Section 9.2.2 of Chapter 9). However, it can be reasonably speculated on the chemical nature of these paths. It is

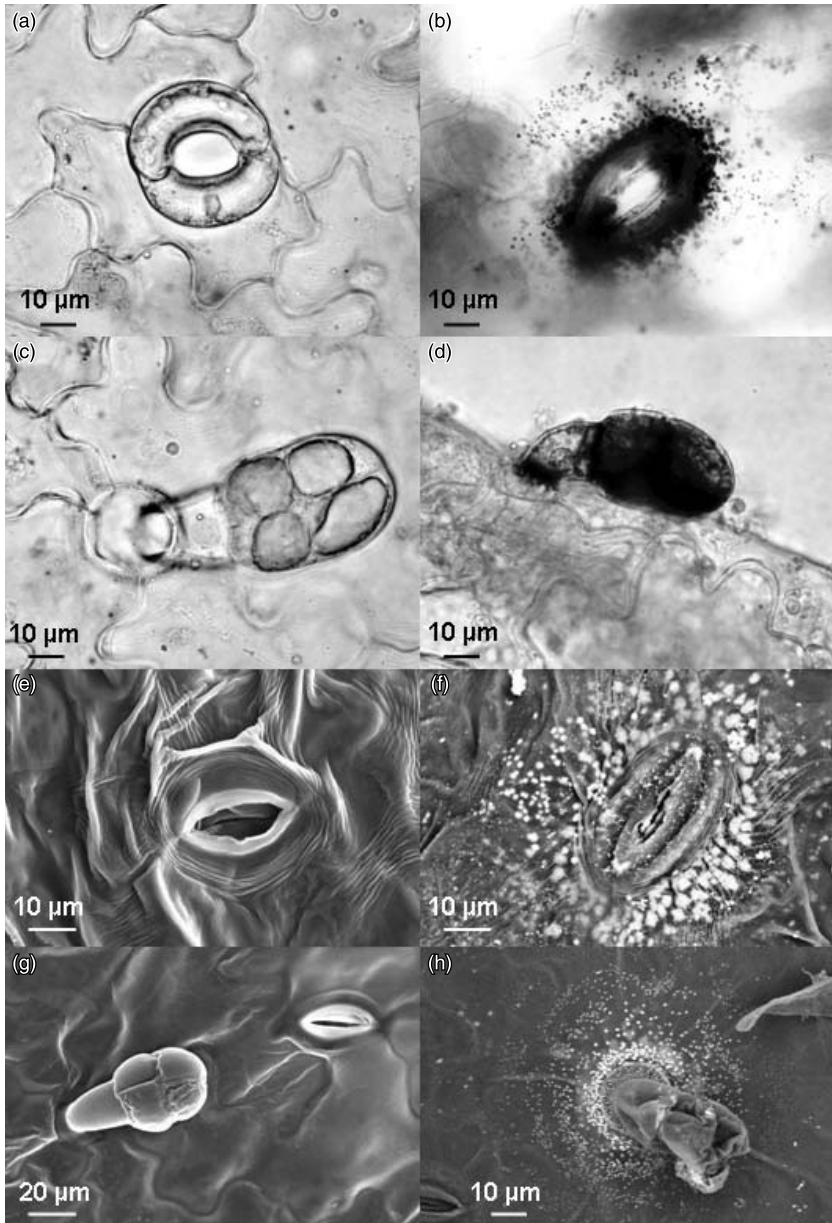


Figure 8.6 (a–d) Light (LM) and (e–f) scanning electron microscopic (SEM) investigation of silver (Ag) deposits around stomata and in trichomes of *V. faba* leaves after treatment with AgNO₃. (a and e) Stomata of untreated leaf surfaces serving as control. (b and f) Stomata of AgNO₃-treated leaf surfaces with characteristic silver deposits surrounding the stomatal pore. (c and g) Trichomes of untreated leaf surfaces serving as control. (f and h) Trichomes of AgNO₃-treated leaf surfaces with characteristic silver deposits in the base and head of the trichome. Data from Schlegel *et al.* (2005). This figure is produced in colour in the colour plate section, which follows page 249.

known that plant cuticles contain polar functional groups which may form the basis for polar aqueous pores. Either non-esterified carboxyl and/or hydroxyl groups of cutin monomers (see Chapter 3) or wax molecules (see Chapter 4) could contribute to the formation of the polar transport paths. Alternatively, polar carbohydrates known to be associated with isolated cuticles (see Chapter 1), could form the basis for the occurrence of polar transport paths. If carbohydrates would extend from the epidermal cell wall through the lipophilic cuticle to the outer surface, they could form preferential polar sites for the diffusion of polar compounds.

8.3.2 *Relevance of polar paths of transport in cuticles*

Increasing evidence, as presented here, for the occurrence of polar paths of diffusion across isolated plant cuticles significantly improves our knowledge on the structure and the function of the plant cuticle forming the major interface between plants and their environment. However, in addition to this substantial scientific progress, the existence of polar paths of transport in plant cuticles is also of relevance for a number of further important scientific as well as applied questions. Chapters 11 and 12 of this book deal with bacteria, yeasts and fungi living on leaf surfaces. Polar water-soluble nutrients like sugars, amino acids and inorganic ions leaching from the apoplast of the epidermis to the leaf surface via the polar paths of transport could significantly improve the nutritional status of epiphytic microorganisms (Leveau and Lindow, 2001). Only lipophilic uncharged agrochemicals, when sprayed onto leaf surfaces, can diffuse into the leaf via the lipophilic path of transport as described in Chapter 7 of this book. Inorganic ions applied for leaf fertilisation as well as charged organic agrochemicals applied to leaf surfaces in plant protection have to penetrate the cuticle *via* polar paths of transport. Thus, it is of major practical importance to have a solid knowledge of these polar paths of transport in order to improve the foliar uptake of ionised substances.

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9 Cuticular transpiration

Markus Burghardt and Markus Riederer

9.1 Introduction

One of the main physiological functions of the plant cuticle is the reduction of uncontrolled water loss to the atmosphere. Cuticles are very efficient transport barriers, but they are not impermeable to the passage of water. In the present chapter the following questions will be addressed: (1) What structural characteristics of cuticular membranes are responsible for their barrier properties and (2) which pathways are available for the permeation of water? (3) How is cuticular transpiration influenced by the environmental factors relative humidity and temperature? (4) What is the physiological significance of cuticular transpiration with regard to stomatal closure? (5) How can the role of the cuticular transpiration barrier be assessed as a mechanism of a drought avoidance strategy?

9.1.1 Definition of transport parameters

Water movement across a membrane like the plant cuticle can be quantitatively characterised by a few simple parameters when steady state conditions are assumed. According to the First Fick's law, the transpiration or flow rate of water J [$\text{g s}^{-1} \text{m}^{-2}$] across the membrane is equal to the amount of water lost W [g] per unit time t [s] and exposed area A [m^2]:

$$J = \frac{\Delta W}{\Delta t A} \quad (9.1)$$

The transpiration rate is related to the permeance P [m s^{-1}], which describes the cuticular barrier properties, and to the driving force, which can be expressed as the concentration difference of water between the outer epidermal cell wall and the atmosphere Δc [g m^{-3}] according to:

$$J = P \Delta c = P \Delta a \rho \quad (9.2)$$

The concentration of water is given by the product of the water activity (a) and the density of water ρ [g m^{-3}]. The water status of plant tissues is commonly described by the water potential Ψ [Pa]. The corresponding water activity can be calculated by

$$\Psi = \frac{RT}{V_w} \ln a \quad (9.3)$$

Table 9.1 Water densities in the liquid (ρ_{liquid}) and the vapour (ρ_{vapour}) state and saturation vapour pressure (SP_V) as a function of temperature (T) (data taken from Nobel, 1991 and Pearcy *et al.*, 1991)

T [°C]	ρ_{liquid} [g cm^{-3}]	ρ_{vapour} [g m^{-3}]	SP_V [kPa]
5	0.99999	6.80	0.873
10	0.99973	9.41	1.228
15	0.99913	12.84	1.706
20	0.99862	17.31	2.339
25	0.99823	23.07	3.169
30	0.99707	30.40	4.246
35	0.99567	39.65	5.627
40	0.99224	51.21	7.381
45	0.99025	65.52	9.590

where R is the ideal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T the absolute temperature [K] and V_w the molar volume of water ($1.80 \times 10^{-5} \text{ mol m}^{-3}$). A water potential of -1.5 MPa in the interior tissues of leaves or fruits leads to a water activity of 0.99, and a water potential of -3.0 MPa corresponds to a water activity of 0.98. Therefore, only a negligible error is introduced when water saturation of the internal tissues ($a = 1.0$) is assumed for the calculation of permeances (Pearcy *et al.*, 1991).

The driving force can be expressed either on the basis of the water density in the liquid state or on the basis of the density in the vapour state (Table 9.1). Assuming a cuticular transpiration rate of $1 \times 10^{-4} \text{ g m}^{-2} \text{ s}^{-1}$ at 25°C , 60% relative humidity and an internal water potential of -1.5 MPa , the permeance calculated on the basis of the liquid state of water would amount to $2.57 \times 10^{-10} \text{ m s}^{-1}$. If the driving force is expressed on the basis of the vapour state of water, a permeance of $1.11 \times 10^{-5} \text{ m s}^{-1}$ is obtained. Correspondingly, the conversion factor is given by the ratio of the densities of water in the liquid and the vapour states amounting to 43 270 at 25°C . As discussed and recommended by Kerstiens (1996a,b), cuticular permeances should always be calculated on the basis of the density of water vapour, especially when evaluating the effect of temperature on the barrier properties of cuticular membranes is intended.

Transpiration rates obtained from gas exchange measurements in the field are generally given in molar units [$\text{mol m}^{-2} \text{ s}^{-1}$]. Mol-fraction-based conductances (g) are obtained using the vapour pressure difference (ΔP_V) as driving force:

$$J = g \frac{\Delta P_V}{P_A} \quad (9.4)$$

where P_A denotes the atmospheric pressure (101.3 kPa for standard conditions). Applying this approach to the example given earlier would yield a mol-fraction-based conductance of $4.56 \times 10^{-4} \text{ mol m}^{-2} \text{ s}^{-1}$. Correspondingly, mol-fraction-based conductances g [$\text{mol m}^{-2} \text{ s}^{-1}$] can be converted to vapour-based permeances

P [m s^{-1}] according to:

$$P = g \frac{RT}{P_A} \quad (9.5)$$

The conversion factor amounts to 0.0245 at 25°C and standard atmospheric pressure which can be used to compare *in vitro* measured cuticular permeances with conductances obtained in the field.

9.1.2 Experimental methods

The experimental methods for the determination of cuticular water permeances and potential errors associated with these techniques have been reviewed previously in detail (Kerstiens, 1996a,b). It has been recommended that the term cuticular transpiration should only be used when completely astomatous systems are studied. As the most prominent approach, isolated cuticular membranes obtained from the adaxial sides of hypostomatous leaves or from fruits were used (Riederer and Schreiber, 2001). Isolated cuticular membranes are mounted in transport chambers and the water flow from an aqueous donor into the dry atmosphere is measured gravimetrically (Schönherr and Lenzian, 1981). Alternatively, water flow across cuticular membranes can be measured with tritium-labelled water – an approach which is equivalent to that used for permeation experiments with lipophilic non-electrolytes (Chapter 7). This method allows for the determination of cuticular water permeances even at the minimum overall driving force (100% relative humidity). The driving force in this case is the concentration difference in the tritium label. This method is especially suitable for measuring the effect of water activity on cuticular permeances (Schönherr and Schmidt, 1979; Schreiber *et al.*, 2001).

Enzymatic isolation of cuticles is only possible for a limited number of plant species. Therefore an alternative method was devised which permits to obtain cuticular permeances from transpiration rates of the astomatous side of leaf discs (Hoad *et al.*, 1996; Schreiber *et al.*, 2001; Burghardt and Riederer, 2003). The comparison of isolated cuticular membranes and leaf discs showed that the isolation process did not influence the cuticular transport properties (Schreiber, 2001). There is only little information about the cuticular transport properties of abaxial leaf sides of hypostomatous leaves. Recently a new method for the determination of cuticular permeances of the stomatous sides of leaves was proposed which is based on the assumption that water transport in the gas phase of stomatal pores can be manipulated by the flow of different gases while the transport across the solid phase of the cuticular membrane is not affected (Santrucek *et al.*, 2004).

In all cases where water loss through incompletely closed stomata may contribute to the overall transpiration rate, the term minimum leaf conductance (g_{min}) should be used (Kerstiens, 1996a,b). The minimum leaf conductance refers to the minimum value a detached leaf can reach when the stomata are completely closed as a result of desiccation stress. The term lowest conductances (g_{low}) is restricted to attached leaves of intact plants in the field. They are almost in all cases higher than the corresponding minimum conductances (Körner, 1994).

9.2 Mechanisms of water transport through the cuticle

The cuticular membrane is composed of the polymer cutin (Chapter 3) constituting the matrix for embedded intracuticular waxes and forming the basis for the deposition of epicuticular wax films and crystals (Chapter 4). Though cuticular membranes are mainly considered as a lipid barrier, there are also hydrophilic structures present (Chapter 8). This probably is due to the fact that the polyester-type polymer cutin contains non-esterified hydroxy and carboxy groups (Schönherr and Huber, 1977) and polysaccharides like pectin and cellulose (Jeffree, 1996). Cuticular membranes contain on an average 21% of non-lipid material (Riederer and Schönherr, 1984). For the leaf cuticle of *Cirsium horridulum* even a ratio of carbohydrates to lipids of 2:1 was reported (Marga *et al.*, 2001). In accordance with the heterogeneous composition of the cuticle two pathways for the permeation of water are discussed: a lipophilic route through the cutin and amorphous wax fraction and a hydrophilic pathway across polar pores of molecular dimensions (Chapter 8). Both pathways can be considered to be arranged as parallel resistances leading to the overall permeance of the cuticular membrane P_{CM} (Schreiber *et al.*, 2001):

$$P_{CM} = P_{lipid} + P_{polar} = \frac{D_{lipid} K_{lipid}}{\Delta x_{lipid}} + \frac{D_{polar} K_{polar}}{\Delta x_{polar}} \quad (9.6)$$

The permeance (P) of each route is a composite quantity and can be related to the diffusion coefficient (D), the partition coefficient (K) and the diffusional path length (Δx) of the lipid and polar pathway (indices lipid or polar), respectively. Present knowledge strongly implies that the participation of either only the polar or of both pathways has to be taken into account when analysing the water permeation through cuticular membranes.

9.2.1 The lipophilic pathway

There is some evidence that water movement across the plant cuticle may take a lipophilic pathway. Cuticular water permeability was found to be correlated with the diffusion coefficients of octadecanoic acid in reconstituted cuticular waxes (Schreiber and Riederer, 1996) and with the cuticular permeances of the lipophilic organic compounds 2,4-dichlorophenoxyacetic acid, benzoic acid and salicylic acid (Schönherr and Riederer, 1989; Niederl *et al.*, 1998; Schreiber, 2002). These findings have been taken as evidence that water permeates mainly across the lipophilic pathway of cuticular membranes made out of cutin and wax domains. Furthermore, cuticular water permeability is enhanced by the removal of cuticular waxes in the presence of accelerator adjuvants (Riederer and Schönherr, 1990) and with increasing temperature (Schreiber, 2001). These effects are also observed for the permeation of lipophilic organic compounds but not for polar dissociated calcium salts (Schönherr, 2000).

The transport across cuticular membranes can be described as a sorption-diffusion process (Schönherr and Riederer, 1989). The permeance is given by

the diffusion coefficient, the partition coefficient, the membrane thickness (l) and the tortuosity factor (τ), which takes into account the lengthening of the diffusional path due to impermeable crystalline wax domains:

$$P = \frac{DK}{l\tau} \quad (9.7)$$

Sorption to cuticular membranes is characterised by the cuticle/water partition coefficient (K_{CW}), which is defined as the ratio of the equilibrium concentrations in the cuticle (c_c) and in an aqueous solution (c_w):

$$K_{CW} = \frac{c_c}{c_w} \quad (9.8)$$

The sorption–diffusion model has been applied successfully in order to describe the permeation of lipophilic compounds across cuticular membranes of *Citrus aurantium* (Kerler and Schönherr, 1988a,b) and cuticular permeances were found to be related to the cuticle/water partition coefficient (Chapter 7):

$$\log P_{CM} = 0.734 \log K_{CW} - 11.26 \quad (9.9)$$

Assuming that water indeed permeates across the same pathway as lipophilic compounds, cuticular water permeability should be predictable from the cuticle/water partition coefficient of water. From the sorption of water vapour from a saturated atmosphere to isolated cuticular membranes (Lendzian and Kerstiens, 1991) the cuticle/water partition coefficient of water can be obtained (Table 9.2). Prediction of the permeance according to Equation 9.9 yields a value of $4.4 \times 10^{-9} \text{ m s}^{-1}$, which underestimates the experimentally determined permeance by a factor of 3000 (Table 9.2).

Dividing the cuticular permeance by the cuticle/water partition coefficient yields a measure for the solute mobility which according to Equation 9.7 is directly proportional to the diffusion coefficient. The slope of the regression line from a plot of the mobility versus the molar volume of the permeating compound represents the size selectivity of diffusion according to the free-volume theory (Schönherr and Baur, 1994; Chapter 7). The mobility of water is significantly higher than expected from its molar volume (Figure 9.1). In conclusion, the prediction models for the

Table 9.2 Partition coefficients for the sorption of water vapour to cuticular membranes (K_{CW}) and experimentally determined cuticular permeances (P_{CM}) (Kerstiens and Lendzian, 1989)

Species	K_{CW}	$P_{CM} [\text{m s}^{-1}]$
<i>Citrus aurantium</i>	8950	1.3×10^{-5}
<i>Ficus elastica</i>	2480	2.8×10^{-6}
<i>Hedera helix</i>	5500	5.6×10^{-6}
<i>Lycopersicon esculentum</i>	3940	5.3×10^{-5}

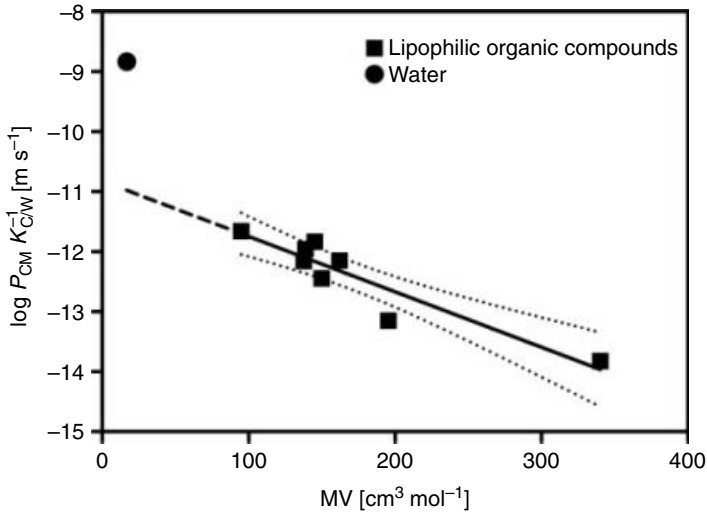


Figure 9.1 Correlation between cuticular permeances (P_{CM}) of lipophilic organic compounds (Kerler and Schönherr, 1988b) corrected by the cuticle/water partition coefficient ($K_{C/W}$) (Kerler and Schönherr, 1988a) and the molar volume (MV, calculated according to Abraham and McGowan, 1987). The cuticular permeance and the cuticle/water partition coefficient of water (Kerstiens and Lenzian, 1989) deviated significantly from the regression line of the lipophilic compounds.

permeation of lipophilic organic compounds across isolated plant cuticles cannot be extrapolated to the description and analysis of cuticular water permeability. Hence, it is questionable whether a significant fraction of the water diffusing across a cuticle actually takes the lipophilic pathway.

9.2.2 The hydrophilic pathway

The permeation of water through polar pores in the plant cuticle was first demonstrated for dewaxed cuticles (polymer matrix membranes) of *C. aurantium* by comparison of the osmotic water permeability (P_f) and the diffusional water permeability (P_d) (Schönherr, 1976a). A ratio equal to unity is indicative for simple lipid membranes while an osmotic water permeability higher than the diffusional one provides evidence for the existence of polar pores in lipid membranes (Verkman, 2000).

The mean radius of polar pores (r_p) can be estimated according to:

$$r_p = 0.36 \sqrt{\frac{P_f - P_d}{P_d}} \quad (9.10)$$

A value of 0.46 nm was obtained for *C. aurantium* polymer matrix membranes (Schönherr, 1976a). Recently this approach was applied to native cuticular membranes from fruits of *Lycopersicon esculentum* and *Capsicum annuum*

Table 9.3 Diffusional (P_d) and osmotic (P_f) water permeability of cuticular membranes from fruits of *Lycopersicon esculentum* and *Capsicum annuum* (Beyer *et al.*, 2005)

Species	P_d [m s^{-1}]	P_f [m s^{-1}]	$P_f P_d^{-1}$
<i>Lycopersicon esculentum</i>	5.6×10^{-5}	6.6×10^{-4}	12
<i>Capsicum annuum</i>	8.2×10^{-5}	5.7×10^{-4}	6.9

(Beyer *et al.*, 2005; Table 9.3). Analysis of these data according to Equation 9.10 yields an average pore radius of 1.2 nm for *L. esculentum* and 0.87 nm for *C. annuum*. The diffusion of water in narrow aqueous pores of molecular dimensions is hindered by steric restriction at the pore entrance and by the friction at the pore wall (Mitragotri, 2003). These effects are taken into account by the hindrance factor (H), which establishes a relationship between the diffusion coefficients in an aqueous pore (D_p) and in bulk water (D_w) according to:

$$D_p = H D_w \tag{9.11}$$

As a first approximation, H can be calculated from the molecular radius ($r_m = 0.19$ nm for water) and the pore radius (r_p):

$$H = \left(1 - \frac{r_m}{r_p}\right)^4 \tag{9.12}$$

Assuming a pore radius of 1.2 nm (*L. esculentum* fruit cuticular membrane) the hindrance factor would amount to 0.48. Correspondingly, the diffusion of water in an aqueous pore of this dimension would be two-fold slower than self-diffusion in bulk water. Analogous to a model describing skin permeability of hydrophilic compounds (Mitragotri, 2003) the permeance of plant cuticular membranes (P_{CM}) for water can be formulated as follows:

$$P_{CM} = K_{CW} \frac{\epsilon}{l\tau} D_w H \tag{9.13}$$

Cuticular water permeability is related to the cuticle/water partition coefficient (K_{CW}), the porosity (ϵ , ratio of the pore area to total area), the membrane thickness, the tortuosity factor, the self-diffusion coefficient of water (D_w) and the hindrance factor. This approach can be applied to analysing cuticular water permeability as follows: if the cuticular permeance of *L. esculentum* ($P_{CM} = 5.3 \times 10^{-5} \text{ m s}^{-1}$; Table 9.2) is multiplied by the thickness of the cuticular membrane ($l = 8 \text{ }\mu\text{m}$, Becker *et al.*, 1986) and divided by the cuticle/water partition coefficient ($K_{CW} = 3940$, Table 9.2), by the diffusion coefficient of water in an aqueous medium ($D_w = 2.4 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$) and by the hindrance factor ($H = 0.48$), a value of 8.8×10^{-5} is obtained for the quotient of porosity and tortuosity. Assuming that the polar pathway takes a straight course through the membrane (tortuosity equal to unity: $\tau = 1$), the porosity would amount to 0.01%. This low value implies that water movement across the cuticular pathway

takes place due to rare and tiny cracks and imperfections in the continuity of the cuticular transpiration barrier. Further evidence that cuticular transpiration results from permeation through polar pores can be derived from the effects of relative humidity (see Section 9.3.1) and temperature (see Section 9.3.2) on cuticular water permeability.

9.2.3 Relationship between cuticular transpiration and cuticle structure and composition

No correlation between cuticular water permeability and the thickness of cuticular membranes or the amounts of cuticular waxes was found for 23 species (Riederer and Schreiber, 2001). Comparison of sun and shade leaves of *Hedera helix* (Table 9.4) adds further evidence that these cuticular properties are not indicators for the barrier properties of the plant cuticle. Both types of leaves have approximately the same cuticular permeance, though cuticle thickness and amount of cuticular waxes are twice as high for the sun leaves. Only after complete removal of cuticular waxes is the permeance of polymer matrix membranes of the shade leaves ten-fold higher compared to those of the sun leaves. The predominating role of cuticular waxes for the transport-limiting barrier of cuticular membranes is evident, because cuticular water permeability increases by one to two orders of magnitude after complete removal of waxes by organic solvents (Tables 9.4 and 9.5).

Table 9.4 Cuticle thickness, amount of cuticular waxes, cuticular permeance (P_{CM}) and permeance of the polymer matrix membrane (P_{MX}) at 25°C of mature *Hedera helix* sun and shade leaves (Burghardt, unpublished results)

	Sun leaf	Shade leaf
Cuticle thickness [μm]	5.3	2.6
Wax amount [$\mu\text{g cm}^{-2}$]	65	33
P_{CM} [m s^{-1}]	3.3×10^{-6}	4.2×10^{-6}
P_{MX} [m s^{-1}]	4.9×10^{-5}	5.2×10^{-4}
$P_{MX} P_{CM}^{-1}$	13	116

Table 9.5 Effect of wax extraction on water permeability: cuticular permeances (P_{CM}) and permeances of polymer matrix membranes (P_{MX}) at 25°C (Schönherr and Lenzian, 1981; Schreiber *et al.*, 2001)

Species	P_{CM} [m s^{-1}]	P_{MX} [m s^{-1}]	$P_{MX} P_{CM}^{-1}$
<i>Citrus aurantium</i>	3.6×10^{-5}	6.8×10^{-3}	188
<i>Lycopersicon esculentum</i>	8.2×10^{-5}	1.6×10^{-3}	20
<i>Prunus laurocerasus</i>	1.1×10^{-5}	2.8×10^{-4}	25
<i>Pyrus communis</i>	2.1×10^{-5}	4.7×10^{-3}	219

Cuticles are asymmetrical membranes with the wax barrier located at the morphologically outer surface (Chapters 2 and 7). The polymer matrix (MX) and the cuticular waxes (wax) have been considered as two resistances in series (Schönherr, 1976b):

$$\frac{1}{P_{CM}} = \frac{1}{P_{MX}} + \frac{1}{P_{wax}} \quad (9.14)$$

The comparison of cuticular membranes and polymer matrix membranes shows that the overall resistance is completely determined by the resistance of the wax barrier. A further differentiation can be made between epicuticular waxes and intracuticular waxes. Selective removal of the epicuticular wax layer had only a slight effect on the water permeability of cuticular membranes from fruits of *L. esculentum* (Vogg *et al.*, 2004) and *Prunus avium* (Knoche *et al.*, 2000) revealing that the main barrier properties in these cases can be attributed to the intracuticular wax layer.

A considerable effort has been undertaken in order to find a relationship between chemical wax composition and cuticular water permeability. *C. aurantium* plants were grown under varying environmental conditions. Though the amounts and the composition of the cuticular waxes varied considerably between the different treatments (Riederer and Schneider, 1990), no significant effect on cuticular transpiration was detected (Geyer and Schönherr, 1990). During ontogenetic development of *H. helix* leaves cuticular transpiration rapidly decreased within the first 30 days, which was also the period where the most prominent changes in wax amount and composition occurred. However, again no relationship between wax composition and transport properties for water could be deduced from this study (Hauke and Schreiber, 1998). A wax mutant of *L. esculentum* deficient in a very long-chain fatty acid elongase showed a reduction of the amounts of intracuticular aliphatic compounds by 50% and a four-fold higher cuticular water permeability (Vogg *et al.*, 2004).

All these studies imply that the cuticular barrier properties can mainly be attributed to long-chain aliphatic wax compounds forming crystalline structures, which are not accessible to permeating water molecules (Riederer and Schreiber, 1995). However, no structure–property relationships has been established on a quantitative level so far. The efficiency of different chain lengths in reducing diffusional water transport across the plant cuticle was estimated from the resistance of fatty acid monolayers against evaporation of water. Perfect monolayers of fatty acids with chain lengths in the range of C₂₆–C₃₂ were estimated to be sufficient for obtaining resistances equivalent for those found in cuticular membranes (Schönherr and Riederer, 1989). Obviously, the aliphatic compounds of cuticular waxes are not arranged as perfect monolayers but form a multi-component, semi-crystalline wax.

Assuming that water permeates mainly across the hydrophilic pathway, a promising approach would be to consider cuticular water permeability with regard to ultrastructural properties. The hydrophilic pathway probably consists of a reticulum of polysaccharide microfibrils ramifying and stretching through the cuticular membrane (Jeffree, 1996). Strands of polysaccharides might reach the outer surface

of cuticular membranes and thus may constitute effective pathways for water diffusion. A survey of water permeability of cuticular membranes of known ultrastructure (as depicted by transmission electron microscopy) revealed that the lowest permeances were observed for cuticles with a non-reticulate outer layer while the highest permeances were associated with all-reticulate cuticular membranes (Kerstiens, 1994).

9.3 Environmental effects on transpiration

9.3.1 Relative humidity

The relative humidity of the atmosphere is very variable and thus may influence cuticular transpiration. It has been shown that with relative humidity increasing from 0 to 100% cuticular permeances for water increase by factors between 2 and 3 (Schönherr and Schmidt, 1979; Hoad *et al.*, 1997; Schreiber *et al.*, 2001). At 100% relative humidity the gravimetric method for the determination of cuticular water permeability fails since the driving force is equal to zero. In order to examine the maximum effect of humidity, the self-diffusion of tritium-labelled water represents a suitable method (Schreiber, 2002). Comparison of cuticular permeances determined for dry air and at water saturation of the atmosphere nicely illustrates the humidity effect on cuticular water permeability (Table 9.6).

The humidity effect on cuticular water permeability can be explained by the presence of aqueous polar pores and be used as a further argument for their existence and importance. Swelling of the cuticular membrane with increasing relative humidity may lead to the formation of additional polar pathways and to a further opening of the initially present ones. Water sorption isotherms exhibit a significant sigmoidal shape with a progressive increase of sorption when the relative humidity is raised above 60% (Luque *et al.*, 1995). This is the same region where the most pronounced humidity effects on cuticular water permeability

Table 9.6 Cuticular permeances (P_{CM}) at maximum driving force (0% relative humidity) determined by the gravimetric method (Schreiber and Riederer, 1996) and at minimum driving force (100% relative humidity) determined by measurement of the permeation of ^3H -labelled water (Niederl *et al.*, 1998)

Species	P_{CM} [m s^{-1}] (0% r.h.)	P_{CM} [m s^{-1}] (100% r.h.)
<i>Euonymus japonica</i>	1.5×10^{-5}	2.2×10^{-5}
<i>Ginkgo biloba</i>	2.3×10^{-5}	6.7×10^{-5}
<i>Hedera helix</i>	2.5×10^{-6}	5.6×10^{-6}
<i>Juglans regia</i>	2.0×10^{-5}	1.7×10^{-4}
<i>Liriodendron tulipifera</i>	1.8×10^{-5}	8.1×10^{-5}
<i>Lycopersicon esculentum</i>	2.7×10^{-5}	2.9×10^{-4}
<i>Prunus laurocerasus</i>	5.8×10^{-6}	3.4×10^{-5}

occur (Schreiber *et al.*, 2001). Water sorption has been attributed mainly to the polysaccharide fraction of cuticular membranes (Kerstiens and Lendzian, 1989; Dominguez and Heredia, 1999). Therefore, it seems reasonable that the high hydration capacity of polysaccharides is responsible for the humidity effect on cuticular water permeability.

9.3.2 Temperature

Temperature is another environmental factor which undergoes large diel and annual changes. The effect of temperature on cuticular water permeability is generally analysed by Arrhenius plots (plotting the natural logarithm of the cuticular permeance versus the reciprocal of the absolute temperature). The activation energy of water penetration across the cuticle can be obtained from the slope of the regression line by multiplication with the ideal gas constant. For cuticular membranes of five species the effect of temperature was examined in the temperature range from 10 to 55°C (Schreiber, 2001). A phase transition was detected in the temperature range between 30 and 39°C. Below the phase transition only moderate effects were observed while above the phase transition a steep increase of cuticular transpiration occurred. However, in this study, cuticular permeances were calculated on the basis of the water density in the liquid state as driving force. Therefore, the temperature effects calculated integrate both the increase of the driving force due changing saturation vapour concentrations and a decrease of the cuticular barrier properties (Kerstiens, 1996b). The Arrhenius plot of cuticular permeances, which were calculated on the basis of the water density in the vapour state (Riederer and Schreiber, 2001), still shows a phase transition (Figure 9.2). At temperatures below the phase transition, a comparably low activation energy is obtained while above the phase transition a drastic effect of temperature on cuticular water permeability is reflected by higher activation energies (Table 9.7).

The activation energy allows drawing conclusions about the corresponding transport pathway. The permeation of water in the hydrophobic environment of a lipid membrane by a solubility–diffusion mechanism is characterised by a comparably high activation energy ($E_a > 42 \text{ kJ mol}^{-1}$). Water transport mediated in a hydrophilic environment mediated by polar pores penetrating a lipid membrane is characterised by a comparably low activation energy ($E_a < 25 \text{ kJ mol}^{-1}$) and the activation energy for the self-diffusion of water amounts to 19 kJ mol^{-1} (Elmoazzen *et al.*, 2002). The low activation energy of cuticular water permeability at temperatures below the phase transition can be taken as further evidence that water movement preferably takes place in a more or less aqueous environment.

The steep increase of cuticular permeances above the phase transition temperature is probably associated with structural changes of the cuticular waxes, since for polymer matrix membranes no phase transition in this temperature range was observed (Schönherr *et al.*, 1979; Schreiber, 2002). Correspondingly, the regression lines of Arrhenius plots for the water permeance of cuticular and matrix membranes tend to converge at high temperatures. The intersection of both lines represents

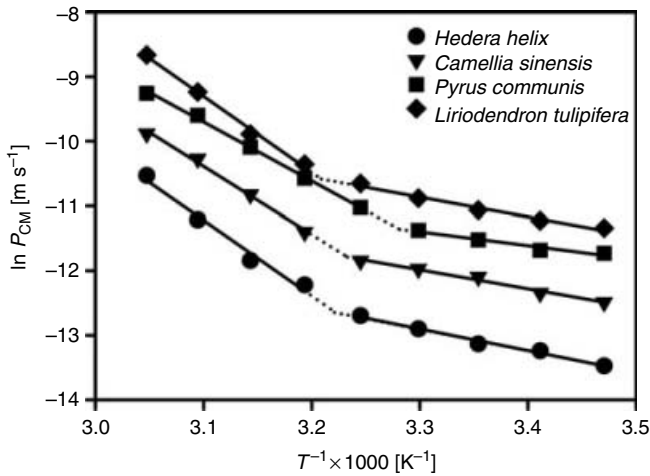


Figure 9.2 Arrhenius plots of cuticular water permeability (recalculated from Riederer and Schreiber, 2001). The natural logarithm of the vapour-phase-based cuticular permeance (P_{CM}) is plotted versus the reciprocal value of the absolute temperature (T).

Table 9.7 Phase transition temperature (T_P) and activation energy (E_a) for the permeation of water across isolated cuticular membranes (Riederer and Schreiber, 2001)

Species	T_P [$^{\circ}\text{C}$]	E_a [kJ mol^{-1}] below T_P	E_a [kJ mol^{-1}] above T_P
<i>Hedera helix</i>	37	28	97
<i>Camellia sinensis</i>	36	25	87
<i>Pyrus communis</i>	32	18	76
<i>Liriodendron tulipifera</i>	38	22	98

the temperature where the effectiveness of the wax barrier is eliminated. This effect occurs for cuticular membranes from leaves of *Juglans regia* at a temperature of 69°C (Figure 9.3). This value is in good correspondence with the temperature range ($66\text{--}74^{\circ}\text{C}$) where complete melting of the cuticular wax was observed by visible detection using a melting point microscope (Schreiber and Riederer, 1996). In addition to the onset of wax melting at the phase transition temperature (Merk *et al.*, 1998), an increased volume expansion of the cutin polymer was assumed to cause defects in the wax barrier, which may contribute to an enhancement of the cuticular water permeability with increasing temperature (Schreiber and Schönherr, 1990).

9.4 Physiology of cuticular transpiration in relation to stomatal closure

So far, this chapter has concentrated on the water permeability of the plant cuticle. The cuticle, however, is part of a system to which the stomata also belong. Therefore,

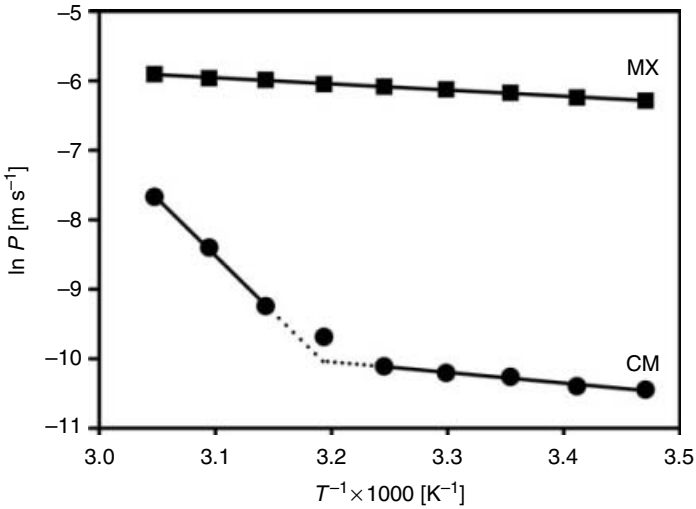


Figure 9.3 Arrhenius plots for the cuticular membrane (CM) and the polymer matrix membrane (MX) of leaves from *Juglans regia*. The natural logarithm of the permeance (P) is plotted versus the reciprocal value of the absolute temperature (T) (Burghardt, unpublished results).

it should be asked what role the cuticle plays in this system as far as drought resistance of the leaf or the whole plant is concerned. Minimum leaf conductances at maximum stomatal closure can be obtained from leaf drying curves by measuring the mass loss of detached leaves as a function of time under controlled environmental conditions. Since even a low fraction of open stomatal pore area may contribute significantly to the overall transpiration rate, a clear differentiation between cuticular permeances of stomata-free systems (adaxial leaf surfaces of hypostomatous leaves) and minimum conductances should be made (Kersties, 1996a,b). Transpiration rates of detached leaves are closely related to the leaf water potential and the minimum transpiration rate is reached only at the turgor loss point, which can be considered as the main indicator for maximum stomatal closure (Burghardt and Riederer, 2003). The minimum transpiration rate remains nearly constant up to a relative water deficit equivalent to the symplastic water content (Figure 9.4). In some cases a slight decrease of the minimum conductance with declining leaf water content has been observed, which may be attributed to a decrease of the hydration state of the cuticular membrane itself (Van Gardingen and Grace, 1992; Hoard *et al.*, 1996).

For a number of species the cuticular permeances of the adaxial leaf surfaces and the minimum conductances of leaves detached from the whole plant were not significantly different (Figure 9.5). *H. helix* is the only exception since minimum conductance is three-fold higher than cuticular permeance (Burghardt and Riederer, 2003). This deviation may be caused by residual transpiration due to incomplete stomatal closure. Model calculations show that stomata may contribute significantly to the minimum conductance even when stomatal pores visibly appear completely

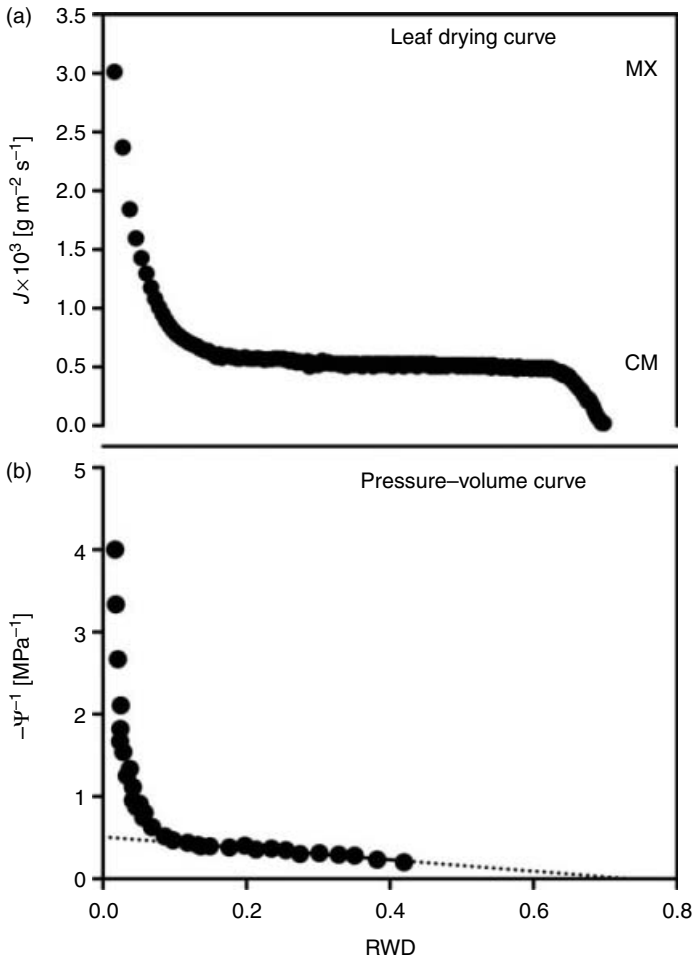


Figure 9.4 (a) Representative leaf drying curve for *Vinca minor*. The transpiration rate (J) is plotted versus the relative water deficit (RWD). (b) Pressure–volume curve of *V. minor*. The reciprocal value of the leaf water potential (Ψ) is plotted versus the relative water deficit (RWD) (data from Burghardt and Riederer, 2003, and unpublished results).

closed (Kerstiens, 1996b). An alternative explanation was recently offered by the development of a new technique for the measurement of water permeability of stomatous cuticular membranes. In this study the cuticular water permeability of the abaxial side of leaves from *H. helix* was measured to be eleven-fold higher than the permeability of the adaxial side (Santrucek *et al.*, 2004). This finding may be related to early reports that the cuticle covering the guard cells may have a higher water permeability ('peristomatal transpiration') than the cuticle of the epidermis (Maier-Maercker, 1983).

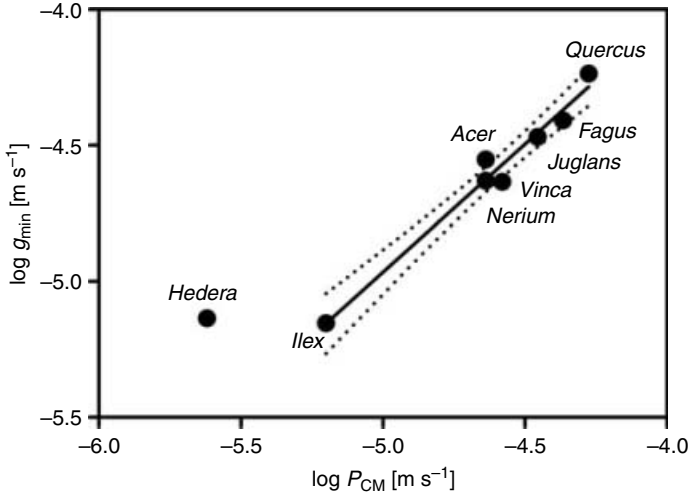


Figure 9.5 Plot of the minimum conductance (g_{min}) versus the cuticular permeance (P_{CM}) (*Acer campestre*, *Fagus sylvatica*, *Quercus petraea*, *Ilex aquifolium*, *Hedera helix*: Burghardt and Riederer, 2003; and *Juglans regia*, *Nerium oleander*, *Vinca minor*, unpublished results).

Though under extreme experimental conditions (0% relative humidity, darkness, leaf water potential below the turgor loss point) residual stomatal transpiration does not contribute significantly to the overall transpiration of detached leaves, the question remains open, if, under realistic environmental conditions in the field, attached leaves of intact plant behave in the same way. The lowest conductances of plants observed in the field (Körner, 1994) are generally higher than minimum conductances and cuticular permeances (Kerstiens, 1996a,b). Comparison of daytime and nighttime conductances indicate that some species maintain substantial stomatal conductances at night (Snyder *et al.*, 2003). This implies that under typical environmental conditions plants are only rarely forced to a strict drought avoidance strategy. However, so far there is no study available considering cuticular permeances, minimum conductances and lowest conductances under controlled experimental conditions in an integrative approach.

9.5 The cuticular transpiration barrier as a mechanism of the drought avoidance strategy

A survey of cuticular permeances of a broad spectrum of plant species has been published recently (Riederer and Schreiber, 2001). Cuticular permeances of fruits (median: $8.6 \times 10^{-5} m s^{-1}$) were on the average 15-fold higher than permeances of leaves (median: $5.8 \times 10^{-6} m s^{-1}$). Analysis of cuticular water permeability of leaves with regard to lifeform and climate of origin shows that the highest permeances are

found within the group of mesomorphic leaves of deciduous species growing in temperate climates. Cuticles from evergreen leaves from epiphytic plants growing in tropical climates have the lowest permeances. However, the classification is not conclusive in all cases. The group of xeromorphic leaves of evergreen species growing in mostly mediterranean non-tropical climates has cuticular permeances ranging from 10^{-4} to 10^{-6} m s^{-1} , which covers nearly the whole spectrum of leaf cuticular permeances measured so far. Since cuticular transpiration was generally examined at 25°C and at 0% relative humidity, a more differentiated picture may emerge, if water permeability is measured under environmental conditions which mimic the real climatic conditions of the natural habitats.

If the water supply from the soil is restricted, plants reduce the transpirational water loss by midday or even by permanent stomatal closure (Larcher, 1972). Under such conditions survival depends on the efficacy of the transpiration barrier to conserve the scarce water resources and to counteract the inevitable decline of the leaf water content. The survival time has been introduced as a useful parameter in order to assess the ability of leaves to avoid drought stress (Pisek and Winkler, 1953):

$$\text{Survival time} = \frac{(\text{RWD}_{\text{SLD}} - \text{RWD}_{\text{SC}})S_{\text{U}}}{J_{\text{min}}} \quad (9.15)$$

The available water content depends on the sublethal water deficit (RWD_{SLD} , relative water deficit where irreversible leaf damage occurs), the relative water deficit at maximum stomatal closure (RWD_{SC}) and the degree of succulence (S_{U} , total amount of water per leaf area). The minimum transpiration rate (J_{min}) is given according to Equation 9.2 by the cuticular permeance (or minimum conductance) and the driving force of transpiration. Model calculations for 50% relative humidity and 25°C (Figure 9.6) yield survival times in the range from 12 (*Fagus sylvatica*) to 19 h (*Acer campestre*) for deciduous leaves growing in temperate climates and 51 h for the xeromorphic leaf of *Nerium oleander* growing in mediterranean-type climates. The highest survival times with 120 h (*H. helix*) and 130 h (*Ilex aquifolium*) are obtained for evergreen leaves growing in temperate climates (Figure 9.6; Burghardt and Riederer, 2003).

9.6 Conclusions

The mechanism of water transport in cuticular membranes is still a matter of debate. Co-permeation of water with lipophilic organic compounds has been interpreted as evidence that water movement predominately takes place across the lipophilic cuticular pathway (Schönherr and Riederer, 1989; Niederl *et al.*, 1998; Schreiber, 2002). However, permeation of water does not fit the prediction models established for lipophilic organic compounds according to the mechanism of a sorption–diffusion process and the free-volume theory (see Section 9.2.1 earlier). Further studies on a quantitative basis are necessary in order to decide if water permeation rather is related to the lipophilic cutin–wax region or primarily to aqueous polar pores as it

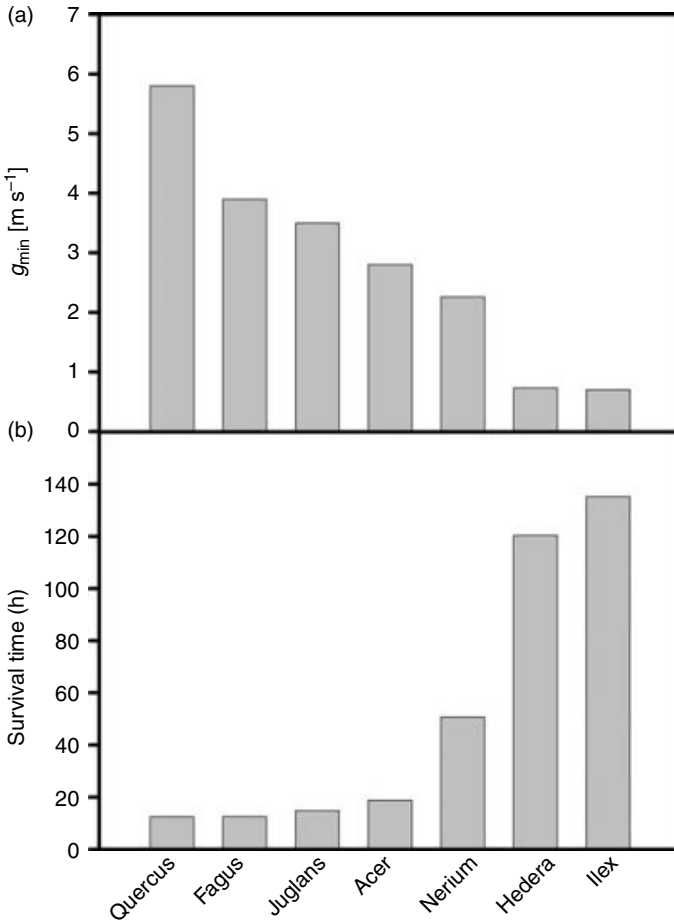


Figure 9.6 (a) Minimum conductance (g_{min}) and (b) survival time of deciduous leaves (*Quercus petraea*, *Fagus sylvatica*, *Juglans regia*, *Acer campestre*) and evergreen leaves (*Hedera helix*, *Ilex aquifolium*) growing in temperate climates and the xeromorphic leaf of *Nerium oleander* growing in mediterranean-type climates (data from Burghardt and Riederer, 2003, and unpublished results).

was described for calcium salts (Schönherr, 2000). This chapter presents evidence from diverse experimental approaches that the predominance of the latter pathway is probable. A further open question is how the contribution of the lipophilic and hydrophilic pathway to the overall water permeance of the cuticle varies among different plant species.

From a review of pooled data of plants growing in different climatic regions it was concluded that complete stomatal closure is rather an exception (Körner, 1994; Kerstiens, 1996a,b). Therefore, the role of the cuticular transpiration barrier as an adaptive strategy with regard to drought avoidance is restricted under

standard growing conditions except for astomatous fruits. The relative importance of the cuticular transpiration barrier increases when stomatal closure increases due to turgor loss under prolonged drought conditions. Therefore, leaf cuticular permeances, minimum conductances and lowest conductances should be compared in a coordinated experimental approach in order to assess under which environmental conditions complete stomatal closure is achieved and to what extent cuticular transpiration and residual stomatal transpiration contribute to the overall transpiration in times of water stress.

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10 The cuticle and cellular interactions

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10.1 Introduction

The epidermis of the aerial parts of plants plays an important role in water retention (Chapter 9), defence against pathogens (Chapters 11, 12 and 13) and gas exchange. The cuticle covering the epidermis is believed to be important for such epidermal functions and to protect the plant from the outer environment. In addition, genetic as well as biochemical studies have suggested that the cuticle is also essential for proper organ formation and fertilisation. In these processes, the cuticle appears to be involved, either positively or negatively, in communication between plant cells. In this chapter, advances in our understanding of the roles of the cuticle in such cellular communications and the genes required for proper cuticle generation are described.

10.2 Essential roles of the cuticle in post-embryonic development

The aerial parts of plants are mainly derived from the shoot meristem, which maintains itself and successively generates organ primordia. In the shoot meristem and the floral meristem, boundaries between primordia for lateral organs such as leaves and the floral organs are established (for a review see Fleming, 2005). Subsequently, despite contact between adjacent organ primordia during their growth, each primordium remains separate. In the past decade, identification and characterisation of mutants and responsible genes have shed light on the mechanisms underlying this prevention of lateral organ fusion during development.

10.2.1 *The cuticle is required for prevention of organ fusion*

To date, a number of mutants that produce fused organs have been described in *Arabidopsis thaliana* (Table 10.1), maize and other plants. In this section, the recent progress in identifying *A. thaliana* mutants and their phenotypic characterisation will be reviewed.

Lolle *et al.* (1992) reported the characterisation of the *fiddlehead* (*fdh*) mutant, which generates fused floral organs and leaves. Histological analysis indicated

Table 10.1 *Arabidopsis thaliana* mutants producing fused organs

Mutant name	Abbreviation	Locus id or chromosome	Organ fusion			References*
			Leaves	Flowers	Ovules	
<i>abnormal leaf-shape1</i>	<i>ale1</i>	At1g62340	Yes	No	No	12, 15
<i>acetyl-CoA carboxylase1/pasticinno3/gurke</i>	<i>accl/pas3/gk</i>	At1g36160	Yes	Yes	N.D.	1, 2, 4, 16
<i>airhead</i>	<i>ahd</i>	chr. 5	No	Yes	No	10
<i>arabidopsis homologue of Crinkly4</i>	<i>acr4</i>	At3g59420	No	No	Yes	3, 5, 13, 17
<i>bulkhead</i>	<i>bud</i>	chr. 1	No	Yes	Yes	10
<i>conehead</i>	<i>cod</i>	chr. 2	Yes	Yes	No	10
<i>deadhead</i>	<i>ded</i>	chr. 1	Yes	Yes	Yes	10
<i>eceriferum10</i>	<i>cer10</i>	chr. 3	No	Yes	No	10
<i>fiddlehead</i>	<i>fdh</i>	At2g26250	Yes	Yes	Yes	9, 11, 19
<i>hothead/adhesion of caryx edges</i>	<i>hth/ace</i>	At1g72970	No	Yes	Yes	7, 10
<i>lacerata</i>	<i>lcr</i>	At2g45970	Yes	Yes	N.D.	18
<i>permeable leaves1</i>	<i>pel1</i>	chr. 1	Yes	Yes	N.D.	14, 15
<i>permeable leaves2</i>	<i>pel2</i>	chr. 1	Yes	Yes	N.D.	14, 15
<i>permeable leaves3</i>	<i>pel3</i>	chr. 5	Yes	Yes	N.D.	14, 15
<i>pothead</i>	<i>phd</i>	chr. 5	No	Yes	No	10
<i>thunderhead</i>	<i>thd</i>	chr. 3	Yes	Yes	Yes	10
<i>wax1</i>	<i>wax1</i>	N.D.	Yes	Yes	N.D.	6
<i>wax2/yore-yore/pel6</i>	<i>wax2</i>	At5g57800	Yes	Yes	N.D.	6, 8, 14

Notes: N.D., not determined.

* 1. Baud *et al.* (2003), 2. Baud *et al.* (2004), 3. Cao *et al.* (2005), 4. Faure *et al.* (1998), 5. Gifford *et al.* (2003), 6. Jenks *et al.* (1996), 7. Krolkowski *et al.* (2003), 8. Kurata *et al.* (2003), 9. Lolle *et al.* (1992), 10. Lolle *et al.* (1998), 11. Pruitt *et al.* (2000), 12. Tanaka *et al.* (2001), 13. Tanaka *et al.* (2002), 14. Tanaka *et al.* (2004), 15. Tanaka *et al.* unpublished observations, 16. Torres-Ruiz *et al.* (1996), 17. Watanabe *et al.* (2004), 18. Wellesen *et al.* (2001), 19. Yephremov *et al.* (1999).

that the epidermal cell layers remained intact and that fusion occurred without cytoplasmic union (Lolle *et al.*, 1992). It was also reported that when leaves and inflorescences of wild-type and *fdh* plants are immersed in alcoholic solution, the rate of chlorophyll leaching is higher in *fdh* plants (Lolle *et al.*, 1997). Based on this observation, the authors speculate that the higher rate of chlorophyll release is due to altered properties of the cell wall and/or the cuticle.

Mutations in several other loci that cause phenotypes similar to that of *fdh* have since been identified by large-scale genetic screening (approximately 15 000 M₁ family) and phenotypic characterisation of *eceriferum* mutants (*ded*, *bud*, *hth*, *fdh*, *cod*, *cer10*, *thd*, *ahd*, *phd*; see Table 10.1, Lolle *et al.*, 1998). In all of these mutants, except *pothead* (*phd*), the rates of chlorophyll leaching are elevated. The correlation between chlorophyll permeability and organ fusion in these mutants is consistent with the hypothesis that changes in permeability play a role in the acquisition of fusion competence by epidermal cells (Lolle *et al.*, 1998). Because organ fusion takes place at the organ surface and because the cuticle might be involved in the restriction of chlorophyll leaching, it is possible that cuticular properties are altered in these mutants, although this notion remains to be tested.

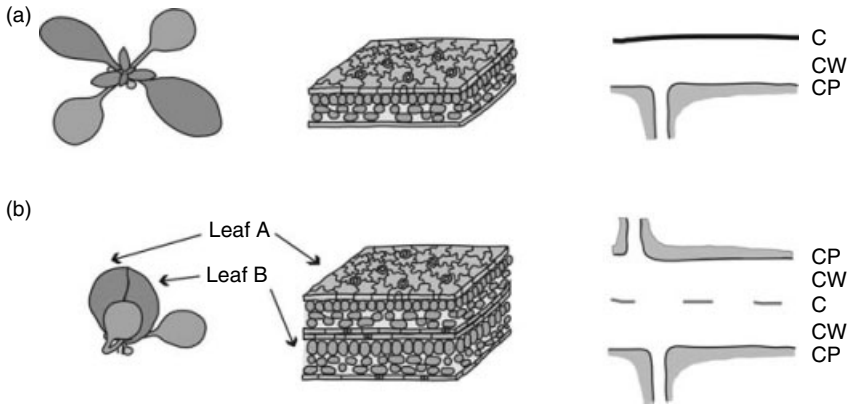


Figure 10.1 Schematic representation of fused leaves and typical cuticle structure. (a) Plant with normal cuticle in which leaf fusion does not occur. (b) Plant with defective cuticle and fused leaves. Schematic diagrams of gross morphology (left), leaf sections (middle) and ultrastructure of outer epidermal surface (right) are shown. Abbreviations: c, cuticle; cw, cell wall; cp, cytoplasm.

It is also noteworthy that the permeability of a water-soluble dye (toluidine blue, TB) across the leaf surface of *fdh* and *cer10* plants differs significantly from that of wild-type; leaves from both mutants allow incorporation of the dye whereas wild-type leaves do not (Tanaka *et al.*, 2004). In principle, this method (TB test) allows the detection of cuticular defects and thus cuticle permeability and organ fusion competence appear to correlate in these cases. In addition, transgenic *A. thaliana* plants expressing a fungal cutinase also exhibit higher rates of chlorophyll leaching and organ fusion (Sieber *et al.*, 2000), again corroborating the correlation between organ fusion and cuticle properties.

Correlation between defects in the leaf cuticle and fusion of organs is also evident in cutinase-expressing plants as well as in *lacerata* (*lcr*) and *abnormal leaf shape1* (*ale1*) mutants; in these transgenic plants and mutants, the electron-dense leaf cuticle is reduced and leaves fuse with one another (Figure 10.1; Sieber *et al.*, 2000; Wellesen *et al.*, 2001; Tanaka *et al.*, 2001). In the *wax2/yore-yore/pel6* mutant, the ultrastructure of the cuticular membrane in the stem is altered (Chen *et al.*, 2003), wax contents of aldehydes and alkanes are markedly reduced (Chen *et al.*, 2003; Kurata *et al.*, 2003) and fusion between leaves and between floral organs is observed (Chen *et al.*, 2003; Kurata *et al.*, 2003; Tanaka *et al.*, 2004). Taken together, these observations provide evidence that the cuticle plays an essential role in the prevention of organ fusion.

10.2.2 Genetic screening for mutants with defective cuticles

Several groups have attempted to systematically identify *A. thaliana* mutants possessing an altered cuticle using criteria such as organ glossiness and physiological

properties of the cuticle. Screening based on different criteria has resulted in the identification of partly overlapping but distinct classes of mutant loci.

The reduction in the epicuticular waxes of the stems of *A. thaliana* leads to a readily visible glossy appearance and 89 independently arising *eceriferum* mutants, which resulted from 21 loci (*CER1* through *CER20* and *TT5*), have been identified (Dellaert *et al.*, 1979; Koornneef *et al.*, 1989). With a few exceptions (e.g. *cer18*), these mutants differ from wild type in the amount and/or morphology of epicuticular waxes on inflorescence stems. Most of the *ECERIFERUM* (*CER*) mutations do not result in organ fusion, but some (*CER1*, *CER2*, *CER3*, *CER6*, *CER8* and *CER10*) affect fertility (Koornneef *et al.*, 1989; Preuss *et al.*, 1993). Interestingly, the *cer10* mutant produces fused floral organs (Lolle *et al.*, 1998).

Biochemical analysis of wax contents revealed that *cer* mutants are associated with altered or reduced wax constituents in stems and leaves (Hannoufa *et al.*, 1993; Jenks *et al.*, 1995). Three other loci (*CER22* through *CER24*) have been identified by genetic screening of 1,229 M2 individual plants derived from EMS-mutagenised *A. thaliana* plants based on non-visual screening by gas chromatography (Rashotte *et al.*, 2004). These *cer* mutants also have drastic morphological defects. It should be noted, however, that such wax reductions having no effect on plant morphology do not necessarily demonstrate that waxes are dispensable in preventing organ fusion and plant growth as none of the *cer* mutants completely lacks epicuticular waxes.

Jenks *et al.* (1996) performed a genetic screening of an *A. thaliana* population mutagenised by T-DNA insertions for mutants with increased leaf surface glaucousness or glossiness. Six glaucous mutants (designated *knb* and *bcf*) and two glossy mutants (designated *wax1* and *wax2*) were identified. Wild-type *A. thaliana* leaves lack epicuticular wax crystals, while *knb* and *bcf* mutants produce flake-like wax crystals on the leaf surface. The *wax1* and *wax2* mutants produce fused organs and glossy inflorescence stems with reduced wax crystals (Jenks *et al.*, 1996; Chen *et al.*, 2003). Further characterisation revealed that the *wax2* mutant is defective in both cuticular membrane structure and wax composition (Chen *et al.*, 2003). Thus, it has been proposed that *WAX2* might be involved in both cutin and wax production (Chen *et al.*, 2003).

Given that mutants producing fused organs include those with cuticle defects, it is reasonable to speculate that a properly functioning cuticle is required for prevention of organ fusion during the early development of leaves. Then, which constituents or properties of the cuticle are responsible for prevention of organ fusion? Tanaka *et al.* (2004) reported a simple method designated the TB test to detect leaves with altered cuticle properties, allowing a water-soluble molecule like TB to permeate across the defective cuticle. In principle, when leaves are dipped into TB solution, the solution is repelled if the leaf surface is covered with a hydrophobic barrier (e.g. wild-type leaf cuticle). However, if the cuticle is permeable to the solution, the dye, having affinity for the cell wall, stains the cell walls in the cuticle-defective regions. The cotyledons of the cuticle-defective mutant *ale1* were intensely stained with TB (Tanaka *et al.*, 2004), while in some *cer* mutants (Table 10.2),

Table 10.2 *Arabidopsis thaliana* mutants defective in the generation of a functional cuticle

Mutant name	Abbreviation	Locus id or chromosome	Permeability of leaf cuticle or cell wall	Hydration of wild-type pollen on leaves	Male sterility	Glossy appearance	Gene product	References
<i>abnormal leaf-shape1</i>	<i>ale1</i>	At1g62340	Increased a	N.D.	Fertile	No	Similar to subtilisin-like serine proteases	25, 28
<i>abnormal leaf-shape2</i>	<i>ale2</i>	chr. 2	Increased a	N.D.	Fertile	No	?	28
<i>acetyl-CoA carboxylase1/pasticum3/gurke</i>	<i>acc1/pas3/gk</i>	At1g36160	N.D.	N.D.	N.D.	N.D.	Acetyl-CoA carboxylase	2, 3, 5, 29
<i>airhead</i>	<i>ahd</i>	chr. 5	Increased b	Yes	Fertile	No	?	17
<i>arabidopsis homologue of Crinkly4</i>	<i>acr4</i>	At3g59420	Increased a	N.D.	Fertile	No	Receptor-like protein kinase	7, 26, 30
<i>bulkhead</i>	<i>bud</i>	chr. 1	Increased b	No	Fertile	No	?	17
<i>conehead</i>	<i>cod</i>	chr. 2	Increased b	Yes	Fertile	No	?	17
<i>deathhead</i>	<i>ded</i>	chr. 1	Increased b	Yes	Reduced	Yes	?	17
<i>eceriferum1</i>	<i>cer1</i>	At1g02205	Not significantly increased a	N.D.	Sterile d, e	Yes	Similar to the sterol desaturase family proteins	1, 9, 12, 27
<i>eceriferum2</i>	<i>cer2</i>	At4g24510	Not significantly increased a	N.D.	Sterile d, e	Yes	A novel protein	1, 19, 21, 27, 32
<i>eceriferum3</i>	<i>cer3</i>	At5g02310	Not significantly increased a	N.D.	Sterile d, e	Yes	Related to alpha-type E3 ubiquitin-protein ligase	8, 9, 11, 12, 27
<i>eceriferum5</i>	<i>cer5</i>	At1g51500	Increased a	N.D.	Fertile	Yes	Similar to the ABC transporters	12, 20, 27
<i>eceriferum6/pop1</i>	<i>cer6/pop1</i>	At1g68530	Not significantly increased a	N.D.	Sterile d, e	Yes	Similar to KCS and FATTY ACID ELONGASE	6, 12, 18, 21
<i>eceriferum10</i>	<i>cer10</i>	chr. 3	Increased a, b	Yes	Reduced	Yes	?	12, 17, 23, 27
<i>eceriferum12</i>	<i>cer12</i>	chr. 4	Increased a	N.D.	Fertile	Yes	?	12, 23, 27
<i>eceriferum14</i>	<i>cer14</i>	chr. 2	Increased a	N.D.	Fertile	Yes	?	12, 23, 27
<i>eceriferum19</i>	<i>cer19</i>	chr. 1	Increased a	N.D.	Fertile	Yes	?	12, 23, 27
<i>fiddlehead</i>	<i>feh</i>	At2g26250	Increased a, b	Yes	Fertile	No	Similar to KCS and FATTY ACID ELONGASE1	15, 16, 22, 27, 33
<i>hothead/adhesion of caryx edges</i>	<i>thlace</i>	At1g72970	Increased b	Yes	Fertile	No	Similar to a group of FAD-containing oxidoreductases	13, 17

<i>lacerata</i>	<i>lcr</i>	At2g45970	N.D.	Yes	N.D.	N.D.	Fatty acid ω -hydroxylase (CYP86A8)	31
<i>lacs2</i>	<i>lacs2</i>	At1g49430	Increased b	Yes	Fertile	No	Long-chain acyl-CoA synthetase	24
<i>peameable leaves1</i>	<i>pel1</i>	chr. 1	Increased a	N.D.	N.D.	Yes	?	27, 28
<i>peameable leaves2</i>	<i>pel2</i>	chr. 1	Increased a	N.D.	N.D.	Yes	?	27, 28
<i>peameable leaves3</i>	<i>pel3</i>	chr. 5	Increased a	N.D.	N.D.	Yes	?	27, 28
<i>permeable leaves4</i>	<i>pel4</i>	chr. 2	Increased a	N.D.	Fertile	No	?	27
<i>permeable leaves5</i>	<i>pel5</i>	chr. 1	Increased a	N.D.	Fertile	No	?	27
<i>pothead</i>	<i>phd</i>	chr. 5	No b	No	Fertile	No	?	17
<i>thunderhead</i>	<i>thd</i>	chr. 3	Increased b	Yes	Fertile	No	?	17
<i>wax1</i>	<i>wax1</i>	N.D.	N.D.	N.D.	Sterile	Yes	?	10
<i>wax2/yore-yore/pel6</i>	<i>wax2</i>	At5g57800	Increased a, b, c	N.D.	Sterile e	Yes	Similarity to the sterol desaturase family and the short-chain dehydrogenase/ reductase family, homologous to Arabidopsis CER1 and maize GL1	4, 10, 14, 27

Notes: Mutants that produce fused organs are indicated in bold letters. N.D., not determined; a, permeability of TB across leaf cuticle that was examined by the TB test; b, rate of chlorophyll leaching from leaves in alcoholic solution; c, rate of water loss; d, defective in pollen hydration; e, the male sterility can be overcome in high humidity.

1. Aarts *et al.* (1995), 2. Baud *et al.* (2003), 3. Baud *et al.* (2004), 4. Chen *et al.* (2003), 5. Faure *et al.* (1998), 6. Fiebig *et al.* (2000), 7. Gifford *et al.* (2003), 8. Hammoufa *et al.* (1996), 9. Hülskamp *et al.* (1995), 10. Jenks *et al.* (1996), 11. Jenks *et al.* (2002), 12. Koormeef *et al.* (1989), 13. Krolkowski *et al.* (2003), 14. Kurata *et al.* (2003), 15. Lolle *et al.* (1992), 16. Lolle *et al.* (1997), 17. Lolle *et al.* (1998), 18. Millar *et al.* (1999), 19. Negruk *et al.* (1996), 20. Pighin *et al.* (2004), 21. Preuss *et al.* (1993), 22. Pruitt *et al.* (2000), 23. Rashotte *et al.* (2004), 24. Schnurr *et al.* (2004), 25. Tanaka *et al.* (2001), 26. Tanaka *et al.* (2002), 27. Tanaka *et al.* (2004), 28. Tanaka *et al.* unpublished observations, 29. Torres-Ruiz *et al.* (1996), 30. Watanabe *et al.* (2004), 31. Wellesen *et al.* (2001), 32. Xia *et al.* (1996), 33. Yephremov *et al.* (1999).

the leaves were stained weakly or in specific regions (e.g. the petiole). Using the TB test as an assay for defective cuticles, a genetic screening of EMS-mutagenised *A. thaliana* population (descended from 3305 M2 plants) for mutants was performed and a total of 19 mutants exhibiting alterations in leaf surface properties were identified.

The results of genetic mapping, examination of allelism and phenotypic classification revealed that these mutations represent at least seven loci [*FDH*, *PERMEABLE LEAVES (PEL1 through PEL6)*]. The *pel6* mutant contained a mutation in the *WAX2/YORE-YORE (YRE)* gene, suggesting that *PEL6* is a new mutant allele of *WAX2/YRE*. The genes responsible for the *PEL1* through *PEL5* mutations have not been cloned to date. Among the mutants identified, *pel1*, *pel2*, *pel3* and *fdh*, which are more intensely stained than *pel4* and *pel5*, produced fused leaves. The *PEL4* and *PEL5* mutations do not affect gross morphology of the plants and appear to be distinct from typical *cer* mutants; the glaucous appearance of inflorescence stems in *pel4* and *pel5* is indistinguishable from that of wild type. The *pel6* mutant exhibits predominant staining of the trichomes, which also fuse to one another (Tanaka *et al.*, 2004). The correlation between the intensity and/or pattern of staining and fusion events implies that the permeability of the cuticle is a critical factor that can determine the competency for organ fusion. Further phenotypic analysis of these mutants and identification of responsible genes as well as understanding of the biochemical roles of the gene products will deepen our understanding of the molecular basis for generation of cuticle as a permeability barrier.

10.2.3 Molecular identification of genes involved in the generation of a functional cuticle

Genes responsible for the mutations described earlier have been identified (Table 10.2). In this section, the structural features and predicted functions of encoded proteins, particularly those apparently involved in the biosynthesis or export of cuticular constituents, are described.

The *FIDDLEHEAD (FDH)* gene was cloned using the transposon-tagging procedure (Yephremov *et al.*, 1999; Pruitt *et al.*, 2000). The predicted FDH protein sequence exhibited significant sequence similarity to various condensing enzymes and most closely resembles those encoded by the *FATTY ACID ELONGASE (FAE)* family, such as β -keto-acyl-CoA synthase (KCS) from *Simmondsia chinensis* (jojoba) (Lassner *et al.*, 1996), and *FAE1*, *KCS1* and *CER6/CUT1* from *A. thaliana* (James *et al.*, 1995; Todd *et al.*, 1999; Millar *et al.*, 1999; Fiebig *et al.*, 2000). There is increasing evidence suggesting that these factors are involved in the generation of very long-chain fatty acids (VLCFA; carbon-chain length > 18). VLCFA are formed by a microsomal fatty acid elongation (FAE) system (for review see Somerville *et al.*, 2000; Jenks *et al.*, 2002). Each elongation reaction involves the addition of a two-carbon unit from malonyl-coenzyme A (CoA) to an acyl primer (e.g. fatty acyl-CoA in various chain length), followed by reduction, dehydration and a final reduction.

Mutations in the *A. thaliana* *FAE1* locus result in reduced VLCFA levels in seeds (James and Dooner, 1990; Lemierx *et al.*, 1990; James *et al.*, 1995), suggesting that *FAE1* is involved in *in vivo*. Overexpression of *FAE1* in tissues of *A. thaliana* and tobacco results in the accumulation of derivatives of VLCFA, as if *FAE1* is the rate-limiting enzyme that catalyzes lipid chain elongation *in vivo* (Millar and Kunst, 1997). Using the *Brassica napus* low erucic (docos-13,14-enoic) acid rapeseed (LEAR) cultivar, which is defective in the FAE system, Lassner *et al.* (1996) demonstrated that the expression of jojoba *KCS* cDNA in the LEAR plants restored *KCS* activity, which is involved in the first step of the FAE system. *CER6/CUT1* is required for the elongation of fatty-acid derivatives (Hannoufa *et al.*, 1993; Preuss *et al.*, 1993; Millar *et al.*, 1999; Fiebig *et al.*, 2000). Thus, the strong similarity in the amino acid sequences of *FDH*, *KCS* and *FAE1* suggest that *FDH* may also be involved in the lipid metabolism required for the generation of a proper cuticle. In addition, consistent with the notion that *FDH* is involved in metabolism of cuticle-related lipids, *FDH* mRNA is detected in epidermis-related tissues such as the L1 layer of inflorescences (Yephremov *et al.*, 1999).

The *LACERATA* (*LCR*) gene encodes a cytochrome P450 family protein (*CYP86A8*) (Wellesen *et al.*, 2001). This raises the possibility that *LCR* acts as an oxygenase, which is involved in the generation of a functional cuticle. Wellesen *et al.* (2001) further demonstrated that microsomes purified from yeast expressing *LCR* displayed significant activity in the ω -hydroxylation of fatty acids. Among several substrates examined, the most effective were hexadecanoate (C16:0) and octa-9,10-decenoate (C18:1). Considering that the chief monomers of cutin are 10,16-dihydroxyhexadecanoic acid, 18-hydroxy-9,10-epoxyoctadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid, fatty acid hydroxylation is likely required for the biosynthesis of cutin monomers (Kolattukudy, 1980, 2001), and thus it is plausible that *LCR* is directly involved in cutin biosynthesis.

The *WAX2/YORE-YORE* (*WAX2/YRE*) gene encodes a predicted protein of 632 amino acids. The sequence of this protein exhibits the highest sequence similarity to putative proteins encoded by *Senecio odora* cDNA *EPI23* (Hansen *et al.*, 1997) and the rice *GLOSSY1* homologue (Hansen *et al.*, 1997) and it is also related to *A. thaliana* *CER1* (Aarts *et al.*, 1995) and maize *glossy1* (Hansen *et al.*, 1997), which is required for wax generation. The *WAX2* protein sequence is related to the sterol desaturase family at the amino terminal region and possesses the three His-rich motifs that are conserved in this family. The sequence near the carboxy terminus of this protein also exhibits similarity to the signature sequence found in the short-chain dehydrogenase/reductase (*SDR*) family. Such similarities are not found in *GLOSSY1* or *CER1*. Based on the similarity with metabolic proteins, *WAX2/YRE* is believed to be involved in a certain metabolic process for the modification of fatty-acid derivatives.

Phenotypic analysis has suggested that the *wax2/yre* mutant is defective in generation of aldehydes and alkanes as well as in cutin formation. Because the two domains are each related to different metabolic enzymes, an interesting hypothesis is that *WAX2/YRE* is a fusion of two functional proteins and this might explain

the defects of the *wax2* mutant in both the generation of the cuticular membrane and cuticular wax. Consistent with the predicted role of WAX2/YRE in cuticle generation, the WAX1/YRE gene is specifically expressed in the protoderm and the epidermis (Kurata *et al.*, 2003). Kurata *et al.* (2003) reported a synergistic effect of CER1 and YRE mutations on the morphology of trichomes, suggesting partially overlapping functions of CER1 and WAX2/YRE.

The major constituents of the cuticle, cutin and waxes, are derived from fatty acids (carbon-chain length 16 and 18) generated in chloroplasts. Elongation of fatty acyl-CoA catalyzed by the FAE system requires malonyl-CoA as a carbon source (for reviews, see Somerville *et al.*, 2000; Jenks *et al.*, 2002). Acetyl-CoA carboxylase is known to catalyze the generation of malonyl-CoA using acetyl CoA as a substrate (Somerville *et al.*, 2000). Recently, *gurke/pasticino3* (*gk/pas3*) mutants of *A. thaliana* were found to have mutations in the ACETYL-COA CARBOXYLASE1 (*ACCI*) gene (Baud *et al.*, 2004). Whereas strong *GK/PAS3/ACCI* mutations cause a specific defect in the embryonic pattern such that cotyledons and shoots fail to develop (Baud *et al.*, 2003), weak *GK/PAS3* mutations have been reported to cause the fusion of lateral organs (Torres-Ruiz *et al.*, 1996; Faure *et al.*, 1998). Thus, it is possible that ACC1 is involved in generating the malonyl-CoA pool, which is used for biosynthesis of cuticle-related lipids such as waxes, although it remains unclear whether the *gk/pas3/acc1* mutant is actually defective in cuticle properties.

The plant cuticle is composed of derivatives of fatty acids, which are generated *de novo* in chloroplasts. The final products of fatty acid synthesis in the chloroplasts are 16:0-ACP, 18:0-ACP and 18:1-ACP. Before export from chloroplasts, they are cleaved from ACP by thioesterases. Subsequently, long-chain acyl-CoA synthetases (LACSs) catalyze the conversion of free fatty acids to fatty acyl-CoAs, which are used for general membrane lipid synthesis as well as wax biogenesis (Chapter 5). *A. thaliana* contains nine LACS isozymes (Shockey *et al.*, 2002). Schnurr *et al.* (2004) demonstrated that one of these nine isozymes, LACS2, is involved in cuticle formation. First, using the LACS2 promoter-GUS reporter gene, LACS2 expression is specifically detected in the epidermis. Second, *in vitro* assays show that 16-hydroxyhexadecanoate is an excellent substrate for LACS2. Third, the *lacs2* mutant exhibits defects in epidermal surface functions, such that the rate of chlorophyll leaching from leaves in alcoholic solution increases and mutant leaves, particularly on abaxial surfaces, support pollen germination. In addition, ultrastructural analysis, visual inspection of inflorescence stems and investigation of wax load and composition have suggested that the cutin layer in the abaxial surface of leaves of *lacs2* mutants is thinner than that in the wild type, but the LACS2 mutation does not substantially affect wax generation (Schnurr *et al.*, 2004). The *lacs2* phenotype is similar to that of *lcr* mutants and transgenic plants expressing a fungal cutinase. The latter appear to have a defect in the cutin layer, although the phenotypes of *lacs2* are distinct from those of LCR and cutinase-expressing plants, which also generate fused organs. The reasons for these phenotypic differences in terms of organ fusion are unclear; they may be the result of a relatively

weak effect of the *LACS2* mutation on cutin formation, as it seems that cutin is still sufficiently formed in the *lacs2* mutant, particularly on the adaxial leaf surface.

The *HOTHEAD* (*HTH*) gene was identified based on its chromosomal location and a candidate approach. It is predicted to encode a protein with 594 amino acids having similarity to the flavoprotein form of α -hydroxynitrile lyase and other oxidoreductases (Krolikowski *et al.*, 2003). Considering that the *hth* mutant exhibits the organ fusion phenotype (Lolle *et al.*, 1998; Krolikowski *et al.*, 2003), the *HTH* gene might be directly or indirectly involved in the generation of a functional cuticle. Further biochemical characterization of the cuticle in the *hth* mutant would shed light on the role of HTH. The *A. thaliana* genome (The Arabidopsis Genome Initiative, 2000) contains seven other *HTH*-related genes, but the functions of these genes remain to be elucidated.

Several *eceriferum* (*cer*) mutants have been shown to affect the properties of cuticle as indicated by the TB test (Tanaka *et al.*, 2004; Table 10.2); most plants homozygous for *CER5*, *CER10*, *CER12*, *CER14* and *CER19* exhibited leaf staining with an aqueous solution of TB. This suggests that permeability of water-soluble molecules through the cuticle is increased in these mutants. The gene responsible for the *CER5* mutation has been cloned (Pighin *et al.*, 2004) and detailed phenotypic analysis involving TEM and cryo-SEM of the *cer5* revealed an unusual inclusion of cytoplasm in the vacuole. Furthermore, unusual sheet-like inclusions appear to be accumulated in this cytoplasm in epidermal cells in the stem. Nile red staining indicated that these inclusions are lipidic and that the cuticular wax load is significantly reduced in the *cer5* mutant, while total epidermal wax (surface plus intracellular) of wild-type and *cer5* tissues do not differ significantly. Thus, *cer5* appears to be defective in the export of lipids related to wax, not in wax biogenesis (Pighin *et al.*, 2004).

The *CER5* gene has been isolated by a map-based approach combined with analysis of insertion mutants. The predicted *CER5* protein contains domains characteristic of ABC transporters, including Walker A and B boxes, the C motif for nucleotide binding and six transmembrane domains. Thus, *CER5* is predicted to be a so-called half-transporter and presumably dimerisation is required for its function. An attractive hypothesis is that *CER5* is directly involved in the transport of wax-related lipids (see Chapter 5). Consistent with this notion, the *CER5* gene is expressed in epidermal cells and the GFP-*CER5* fusion protein is localised in the cell surface, presumably in the plasma membrane (Pighin *et al.*, 2004). *CER5* transcripts have been found in all organs examined, including inflorescence stems, leaves and roots. Incomplete loss of cuticular wax in the stems and leaves in the *cer5* mutant suggest the presence of factors with overlapping function.

The *A. thaliana* genome contains 129 genes encoding putative ABC transporters (Sánchez-Fernández *et al.*, 2001). It would be of interest to test whether other ABC transporters, particularly those with significant sequence similarity to *CER5* (e.g. At3g21090 and At1g51460), have functions overlapping with those of *CER5* *in vivo*.

10.3 Functions of the cuticle in plant reproduction and embryogenesis

10.3.1 *The cuticle as an interface in stigma–pollen interactions*

Successful fertilisation involves pollen adhesion onto the stigma surface, hydration, pollen germination, pollen tube penetration of the stigma surface and pollen tube elongation towards the micropyle of ovule. In plants with a ‘dry’ stigma (Heslop-Harrison, 1977), stigmatic surfaces are covered with a cuticle and, in such plants, it appears that the pollen breaches the cuticle to proceed into stigmatic cell walls (Dickinson and Lewis, 1973; Elleman *et al.*, 1992). On the basis of ultrastructural observation, the electron-dense cuticle disappears prior to penetration of the pollen tube into the stigmatic cell wall, at least in plants including *Raphanus* and *A. thaliana* (Dickinson and Lewis, 1973; Elleman *et al.*, 1992). These observations support the idea that the cuticle might be enzymatically degraded prior to penetration (Figure 10.2), and several physiological experiments reinforce this notion.

Heslop-Harrison and Heslop-Harrison (1975) treated stigmata of *Agrostemma githago* with enzymatic solutions and examined their effect on pollen tube germination and entry. After treatment of stigma surfaces with pronase and subsequent pollination, pollen grains germinated but failed to penetrate implying that the stigmatic cell surface includes a protein that possesses cutinase activity or that may be involved in cutinase activation. Hiscock *et al.* (1994) reported that the pollen of *B. napus* contains an active cutinase. The protein sequence of this cutinase has not been determined to date; however, using an antibody raised against fungal cutinase, they also examined the localisation pattern of the cross-reactive substance in pollen. In their immunocytological experiments, signals were obtained at the site where the pollen tube emerges (Hiscock *et al.*, 1994). This is consistent with the hypothesis that cutinase is localised at the tip of the pollen tube and that it may be involved in penetration of the stigmatic cuticle, although direct evidence is lacking.

Apart from the location of the hypothetical cutinase, both the stigmata and pollen of *B. napus* may possess cutinase, as shown by a biochemical assay using the synthetic substrate *p*-nitrophenyl butyrate (PNB; Hiscock *et al.*, 2002). In addition, such esterase activities were blocked by inhibitors of fungal cutinase, such as diisopropyl fluorophosphates (DIPF) and ebelactone B (Hiscock *et al.*, 2002). Using such inhibitors, a physiological experiment was performed in order to investigate the causal relationship between inhibition of esterase (most likely cutinase) and pollen penetration through the stigmatic surface. Treatment of the *B. napus* stigma with either DIPF or ebelactone B does not seriously impair pollen germination but inhibits penetration by the pollen tube of the dry cuticle-covered *Brassica* stigma (Hiscock *et al.*, 2002). As a result, Hiscock *et al.* (2002) proposed that cutinase is required for the penetration of the stigmatic cuticle by the pollen tube in *B. napus*. Thus, the results of ultrastructural analysis and physiological experiments suggest that the cuticle covering dry stigmatic surfaces functions to prevent pollen tube penetration. However, it is unclear whether degradation of stigmatic cuticle is sufficient for pollen tube penetration.

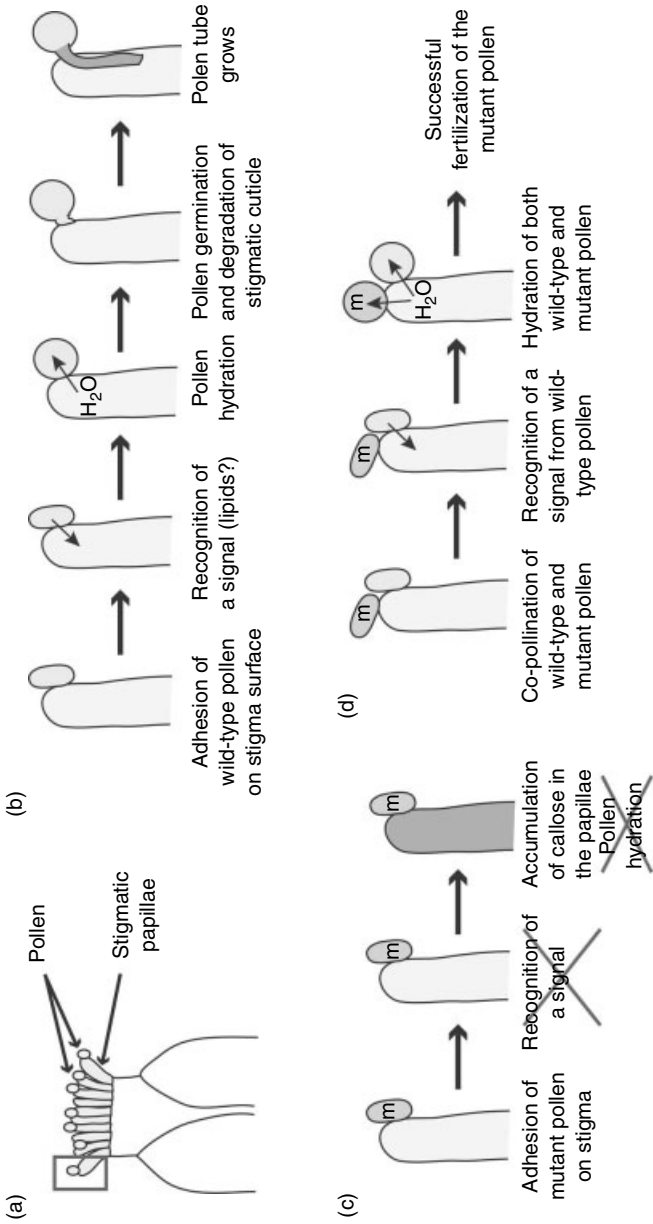


Figure 10.2 Schematic diagram of successive events in compatible pollination. (a) Diagram of stigma of *Arabidopsis thaliana*. (b) Sequential events following compatible pollination. (c) Events following pollination by mutant pollen defective in pollen–stigma recognition. (d) Restoration of mutant. Abbreviation: m, pollen fertility by co-pollination with wild-type pollen. This figure is produced in colour in the colour plate section, which follows pages 249.

The predicted role of the stigmatic cuticle described above is 'protective'; it appears as though the cuticle protects the stigma from invasion by pollen. Molecular genetic studies, however, suggest that cuticle-related materials are actively involved in the recognition of compatible pollen by the stigma. In Brassicaceae, compatible pollination results in successful penetration of pollen through the cuticle and in the subsequent progression and fertilisation. In contrast, incompatible pollen is able to germinate and the stigmatic cuticle disappears upon pollen attachment, but stigmatic papillae cells accumulate callose and the pollen fails to progress (Dickinson and Lewis, 1973).

Preuss *et al.* (1993) isolated a male sterile *A. thaliana* mutant defective in pollen–pistil interactions (*pop1*, also known as *cer6-2*). Pollen generated by *pop1* homozygous plants is able to attach to stigmatic papilla cells but fails to hydrate and germinate on the stigmatic surface. Similarly to incompatible pollination in *Brassica*, pollination with *pop1* pollen induces accumulation of callose in stigmatic cells. Interestingly, *pop1* pollen, when co-pollinated with wild-type pollen, is able to germinate and successfully complete fertilisation (Preuss *et al.*, 1993; Figure 10.2). The reason for this is that in compatible pollination (as in wild-type *A. thaliana*), stigmatic cells perceive a signal from compatible pollen and alter their properties to support pollen hydration. In this scenario, *pop1* pollen is defective in the generation of such a signal. Although the molecular nature of this signal is unknown, it is reasonable to speculate that cuticle-related lipids are involved in such a signalling event (Figure 10.2), as in addition to a defect in fertility, the *pop1* mutant also exhibits a striking defect in the production of long-chain lipids in inflorescence stems and pollen (Preuss *et al.*, 1993).

The *POP1* mutation is allelic to *CER6* and the *CER6/POP1* gene was found to be identical to the *CUT1* gene, which encodes a putative condensing enzyme (Millar *et al.*, 1999; Fiebig *et al.*, 2000). Thus, the *CER6/POP1/CUT1* protein appears to be directly involved in the production of VLCFA, corroborating a causal relationship between loss of VLCFA and the *pop1* phenotype. Analysis of intragenic suppressors of *cer6* mutants has demonstrated that low amounts of VLCFA are sufficient for pollen hydration and germination (Fiebig *et al.*, 2000). The *cer2* mutant which has been shown to have a defect in the generation of long-chain lipids also exhibits defects in pollen hydration (Preuss *et al.*, 1993).

Hülkamp *et al.* (1995) performed genetic screening of an EMS-mutagenised *A. thaliana* population (15 000 M₂ plants) for male-sterile mutants defective in pollen–stigma recognition and identified five independently arising mutants. All five mutants were associated with a bright green stem phenotype, which is typical of *eceriferum* mutants. Based on the results of allelism tests and genetic mapping, these mutants were found to be allelic to *CER1* (three alleles), *CER3* (one allele) and *CER6* (one allele) (Hülkamp *et al.*, 1995). Pollen from these mutants fails to hydrate when placed on the stigma and these defects were restored by co-pollination with wild-type pollen (Hülkamp *et al.*, 1995). The hydration of mutant pollen was facilitated even when wild-type pollen was treated with formaldehyde to prevent germination. Taken together, these results suggest that *CER1*, *CER2*, *CER3* and

CER6 genes in the male parent appear to be involved in pollen recognition by the stigma, and the subsequent transfer of water from the stigma to pollen (Figure 10.2; Table 10.2).

It has been shown that *cer1*, *cer2*, *cer3* and *cer6* are defective in various classes of long-chain lipids (Hannoufa *et al.*, 1993; Preuss *et al.*, 1993; Jenks *et al.*, 1995). Nonacosane (C29) is commonly lacking in these mutants, which is consistent with the notion that this compound and/or compounds related to it are required for pollen–stigma recognition. Another class of lipids – triacylglycerides – is implicated in pollen hydration, as application of trilinolein (a *cis*-unsaturated triacylglyceride) enables pollen grains obtained from *cer1*, *cer3* and *cer6* to produce tubes that penetrate the stigma (Wolters-Arts *et al.*, 1998).

A. thaliana mutants with defective leaf cuticle provide opportunities for testing the causal relationship between the cuticular barrier and the capacity for pollen hydration on the leaf surface. Pollen does not germinate when adhering to the surface of wild-type *A. thaliana* leaves. Interestingly, Lolle and Cheung (1993) reported that pollen attached to leaves of the *fiddlehead* mutant is capable of hydration. In addition to *fdh*, various mutant plants have been reported to allow adhered pollen to hydrate and germinate (reviewed in Lolle and Pruitt, 1999; Wellesen *et al.*, 2001; Chen *et al.*, 2003; Schnurr *et al.*, 2004; Table 10.2). The genes responsible for these mutations include those encoding for a putative KCS (fatty acid elongase), fatty acid ω -hydroxylase and LACS, which appear to be directly involved in the biosynthesis of the cuticle (Table 10.2). Therefore, the cuticle seems to function as a barrier to pollen hydration in leaves. The *lcr* and *lacs2* mutants are primarily defective in cutin biogenesis, suggesting that the cutin layer is essential for preventing pollen hydration on the leaf surface. The cuticle components or structures required for restricting pollen hydration remain elusive in other mutants.

10.3.2 Role of the cuticle during embryogenesis

During embryogenesis, the cuticle is generated to surround developing embryos. A cuticle can be detected in plant embryogenesis as early as the zygote stage in the case of *Citrus jambhiri* (Bruck and Walker, 1985). In other plants, such as maize and *A. thaliana*, electron-dense cuticles, as detected by transmission electron microscopy, apparently are generated on the surface of globular-stage embryos prior to the generation of organ primordia. Why is the cuticle generated so early in embryogenesis? One clue comes from observations of an *A. thaliana* mutant defective in cuticle formation during embryogenesis. The *abnormal leaf shape1* (*ale1*) mutant exhibits a defect in the generation of a continuous cuticle during embryogenesis and in seedlings. In addition to the cuticular defects, embryos of the *ale1* mutant adhere to the endosperm cells that surround the developing embryos (Tanaka *et al.*, 2001). In addition, it has been reported that mutation in *A. thaliana* *ACC1* gene also causes adhesion between embryos and endosperm (Baud *et al.*, 2004). Because *ACC1* is probably involved in the generation of cytosolic malonyl-CoA, which is required for the biosynthesis of VLCFA, it is possible that the adhesion

between embryos and endosperm is caused by a defect in cuticle formation (Baud *et al.*, 2004).

10.4 Regulators of epidermal differentiation and cuticle formation

10.4.1 Intercellular signalling and cuticle formation

The *abnormal leaf-shape1* (*ale1*) mutant is characterised by the partial loss of an electron-dense cuticle on embryos and juvenile plants, increased permeability of TB solution across the leaf surface and sensitivity of seedlings to low humidity. The *ALE1* gene encodes a protein related to subtilisin-like serine proteases (Tanaka *et al.*, 2001). The sequence of the predicted *ALE1* protein includes a signal sequence, which may direct the protein into a secretory pathway. Transcripts from the *ALE1* gene are predominantly accumulated within endosperm cells surrounding developing embryos. This raises the possibility that, during embryogenesis, cuticle formation requires communication between the protoderm and extra-embryonic tissue, and that *ALE1* might be involved in such a biological process. A number of subtilisin-like proteases in yeast and animals have been reported to catalyze the cleavage of various signalling molecules and this cleavage, in combination with other modifications, results in either activation (e.g. a-factor, insulin) or inactivation (e.g. neurotransmitter) of the signalling molecules. It would be of interest to determine whether *ALE1* functions in a similar manner.

Maize *crinkly4* (*cr4*) mutants exhibit various abnormalities in the leaf epidermis such that epidermal cells are deformed and leaves fuse together (Becraft *et al.*, 1996). In the *cr4* mutant, the electron-dense cuticle is reduced (Jin *et al.*, 2000). The *CR4* gene encodes a receptor-like threonine/threonine kinase with a putative extracellular domain containing novel repeats of 39 amino acids and a cysteine-rich region similar to that of the tumornecrosis factor receptor (TNFR) of mammals. Based on these results, *CR4* is believed to transmit an extracellular signal into the cytosol and such signal transduction may promote differentiation of epidermal cells. Later, it has been demonstrated that a role in epidermal differentiation for the *CR4*-related receptor-like protein kinases is conserved between maize and *A. thaliana* (Gifford *et al.*, 2003; Watanabe *et al.* 2004).

An *A. thaliana* homologue of *CRINKLY4* (*ACR4*) has been identified by degenerate PCR on the basis of the similarity to the maize *CRINKLY4* gene (Tanaka *et al.*, 2002). The *A. thaliana* genome contains five genes encoding proteins with putative *CR4*-related extracellular domains. Among these, only *ACR4* contains all of the domains found in *CR4* (Tanaka *et al.*, 2002; Cao *et al.*, 2005). *ACR4* is preferentially expressed in epidermis-related tissues (Tanaka *et al.*, 2002; Gifford *et al.*, 2003) and *acr4* mutants exhibit defects in the organization of cell layers in ovule integuments and the endothelium (Gifford *et al.* 2003; Watanabe *et al.*, 2004) and in the generation of an electron-dense cuticle in the ovule (Watanabe *et al.*, 2004). Furthermore, cuticle permeability in the ovule as well as in the leaves of *acr4* is altered, as demonstrated by TB test.

Epidermal defects in the leaves of *acr4* are frequently observed in leaf petioles and the abaxial surfaces of leaf lamina as well as in unusual protrusions of epidermal cells on leaves (Watanabe *et al.*, 2004). However, the *ACR4* mutation has little or no effect on overall leaf morphology. The epidermal defects of the *ALE1* mutant, originally identified in the Landsberg *erecta* (*Ler*) ecotype, are restored when introduced into the Wassilewskaja (*Ws*) ecotype by genetic crossing (Watanabe *et al.*, 2004). The *acr4 ale1* double mutant generated using single mutants crossed into the *Ws* background exhibited severe epidermal defects in the cotyledons and leaves with eventual deformation of overall shoot morphology (Watanabe *et al.*, 2004). These results demonstrate that *ALE1* and *ACR4* are collectively essential for proper cuticle formation as well as organ development in *A. thaliana* (Watanabe *et al.*, 2004).

10.4.2 Transcriptional control of cuticle production

A number of genes involved in the biosynthesis or secretion of cuticle-related substances have been identified, as described earlier. Consistent with previous observations that biochemical activities related to cuticle biosynthesis are detected within the epidermis (Croteau and Kolattukudy, 1974; Evenson and Post-Beittenmiller, 1995), a number of these genes are reported to be predominantly expressed in epidermal and related tissues, such as the meristem L1 layer. It seems likely that differentiation of the epidermis and appropriate gene expression in epidermal cells is a prerequisite for cuticle generation. Two distinct classes of transcription factors that are probably involved in the expression of genes for cuticle biosynthesis have recently been described (Abe *et al.*, 2003; Aharoni *et al.*, 2004; Broun *et al.*, 2004).

The *PROTODERMAL FACTOR1* (*PDF1*) gene of *A. thaliana* encodes for a putative extracellular proline-rich protein that is exclusively expressed in the L1 layer of shoot apices and the protoderm of organ primordia (Abe *et al.*, 1999). A 1.5-kb region upstream of the *PDF1* gene has been shown to direct L1-layer-specific expression of the β -glucuronidase (*GUS*) reporter gene. By analysis of progressive deletions of the *PDF1* gene promoter, Abe *et al.* (2001) identified an 8-bp *cis*-regulatory element [designated L1 box, 5'-TAAATG(C/T)A-3'] that is conserved in various L1-specific genes (e.g. *FDH*: Yephremov *et al.*, 1999; *SCR*; Wysocka-Diller *et al.*, 2000; *ATLTP1*; Thoma *et al.*, 1994) and is critical for L1 layer-specific expression of the *GUS* reporter gene under control of the *PDF1* promoter (Abe *et al.*, 2001). It has been shown that two related homeo domain proteins, *ATML1* and *PDF2*, can bind to a 21bp DNA containing the L1-box sequence *in vitro* and a mutation in the L1-box sequence abolishes this binding (Abe *et al.*, 2001; Abe *et al.*, 2003). Transcripts from *ATML1* and *PDF2* predominantly accumulate in the L1 layer and the protodermal cells of embryos (Lu *et al.*, 1996; Abe *et al.*, 2003). In addition, Abe *et al.* (2003) further demonstrated that *A. thaliana* plants doubly homozygous at T-DNA insertion of *ATML1* and *PDF2* alleles failed to generate typical epidermal pavement cells and to express the L1-layer-specific genes such as *PDF1* and *ACR4*, although single mutants did not exhibit obvious defects.

In transgenic *A. thaliana* plants with *PDF2* co-suppression, fusion between sepals along their edges is often observed (Abe *et al.*, 2003). This suggests that *ATML1* and *PDF2* have a redundant function and regulate the expression of L1- and protoderm-specific genes in an L1-box-dependent manner. Considering the tight correlation between organ fusion and cuticle properties described earlier, organ fusion in the *PDF2* co-suppressed plants might have been caused by altered expression of the genes involved in cuticle formation, although further experimental data are necessary. In this regard, the presence of the L1 box in the *FDH* promoter is of particular interest because loss of *FDH* expression would result in the fusion of organs, including sepals. Several other genes responsible for preventing organ fusion also include the L1 box and related sequences in upstream regions (Tanaka *et al.*, unpublished results).

The production of epicuticular wax involves various genes that are predominantly expressed in epidermis-related tissues (Chapter 4). For example, transcripts of the *CER2*, *CER5*, *CER6/POPI* and *WAX2/YRE/PEL6* genes are preferentially detected in the L1 layer of the shoot apical meristem, protodermal cells of organ primordia and epidermal cells, and mutations in these genes result in apparent modification of and/or reductions in wax content (Xia *et al.*, 1996; Millar *et al.*, 1999; Kurata *et al.*, 2003; Pighin *et al.*, 2004). In addition, using the β -glucuronidase (*GUS*) reporter, it has been demonstrated that genomic DNA fragments containing the 5'-upstream regulatory sequences from genes such as *CER2*, *CER6*, *YRE* and *CER5* (Xia *et al.*, 1996; Hooker *et al.*, 2002; Kurata *et al.*, 2003; Pighin *et al.*, 2004) are sufficient to direct the expression of the reporter gene in an epidermis-specific manner. These results indicate that the production of epicuticular wax is controlled at the transcription level, although other controlling mechanisms may also be involved.

In an effort to functionally characterise *A. thaliana* transcription factors, Broun *et al.* (2004) performed functional screening of transgenic plants harbouring genes for transcription factors under the control of the *CAMV35S* promoter. They identified a phenotype typical of wax overproduction in transgenic plants overexpressing the full-length sequence of an *ERF/EREBP*-type transcription factor gene, *AT1G15360* [named *WAX INDUCERI (WINI)*]. In these plants, leaves were glossier than those of control plants and more alkanes were accumulated in both the leaves and the stem, while marked changes in nonacosane in leaves and stems and hentriacontane in leaves were seen. Primary alcohol content was lower in leaves and higher in stems when compared with the wild type. Thus, *WINI* overexpression results in a preferential increase in the products of the decarbonylation pathway.

The fact that *WINI* is a putative transcription factor whose overexpression caused increased production of alkanes led the authors to examine the expression of genes potentially involved in wax biosynthesis. Among the genes examined, transcripts of *CER1*, *KCS1* and *CER2* were more abundantly accumulated in the leaves of transgenic plants expressing *WINI* than in wild-type leaves. Thus, it seems that *WINI* either directly or indirectly promotes the expression of these genes. Aharoni *et al.* (2004) independently identified *WINI/SHAINI (SHN)* overexpressing plants

by screening for an activation tagging line. In leaves of *WIN/SHN*-overexpressing plants, the contents of alkanes, secondary alcohols and ketones, which are produced by the decarbonylation pathway, were increased when compared with wild-type leaves (Aharoni *et al.* 2004). The CER1 protein appears to be positively involved in the production of alkanes, as the *cer1* mutant is defective in alkane production (Hannoufa *et al.*, 1993; Jenks *et al.*, 1995), although compounds such as primary alcohols are also decreased in the *cer1* mutant. Induction of *CER1* expression by *win1* and accumulation of alkanes is thus consistent with the observed phenotype and the hypothesis that CER1 facilitates decarbonylation.

Transcriptional factors regulating the expression of the metabolic enzymes responsible for cuticle biogenesis have been emerging for some time, as describe earlier. Cuticle biogenesis is tightly linked with the differentiation of the protoderm and the epidermis during development as well as with the outer environment. Elucidation of target genes as well as the regulation mechanisms for the expression and activation of these transcription factors would help our understanding of cuticle biogenesis during the plant life cycle.

10.5 Concluding remarks

Recent genetic and biochemical analyses have highlighted the physiological roles of the cuticle in intercellular and intertissue communication, including the prevention of fusion between organs and tissues, and the hydration and penetration of pollen during fertilisation. There is increasing evidence supporting the notion that permeability of molecules across the cuticle and a proper cutin layer are essential for preventing organ fusion, although it is still unclear to what extent wax is involved in these traits. The genes involved in the various steps of functional cuticle formation have been identified, including those apparently involved in the differentiation of epidermis-related tissues, transcriptional control, cuticle biogenesis and export of cuticle-related lipids. The plant genome presumably contains a family of genes involved in the biogenesis and export of cuticle-related lipids. Although unclear at present, the genetic and biochemical relationships between the regulators and the genes themselves appear to be directly involved in cuticle biogenesis. Further efforts using genetic materials as well as the information obtained to date, may reveal the causal relationship between loss of specific constituents or physiological alteration of cuticle and cellular interactions as well as the molecular mechanisms regulating cuticle biogenesis.

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11 Microbial communities in the phyllosphere

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11.1 Introduction

The term phyllosphere was coined by Last (1955) and Ruinen (1956) to describe the plant leaf surface as an environment that is physically, chemically and biologically distinct from the plant leaf itself or the air surrounding it. The term phylloplane has been used also, either instead of or in addition to the term phyllosphere. Its two-dimensional connotation, however, does not do justice to the three dimensions that characterise the phyllosphere from the perspective of many of its microscopic inhabitants. On a global scale, the phyllosphere is arguably one of the largest biological surfaces colonised by microorganisms. Satellite images have allowed a conservative approximation of 4×10^8 km² for the earth's terrestrial surface area covered with foliage (Morris and Kinkel, 2002). It has been estimated that this leaf surface area is home to an astonishing 10^{26} bacteria (Morris and Kinkel, 2002), which are the most abundant colonisers of cuticular surfaces. Thus, the phyllosphere represents a significant refuge and resource of microorganisms on this planet.

Often-used terms in phyllosphere microbiology are epiphyte and epiphytic (Leben, 1965; Hirano and Upper, 1983): here, microbial epiphytes or epiphytic microorganisms, which include bacteria, fungi and yeasts, are defined as being capable of surviving and thriving on plant leaf and fruit surfaces. Several excellent reviews on phyllosphere microbiology have appeared so far (Beattie and Lindow, 1995, 1999; Andrews and Harris, 2000; Hirano and Upper, 2000; Lindow and Leveau, 2002; Lindow and Brandl, 2003). This chapter aims to provide a brief but broad and updated synthesis of research activities and findings related to phyllosphere microbiology. As the next chapter in this volume (Chapter 12) will focus in greater detail on phyllosphere fungi, the present chapter is biased, in some sections at least, towards bacterial colonisers of the leaf surface.

11.2 Methodologies in phyllosphere microbiology

11.2.1 Sampling techniques

Sampling the phyllosphere is at the basis of many observations or experiments in leaf surface microbiology, and thus deserves its own and in-detail section

at the beginning of this chapter. Several reviews are available that describe the advantages and disadvantages of different methods for sampling and quantification of microorganisms from the phyllosphere (Donegan *et al.*, 1991; Jacques and Morris, 1995; Dandurand and Knudsen, 1996).

One of the simplest techniques is leaf printing (Corpe, 1985), by which a plant leaf is pressed onto the surface of an agar plate for a defined period of time and carefully removed again. The agar plate is then incubated to allow growth of the microorganisms that transferred from the leaf surface. Depending on the medium and the abundance and composition of the microflora, the result is a collection of bacterial and fungal growth foci ('colonies') that often follow the contours of the leaf. Obvious disadvantages of the method are its low resolution of observation and its limited ability to provide a quantitative appreciation for the phyllosphere community composition. However, leaf printing can provide a first indication that the distribution of microorganisms on leaf surfaces is not uniform (Leben, 1998). For example, more growth often occurs where the veins of the leaf touch the agar, suggesting that these structures are more densely populated areas on the leaf (Manceau and Kasempour, 2002).

Furthermore, leaf printing has been an extremely useful and popular laboratory experiment for teaching purposes (Holland *et al.*, 2000). The outcome of a leaf printing experiment will depend in large part on the medium that is used to prepare the agar plate. Medium composition can be varied and exploited to look only at a subset of the microbial community on the leaf. By incorporation of compounds with antifungal (e.g. cycloheximide) or antibacterial (e.g. penicillin) activity, fungal or bacterial colonisers, respectively, can be excluded from the analysis. As microorganisms differ in their nutritional capabilities, it is also possible to include specific nutrient sources in the medium to select for a nutritionally defined subpopulation of the phyllosphere. For example, agar plates that contain methanol as the sole source of carbon have been used to demonstrate the ubiquity of methylotrophic bacteria on plant leaf surfaces (Corpe, 1985).

More quantitative than leaf printing is the method of leaf washing. One or more leaves are placed in a tube or flask containing a specified volume of wash solution (e.g. saline solution or phosphate buffer), and microbes are removed from the cuticular surface by vortexing and/or sonication. The number of bacteria in the wash solution is then determined by the most probable number (MPN) technique (Oblinger and Koburger, 1975) or by plating, directly or after dilution of the wash solution, onto nonselective or selective solid medium to determine the number of colony-forming units (CFUs). The plating can be done using, for example, a Drigalski spatula, glass beads or a spiral plater (Gilchrist *et al.*, 1973). The latter has the advantage that quantitation of CFUs is possible without prior dilution of the leaf wash sample. As with the leaf printing technique, medium composition of the agar plate determines what subpopulation of the phyllosphere will be analysed. This analysis should also take into consideration the fact that different organisms from the leaf surface grow at different rates, so that those bacteria or fungi that grow fast and appear on agar plates first will be more likely to be included in the analysis

than those that grow slowly and appear late or not at all due to overgrowth by fast appearing strains.

From the number of CFUs per plate the number of bacteria that were present on the leaf can be estimated quantitatively by taking into account the volume of the leaf washing solution, the dilution factor and the volume of the aliquot that was spread onto the agar plate or used to inoculate the medium in the MPN method. CFU numbers are usually expressed as the number of bacteria per leaf, per gram of leaf weight or per square centimetre of leaf surface. Often the number is expressed as $^{10}\log(\text{CFU})$, as this allows comparison of population counts from different leaves, which are often distributed not normally but lognormally (Hirano *et al.*, 1982; Kinkel *et al.*, 1995; Woody *et al.*, 2003).

The leaf washing method can be modified to obtain answers to different questions. For example, by not vortexing or sonicating the leaf in a wash solution, bacteria that occupy the leaf surface as aggregates can be removed as intact aggregates and be separated by filtration from those bacteria that live solitary. In this way, Morris *et al.* (1998) were able to estimate the fraction of bacteria that live in bacterial aggregates on the leaf surface. Incidentally, this aggregated lifestyle of bacteria may be a reason for underestimation of total bacterial populations on leaf surfaces: an aggregate of two or more bacterial cells will produce a single CFU on plates, which stresses the need for breaking up of these aggregates as much as possible (Miller *et al.*, 2000).

Another reason for underestimating microbial populations from leaf washing data is the resistance of some microorganisms to be readily removed from the leaf surface by leaf washing (Romantschuk, 1992). A good example is the group of pink-pigmented facultatively methylotrophic bacteria (Holland, 1997). This is probably one of the reasons why these particular bacteria are often missed in phyllosphere composition studies (Holland and Polacco, 1994). Also, plant-pathogenic fungi, such as downy and powdery mildews, produce structures that anchor the fungal hyphae into the plant leaf making them hard to remove (Section 11.3.2, and Chapter 12). To check the efficiency of any leaf washing method, one can use microscopy (Section 11.2.3) to validate the degree of removal from the leaf surface, for example, by staining with the fluorescent DNA stain 4,6-diamidino-2-phenylindole (DAPI). In one instance this was done to check the removal efficiency of a less common sampling method which involves placing a leaf on sterile water, rapidly freezing the water, then carefully removing the leaf and collecting the ice which contains the transferred leaf microflora (Heuser and Zimmer, 2002). Whereas washing by vortexing and sonication usually leaves the cuticular surface covered by residual microorganisms (Jacques and Morris, 1995; Heuser and Zimmer, 2002), the freezing method almost completely removes microorganisms from the leaf (Heuser and Zimmer, 2002).

A method with similar removal efficiency is leaf maceration, by which a single leaf or a collection of leaves is macerated, for example, with a mortar and pestle, in a defined volume of buffer, which is then analysed by plate counting. However, since leaf maceration liberates in addition to those microorganisms that live on

the leaf surface also those that grow inside the plant tissue, phyllosphere population sizes may be overestimated by this method.

One final point of consideration when using methods such as MPN or plate counts is that they provide estimates only for the culturable subset of the microbial population. Microorganisms that resist growing under laboratory conditions are not included in these analyses (also see Section 11.4.1). Furthermore, viable-but-not-culturable-microbial cells (VBNC) that experience prolonged periods of starvation may enter a physiological state which renders them not-culturable, that is, they cannot form a colony on an agar plate, but they are still viable (Bogosian and Bourneuf, 2001). This concept also applies to cuticular surface colonisers. For example, Wilson and Lindow (1992) showed that up to 75% of the bacteria on inoculated bean leaves were VBNC, demonstrating that plate counts can grossly underestimate the viable fraction of bacteria on a leaf surface.

All of the sampling techniques described so far are in essence destructive, that is, the population size of a single leaf can be measured only once, at which time the experiment for that leaf ends. This makes it impossible to follow the temporal dynamics of a microbial population for a single leaf, only for leaves picked at different time points. But because the variation among leaves at any time point can be substantial (Hirano *et al.*, 1982; Jacques and Morris, 1995; Hirano and Upper, 2000), sample sizes necessarily have to be large (i.e. many leaves should be analysed for each time point) in order to obtain statistically significant and precise estimates for changes in microbial population sizes over time. To circumvent the collection of large sample sizes, a semi-destructive sampling method can be used such as the one developed by Woody *et al.* (2003) for studying the temporal population dynamics of the phyllosphere yeast *Aureobasidium pullulans*. Its success is based on the prerequisites that (1) sampling of a leaf segment does not affect the yeast population on other parts of the same leaf, and (2) the distribution of yeast cells is similar for different segments of the same leaf. These prerequisites cannot *a priori* be assumed to be true for every other combination of microorganism and plant (Woody *et al.*, 2003).

Microorganisms that are sampled from the phyllosphere may be used not only for the purpose of estimating phyllosphere population sizes; leaf washings have also been used to inoculate BIOLOG EcoPlates to obtain a carbon-metabolism profile for the microbial community as a whole (Yang *et al.*, 2001). Microorganisms washed from a leaf may be examined microscopically (Section 11.2.3), for example, after staining with DAPI or another DNA dye such as acridine orange, after live/dead staining (Monier and Lindow, 2003a) or after hybridisation with rRNA-specific probe(s) using fluorescent in situ hybridisation (FISH) (Joyner and Lindow, 2000; Brandl *et al.*, 2001; Leveau and Lindow, 2001). Microorganisms that have been modified to express the green fluorescent protein (GFP) can be analysed for individual GFP content using fluorescent microscopy and image analysis (Joyner and Lindow, 2000; Brandl *et al.*, 2001; Leveau and Lindow, 2001; Miller *et al.*, 2001) or flow cytometry (Axtell and Beattie, 2002; Marco *et al.*, 2005). Leaf washings may also be examined for the activity of other reporter genes, such as *inaZ* (Miller

et al., 2001). Furthermore, leaf washings may be the starting material for the isolation of microbial DNA for a culture-independent assessment – either qualitative or quantitative – of leaf surface microbiology (Yang *et al.*, 2001; Heuser and Zimmer, 2002, 2003).

11.2.2 Artificial inoculation

Experimental manipulation of phyllosphere composition and abundance has contributed a great deal to our understanding of the cuticular surface as a microbial habitat. It is achieved by artificial inoculation of plants in the field, greenhouse or laboratory with defined suspensions of bacteria, yeasts or fungal spores. The most common purposes of artificial inoculation are:

- (1) to determine the ability of the inoculated microorganism(s) to survive and thrive in the phyllosphere;
- (2) to follow the growth and/or activity of the introduced strain(s) in relation to factors such as plant and environment;
- (3) to assess the effect of the introduced strain(s) on the abundance and activity of other microorganisms in the phyllosphere; or
- (4) to exploit the introduced microorganism(s) as a biological indicator of the physical, chemical or biological factors that govern the phyllosphere.

In the greenhouse or laboratory, inoculation of plants is achieved by immersing whole plants in a dilute suspension of microorganisms with a known titer, or by spraying them with such a suspension using a device that produces a fine mist or spray. After inoculation, plants are incubated in one of many ways, depending on experimental design and research question. To create an environment that is conducive to microbial growth, it is most common to incubate under conditions of high relative humidity, for example, by covering the plants in bags or placing them in controlled-environment chambers. Plants have been exposed to diverse conditions in order to see if and how these conditions change microbial population sizes and activities. Some examples are different light intensities (O'Brien and Lindow, 1989), low relative humidity (O'Brien and Lindow, 1989; Andersen *et al.*, 1998), or increased ultraviolet (UV) exposure (Kadivar and Stapleton, 2003; Jacobs *et al.*, 2005). In the field, plants are spray-inoculated using knapsack sprayers or scaled-up devices. Most field inoculations are performed to test or exploit the ability of the microorganism(s) in the inoculum to suppress symptoms of disease or microbe-induced freezing (Section 11.9.3). To some extent, environmental conditions in the field can be manipulated, for example by covering plants with meshes to test the effect of rainfall momentum or exposure to sunlight (Upper and Hirano, 2002).

Inoculation of plants can be done with single strains or with mixtures of strains. By mixing two strains in a 1 : 1 ratio and using this mixture as an inoculum, the two strains in the mixture can be directly compared for their relative fitness in

the phyllosphere (Section 11.6.1). By this approach small but significant differences in behaviour between two species can be revealed better than when the two strains are inoculated separately (Lenski, 1992). Mixtures may also be used to test the effect of strains on each other's performance in the phyllosphere. So-called de Wit replacement series, whereby two strains are mixed in various proportions at a constant total inoculum density, have provided important insights into the relative competitive abilities of microbial epiphytes, niche differentiation and resource utilisation (Wilson and Lindow, 1994a, 1995). Factors that have been shown to contribute significantly to the phyllosphere performance of inoculated microorganisms are preparation of the inoculum and inoculum density (Wilson and Lindow, 1994b). Each of these factors should be taken into consideration during the planning of artificial inoculation experiments and interpretation of their results.

One special type of inoculum involves microbial bioreporters for habitat exploration (Leveau and Lindow, 2002). Bioreporters are whole-cell indicators of a specific microbial activity. They are usually bacteria that have been manipulated to carry a reporter gene such as the one coding for GFP or ice nucleation protein (*InaZ*) downstream of an inducible promoter. This promoter determines the usefulness and specificity of the bioreporter strain. For example, Leveau and Lindow (2001) introduced into the epiphytic bacterium *Erwinia herbicola* a fusion of a fructose-inducible promoter and the gene for GFP. The resulting strain of *E. herbicola* responds to the availability of fructose by the synthesis of GFP and emission of green fluorescent light. Inoculation of this strain onto plants made it possible to explore the leaf surface for the availability for fructose (see also Section 11.5.2). Several other bioreporters, with different specificities, have been applied to the phyllosphere (Joyner and Lindow, 2000; Miller *et al.*, 2001; Axtell and Beattie, 2002), and each has provided unique insights into the microbial perception of the cuticular surface (Sections 11.5.2 and 11.7.3).

The concept of bioreporting has also been used for the identification of genes that contribute to the fitness of epiphytic bacteria in the phyllosphere (Sections 11.6.1 and 11.6.3). Marco *et al.* (2003) developed a screening method for phyllosphere-inducible promoters of *Pseudomonas syringae* which is based on the insertion of random genomic DNA fragments upstream of a promoterless but essential locus *metXW* for methionine biosynthesis. When a mixture of bacteria, each containing a different gene fusion, is inoculated onto plants only those bacteria that carry a phyllosphere-inducible promoter express the *metXW* genes and thus will survive. In other words, by inoculation onto plant leaves, bacteria with phyllosphere-inducible promoters are enriched. This approach has been very useful in the identification of genes that are involved in the adaptation of bacterial colonisers to the phyllosphere (Marco *et al.*, 2005; also see Section 11.6.3).

11.2.3 Microscopy

The openness of the phyllosphere makes it extremely suitable for direct observation by microscopy. In this respect, the phyllosphere differs dramatically from

the rhizosphere, which essentially is a hidden environment. Microscopical analysis can be done on whole leaves, leaf sections or even isolated leaf cuticles. The highest magnification, that is, 10 000 \times or more, is achieved by electron microscopy, and both transmission and scanning electron microscopy have revealed interesting details of phyllosphere life at the (sub)micrometre scale (Beattie, 2002). Environmental scanning electron microscopy is a modification of the latter technique which allows 'wet-mode' imaging of phyllosphere samples (Monier and Lindow, 2004). Atomic force microscopy has also been applied to the phyllosphere (Mechaber *et al.*, 1996) to create three-dimensional, high-resolution surface maps of the leaf (see Section 11.5.1).

More accessible to most phyllosphere researchers is light and fluorescence microscopy which generally have a maximum magnification of 1000 \times and a resolution of about 0.2 micrometres. One of the most exciting developments in phyllosphere research has been the combination of fluorescent microscopy and fluorescent proteins such as GFP (Brandl *et al.*, 2001; Leveau and Lindow, 2001; Axtell and Beattie, 2002; Brandl and Mandrell, 2002; Monier and Lindow, 2003a) but also red-fluorescent protein (Brandl and Mandrell, 2002). Bacteria or fungi that have been modified to express GFP constitutively are easily recognised in situ on the leaf surface by their green fluorescence. Thus, colonisation patterns can be related to leaf surface structures. Furthermore, the activity of GFP-based bioreporters (as discussed in Section 11.2.2) can be interpreted in light of their location on the leaf surface (Leveau and Lindow, 2001). With confocal laser scanning microscopy (CLSM), individually sliced views in the z -axis can be viewed and stacked. CLSM has been used quite successfully to reconstruct the three-dimensional arrangement of microorganisms on the leaf surface (Figure 11.1).

For epiphytic microorganisms that occur naturally on leaves or that have not been modified to express fluorescent proteins, different stains or dyes are available

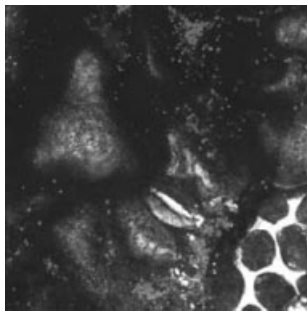


Figure 11.1 View of a colonised bean leaf using confocal laser scanning microscopy. Individual bacterial colonisers producing GFP are visible as green fluorescent dots. The centre shows a stoma; the cuticular top of an epidermal plant cell is visible on the left top corner while at the bottom right the picture slices into the leaf's palisade parenchyma. Chlorophyll is coloured red in this picture. This figure is produced in colour in the colour plate section, which follows page 249.

to facilitate their visualisation. Most practical are those stains that can be used in combination with fluorescence microscopy. These include DNA stains like DAPI and acridine orange, and propidium iodide which stains dead bacteria red (Monier and Lindow, 2003a, 2004). A more specific type of dye is represented by fluorescently labelled oligonucleotides or probes, which are designed to target the rRNA in ribosomes in a procedure that is referred to as FISH (Amann *et al.*, 1995). FISH probes can be labelled with different fluorophores, but carboxytetramethylrhodamine (TAMRA) has been most commonly used in phyllosphere research. TAMRA fluoresces red and its detection is compatible with that of GFP (Joyner and Lindow, 2000; Brandl *et al.*, 2001; Leveau and Lindow, 2001).

11.3 Getting to the phyllosphere (and leaving again)

11.3.1 Immigration

When a leaf appears on the plant, it is generally not sterile, that is, free of microbial inhabitants. Seeds that are naturally or artificially inoculated with bacteria have been shown to develop into plants that carry substantial populations of these bacteria on their leaves (Saettler *et al.*, 1989; Lilley *et al.*, 1997; Upper and Hirano, 2002). Thus, seeds and, indirectly, soil may be potential sources of phyllosphere colonisers. However, most microorganisms in the phyllosphere of naturally occurring plants are considered to arrive only after leaves have emerged by immigration from the air surrounding the leaf (Lindemann and Upper, 1985; Lindow and Andersen, 1996; Kadivar and Stapleton, 2003). The atmosphere is a rich and diverse source of potential microbial colonisers of the phyllosphere. In one study (Lighthart and Shaffer, 1995), the 10-m layer of air over a grass field in Oregon contained an average value of 121 and a peak value of 1369 bacterial CFUs per cubic metre. Plate counts fluctuated greatly between long-term (early, mid and late summer), short-term (morning, afternoon, evening, night) and even very short-term (intervals of 2 min) sampling times. Much of the short- and long-term fluctuation could be attributed to the temporal variation in meteorological conditions (convective wind sweepings, cleansing sea breezes) as they were recorded during the course of the day or season.

Several mechanisms have been proposed for microbial immigration from the air onto the leaf surface (Lindow, 1996), including deposition by aerosol, wind, rain or insects (Lilley *et al.*, 1997). Several studies have produced quantitative data for these processes, which allow a better appreciation for the contribution of immigration to phyllosphere biology. For bean plants in a Wisconsin field, Upper and Hirano (2002) reported an average phyllosphere immigration rate of 11 *P. syringae* bacteria per bean leaf on a rain-free day. Rates of immigration were clearly dependent on the presence of and distance from source plants (i.e. plants carrying high population sizes of *P. syringae*). From airborne insects that were captured in the same field, bacteria could be recovered in numbers that varied from undetectable to 10 000 CFU

per insect (Upper and Hirano, 2002). Dispersal of bacteria by airborne insects appeared to be most effective when leaves were wet and accounted dispersion over up to tens of metres. Leaf-to-leaf migration may also occur through run-off or splash during rains, but this mechanism of dispersal obviously operates at a smaller, local scale within the plant leaf canopy.

Bacterial cells and fungal spores may not arrive on the leaf surface as individuals, but rather in aggregates of more than one individual. The close proximity of these early immigrants to each other on the leaf surface and the interaction that follows between them probably is a first determinant in the fate of these immigrants during the next stages of leaf colonisation.

Much of the success of an immigrant to the phyllosphere also depends on the initial physico-chemical conditions of the cuticular surface (Section 11.5.2; Chapter 4). For example, it has been shown that under conditions of low relative humidity, the chances of survival of *P. syringae* on bean leaves is reduced dramatically compared to conditions of high relative humidity (Monier and Lindow, 2003a). Furthermore, immigrants to a previously colonised leaf surface probably face an environment that is quite different from that of an uncolonised leaf surface. On the one hand, they might enter an environment that, through active modification by the residing microflora, has been made more conducive to their own survival or growth (Section 11.8.1). On the other hand, much of the initially available nutrients on colonised leaves may have been used up by earlier immigrants, leaving less substrate to utilise and to grow on for later immigrants (Section 11.7.3). This principle has been used to explain the relative success of early immigrants in dominating the phyllosphere (Upper and Hirano, 2002) and has been exploited as a biocontrol strategy for the suppression of certain plant pathogens (Section 11.9.3).

11.3.2 Adhesion

Adhesion of epiphytic microorganisms to cuticular surfaces plays several ecological roles. First, it prevents removal from the leaf surface, either immediately after immigration or in the subsequent course of colonisation. It has been suggested, however (Andrews and Buck, 2002), that for many bacteria and yeasts the contribution of adhesion to phyllosphere colonisation is relatively small if the fraction of cells that resists removal and remains on the leaf surface is able to quickly re-populate the leaf surface. A second role of attachment is that it serves as the initial step in the formation of microcolonies, aggregates and biofilms (Andrews and Buck, 2002), which has been implicated in enhanced success of leaf colonisation, for example through niche modification (Section 11.8.1).

The surfaces of leaves and fruits are covered by a hydrophobic cuticle which consists of a complex mix of long-chain aliphatic compounds (Chapters 1 and 4). Many fungi adhere to hydrophobic surfaces, including cuticular surfaces, much better than to hydrophilic surfaces (Clement *et al.*, 1994; Buck and Andrews, 1999; Tucker and Talbot, 2001; Beattie, 2002), although exceptions exist (Buck and Andrews, 1999). Underlying the hydrophobicity mediated adhesion of yeasts, fungi and fungal spores

are hydrophobic interactions between cuticular surface waxes and hydrophobic components in the cell wall such as water-insoluble glycoproteins (Tucker and Talbot, 2001). When leaf waxes are removed, for example, with chloroform, hydrophobicity dependent attachment is reduced (Young and Kauss, 1984). Attachment may also be mediated by fungal-produced components including extracellular polysaccharides (Andrews *et al.*, 1994), hydrophobic mucilage (Hamer *et al.*, 1998) and cutinases and esterases (Deising *et al.*, 1992). The synthesis of such components is often influenced by the availability of water and nutrients (Andrews and Buck, 2002) or temperature (Tucker and Talbot, 2001). Adhesion to the cuticular surface may also be indirect: among phyllosphere yeasts, it is a common trait to be able to attach to fungal spores or hyphae (Allen *et al.*, 2004). Quite a rigorous mode of fungal adhesion to the cuticular surface is through cuticle penetration by specialised hyphae (Chapter 12). Penetration is achieved by secretion of enzymes and/or by sheer physical force. With the subsequent formation of fungal structures inside the leaf (e.g. haustoria), fungal attachment is semi-permanent.

In contrast to fungi, bacteria do not seem to engage in hydrophobic interactions with cuticular surface waxes. Instead, they rely on pili and extracellular polymeric substances for adhesion (Beattie, 2002). Most research has focused on the role of pili (Romantschuk, 1992), which are thin protein tubes originating from the cytoplasmic membrane and which are found in almost all gram-negative bacteria. Mutants of *P. syringae* lacking pili were washed more easily from leaves than the wildtype (Suoniemi *et al.*, 1995), which suggests a role for pili in resisting removal by run-off rain. In field experiments, mutants of *P. syringae* pv. *tomato* lacking type IV pili achieved slightly lower population sizes on leaves of tomato plants than those of the parental wild-type strain DC3000 (Roine *et al.*, 1998). Adhesion of piliated *P. syringae* was not affected by removal of the cuticular surface waxes, suggesting that waxes are not necessary for pili attachment (Romantschuk *et al.*, 1993). Besides pili binding, several other mechanisms have been proposed to explain bacterial adhesion to plant leaf surfaces. Many bacteria produce extracellular polysaccharides on leaves, often as a matrix for biofilms (Romantschuk *et al.*, 1996; Morris *et al.*, 1997; Beattie and Lindow, 1999). Acting as glue, extracellular polysaccharides may help reduce the probability of removal by rain or wind. The same has been suggested for the production of cellulose by *Pseudomonas fluorescens* SBW25 (Gal *et al.*, 2003).

Bacterial attachment to leaves varies with bacterial species and with cuticular surface properties. Immediately after spraying suspensions of *Pantoea agglomerans*, *Clavibacter michiganensis* or *P. syringae* onto maize leaves, all three bacterial species resisted to some extent the removal from the leaf by sonication and vigorous vortexing, but the fraction of non-removable *P. agglomerans* cells was up to ten-fold higher than that of the other two species (Beattie and Marcell, 2002). Furthermore, it was shown with cuticular maize mutants that *P. agglomerans* was less influenced by changes in cuticular wax-dependent surface properties than *C. michiganensis* (Beattie and Marcell, 2002), suggesting that different bacteria use different mechanisms for adhesion. It is unclear whether these differences in mechanisms correlate

with the relative importance of adhesion in the life cycle of any given bacterial epiphyte.

11.3.3 Emigration

Many of the mechanisms by which microorganisms exit the surfaces of leaves are the reverse of those described for immigration (Section 11.3.1). Emigration may account for a substantial reduction in phyllosphere abundance. For example, rainfall may remove as many as 10^5 bacteria from a single bean leaf in the field in a period of 15 min (Lindemann and Upper, 1985). In this process, leaf cuticle properties may play an important role. For example, mutants of corn that differ in wax composition show less retention of sprayed bacteria when cuticular surfaces are more hydrophobic (Beattie, 2002). Microorganisms that run off leaves through rainfall onto the soil usually do not persist there for long. However, they might attach to seeds and end up on the leaves of newly emerging plants (Section 11.3.1).

11.4 Microbial communities in the phyllosphere

11.4.1 Composition

To study the composition of microbial communities in the phyllosphere, the most common procedure is to spread leaf washings onto nonselective or selective solid media (Section 11.2.1), and identify the bacteria, yeasts or fungi that appear on the basis of morphological, physiological or phylogenetic features. Thus, composition analysis is generally biased towards culturable microbial epiphytes only, and in most cases it is not known how representative this subset is for the entire phyllosphere population. The bias towards culturables probably also underlies the common notion that populations of culturable bacteria on leaves are dominated by only a few genera including *Pseudomonas*, *Erwinia* and *Xanthomonas* (Jurkevitch and Shapira, 2000; Lindow and Brandl, 2003). All of these are readily culturable bacteria that grow rapidly on laboratory media. Pink-pigmented facultative methylotrophs of the genus *Methylobacterium* are some of the most abundant bacteria on plants but they are often overlooked due to their comparatively slow growth and special nutrient requirements: they are only found when searched for, and so far, they have been encountered on nearly every plant investigated (Corpe, 1985; Holland and Polacco, 1994).

Very few studies have used culture-independent methods to avoid the bias towards culturables, and their outcome, not surprisingly, shows a much greater diversity in phyllosphere composition (Weidner *et al.*, 2000; Yang *et al.*, 2001; Kadivar and Stapleton, 2003). For example, by denaturing gradient gel electrophoresis (DGGE) of PCR-generated 16S ribosomal RNA gene products, Yang *et al.* (2001) identified 17 unique sequences in DNA collected from the 'Valencia' orange phyllosphere. Only four of these corresponded to bacteria that had been found in

the phyllosphere before, that is, *Acinetobacter* sp., *Bacillus pumilus*, *Enterobacter agglomerans* and a *Cytophagales* species. In contrast, 16S rRNA sequences obtained from leaf washings that were first cultured in BIOLOG EcoPlates showed between 97 and 100% similarity to those of known phyllosphere bacteria such as *Pseudomonas*, *Erwinia* and *Acinetobacter* species, clearly demonstrating that culturing favours the culturable.

Many composition analyses have targeted plants from a single geographical area (Inacio *et al.*, 2002; Pereira *et al.*, 2002) or a single plant species (Austin *et al.*, 1978; Ercolani, 1991; Jurkevitch and Shapira, 2000; de Jager *et al.*, 2001). While such studies may seem rather anecdotal, also given the fact that composition is highly variable (Section 11.4.3), they are instrumental in demonstrating the enormous diversity of microbial life in the phyllosphere. Snap bean plants were shown to carry more than 78 bacterial species representing 37 bacterial genera on their leaves (Beattie and Lindow, 1999). An inventory of phyllosphere fungi and yeasts from Mediterranean plants included a total of 1029 strains of filamentous fungi and 540 strains of yeasts, representing at least 36 and 46 distinct species, respectively (Inacio *et al.*, 2002). Jacques and Morris (1995) presented an overview of bacterial species, representing 29 genera, isolated in 13 independent studies from plants in temperate zones. For at least 3 plants (*Lolium perenne*, *Olea europaea* and *Ranunculus penicillatus*), 3–27% of the bacteria that were isolated and cultured from the phyllosphere could not be identified, showing that our understanding of the microbial composition of the phyllosphere, even for culturable representatives, is still far from complete. Yet, this realisation also comes from culture-independent studies: 5 of the 17 rRNA sequences identified by Yang *et al.* (2001) had a similarity to database entries below 90%, suggesting that they represent previously undescribed taxa.

The degree to which plants can determine the microbial composition in their phyllosphere remains unknown. Using DGGE (Yang *et al.*, 2001), it was shown that the microbial community structure on plants such as green bean, cotton, sugar beet and orange, but not corn, were similar on different individuals of the same plant species, but different from all other plant species. If selection takes place it would probably be on the basis of leaf characteristics such as the presence or absence of protective sites (Section 11.6.2) or the availability of plant-specific nutrients (Section 11.7.3), in combination with environmental factors such as weather conditions or geographical location.

11.4.2 Abundance

In general, microbial abundance in the phyllosphere refers to one of the following: (1) the total average number of bacteria, yeasts or fungi on a leaf, (2) the average number of a specified subset of bacteria, yeasts or fungi on a leaf, (3) the most abundant species of bacteria, yeasts or fungi on a leaf, or (4) the number of bacteria, yeasts or fungi on a dimensional scale smaller than that of a single leaf. As it is the case for composition studies, most estimates for microbial abundances on leaf

surfaces are made on the basis of counts of culturables (Section 11.2.1), although some recent reports have used culture-independent methods such as real-time PCR (Heuser and Zimmer, 2002, 2003) and direct microscopic counting (Andrews and Buck, 2002). To be able to compare between plants, microbial abundances are best described as densities, for example, numbers of microorganisms per square centimetre of leaf surface, but they have also been expressed per leaf or per gram of leaf tissue. Of all microbial epiphytes, bacteria are generally considered to be the most abundant on leaves (Lindow and Brandl, 2003). A typical leaf may contain up to 10^6 – 10^7 bacteria per square centimetre of surface. Scaled up to human dimensions, this translates into population densities that are 10- to 100-fold higher than the most densely populated countries in the world. The number of yeasts and yeast-like organisms may reach 10^7 CFU per gram (fresh weight) of leaf material on some plant species. The most abundant yeasts and yeast-like fungi on the phylloplane of temperate plants are *Aureobasidium pullulans*, *Sporobolomyces* species, *Rhodotorula* species and *Cryptococcus* species (McCormack *et al.*, 1994).

Microbial abundances in the phyllosphere are characterised by high degrees of variability, a factor 1000 and sometimes more, among leaves from the same plant or field (Hirano *et al.*, 1982; Jacques and Morris, 1995; Hirano and Upper, 2000). Leaf densities of microorganisms are therefore generally described and compared after a $^{10}\log$ transformation. The degree of leaf-to-leaf variation may differ between plant species or change as a result of environmental conditions (Kinkel *et al.*, 2000). It has recently been suggested (Woody *et al.*, 2003) that leaf-to-leaf variability is not due to asynchronous temporal changes in population sizes on individual leaves, but rather due to the variation among individual leaves in their ability to promote survival or sustain microbial growth (Sections 11.6 and 11.7).

Variation in population densities is not restricted to the dimensional scale of single leaves. Even on different sections from the same leaf there can exist substantial variation in microbial numbers. For example, among 9-mm² sections of potato leaves total bacterial populations varied by over 100-fold (Kinkel *et al.*, 1995), and on bean leaf sections of 44-mm², *P. syringae* populations even varied 100 000-fold (Monier and Lindow, 2004). Also at the microscopic level bacteria, yeasts and fungi are not uniformly distributed across the leaf surface (Monier and Lindow, 2004). The most commonly and heavily colonised sites on naturally colonised plant leaves are the bases of trichomes, stomata, epidermal cell wall junctions and the grooves along veins (Beattie and Lindow, 1999). It has been hypothesised that these sites are 'protective' sites where microbial growth is favoured more than on other sites on the leaf surface, for example, because of higher nutrient availability (Section 11.7.3). From microscopy studies, it has also become clear that many bacteria occupy the leaf surface in aggregates (Morris *et al.*, 1997). In fact, aggregates can constitute between 10 and 40% of the total bacterial population on leaves of certain plant species (Morris *et al.*, 1998). On leaves from the field, these aggregates are often mixed populations of different species (Morris *et al.*, 1998). Laboratory studies of aggregate formation by phyllosphere bacteria have revealed that aggregate sizes were distributed with a strong right-hand-skewed frequency (Monier and Lindow, 2004). While large

aggregates on these bean leaves were not frequent, they accounted significantly for the majority of cells present on a leaf.

11.4.3 Dynamics

Many studies have documented differences in microbial abundance and composition on leaves from the same plants or field at different points in time or after a defined treatment (O'Brien and Lindow, 1989; Ercolani, 1991; Ellis *et al.*, 1999; Hirano and Upper, 2000; de Jager *et al.*, 2001; Kadivar and Stapleton, 2003). This demonstrates that microbial populations on cuticular surfaces are not static. Changes in abundance or composition are ultimately a sum of immigration, growth, death and emigration, and each of these is directly or indirectly influenced by external factors such as rain, UV radiation exposure and leaf age. Rains have been shown to trigger rapid multiplication of epiphytic bacteria (Hirano and Upper, 2000), probably due to increased nutrient availability as a result of leaching (Section 11.7.2). Exposure of plants to conditions of low relative humidity usually causes bacterial populations to decrease and/or change in composition (O'Brien and Lindow, 1989; Hirano and Upper, 2000). Weather conditions may also determine the most abundant colonisers of leaves: for example, on bean leaves in the field, PPFMs were most abundant when the weather was hot and dry, whereas *P. syringae* was quite abundant during periods of wet and warm weather (Hirano and Upper, 2000).

Changes in UV exposure have been shown to affect microbial populations on leaf surfaces (Newsham *et al.*, 1997; Sundin and Jacobs, 1999; Jacobs and Sundin, 2001, 2002; Hughes *et al.*, 2003; Jacobs *et al.*, 2005). In one instance, UV-exposed phyllosphere samples showed an increase in bacterial diversity (Kadivar and Stapleton, 2003). Leaf age also has a clear effect on microbial composition (Ercolani, 1991). While bacteria, yeasts and filamentous fungi are often found to colonise the same leaf surface, there can be succession in microbial composition as the leaf gets older. In general, bacteria are the pioneers on young leaves, yeasts may dominate as the leaf becomes older and filamentous fungi up to leaf senescence (Blakeman, 1985). Other factors that may influence the density and diversity of microbial populations in the phyllosphere are plant species (Heuer and Smalla, 1997), air pollution (Brighigna *et al.*, 2000), carbon dioxide levels (Magan and Baxter, 1996), leaf position in the canopy (Andrews *et al.*, 1980; de Jager *et al.*, 2001), acid rain (Helander *et al.*, 1993) and insects (Stadler and Müller, 2000).

11.5 Microbial perception of the phyllosphere

11.5.1 Topography

As the dimensions of humans and microorganisms differ by about six orders of magnitude, only a 1 000 000-fold magnification could offer us the best possible impression of leaf surface topography from the perception of an individual microbial

epiphyte. Electron microscopy commonly achieves a 60 000-fold zoom, while light and fluorescence microscopy maximally reaches 1000-fold, offering (only) a bird's eye view of the habitat. There are relatively few studies that provide quantitative data on the topography of leaf surfaces (Mechaber *et al.*, 1996; Monier and Lindow, 2004). This is surprising, since leaf surface structures have been implicated to have a large influence on the microbial biology of the phyllosphere. Structurally and functionally different features of the leaf such as stomata, veins, trichomes and epidermal cells may vary dramatically in water retention capacity, cuticle thickness or release of plant compounds; so their individual ability to harbour and sustain microbial populations may also differ. Monier and Lindow (2004) scanned adaxial surfaces of bean leaves and found them to consist 74% of undifferentiated epidermal cells, while stomates, veins and trichomes accounted for 17, 7 and 2%, respectively.

Using atomic force microscopy, Mechaber *et al.* (1996) mapped the adaxial surfaces of cranberry leaves in three dimensions. Young leaves showed a regular pattern of broad plateaus on the surface of individual epidermal plant cells with drops in elevation of up to 3 μm between the cells. Old leaf surfaces on the other hand appeared much rougher and with a less regular pattern. These differences between young and old leaves indicate that leaf surface topography and thus physical properties are highly variable with age, and the same is true for leaves of different plant species.

11.5.2 Physico-chemical parameters

The phyllosphere represents a unique habitat in terms of physical and chemical parameters (Burrage, 1971; Chapter 4). Water may be available in the form of rain, fog or dew. In addition, the cuticle may in some cases be covered by aqueous deposits which are a result of the interaction between hygroscopic salt crystals on the cuticular surface and water vapour from the atmosphere and the stomata (Burkhardt *et al.*, 1999). The wetness of individual leaf surfaces can be quantified by measuring electrical conductivity between two electrodes clipped to a living plant leaf surface (Klemm *et al.*, 2002). To assess water availability at a smaller, that is, micrometre scale, bacterial bioreporters have been used (Axtell and Beattie, 2002). Cells of *E. herbicola* carrying a *proU-gfp* gene fusion produce GFP in a quantitative manner in response to low water potential. After inoculation onto bean leaves, GFP expression profiles indicated that cells experienced increased osmolarity, probably as a result of evaporation of the available water. However, there was considerable variation among individual bacteria on the leaf surface: those that were located close to veins were less fluorescent, suggesting that such leaf structures can locally increase water retention.

Other physico-chemical parameters are UV exposure and temperature. UV radiation from the sun reaching the phyllosphere consists on the average 95% of UV-A (320–400 nm) and 5% UV-B (290–320 nm; Jacobs *et al.*, 2005). The effects of these types of radiation on microbial epiphytes differ: UV-A exposure leads

to the (sub)lethal formation of reactive oxygen species, whereas UV-B directly damages the DNA. UV exposure of individual leaves differs with geography, climate and canopy structure, but UV-protective measures are common among many phyllosphere microorganisms (Section 11.6.2). Temperatures can differ dramatically in the course of a single day, but also on a single leaf, for example, from the centre to the edge. Temperature may have a direct effect on the growth of microorganisms, but may also act indirectly, for example, through accelerated or delayed evaporation of available water.

11.5.3 *Biological environment*

The area of the leaf surface that is actually covered by microorganisms varies depending on many factors. In a laboratory experiment with *P. syringae*, bean leaves were covered for up to 12% of their surface area with bacteria, whereas some 60% of the cells were located in an aggregate of 100 cells or more (Monier and Lindow, 2004). This means (1) that any new bacterial immigrant to these leaves would have a relatively low probability (up to 12%) of landing next to other bacteria, but (2) that any already present inhabitant has a high probability (60% or more) to be surrounded by many others. Bacteria in an aggregate are presented with a biological environment which is very different from that of a solitary cell and which probably sets the scene for fierce competition for food and space, and also opens the possibility for collaboration through density-dependent communication (Section 11.8.4) or for plasmid exchange (Section 11.8.5). In laboratory studies with single bacterial strains, aggregates mostly or exclusively consist of clonal individuals, whereas in nature, aggregates on leaves are most commonly mixes of different bacterial species (Morris *et al.*, 1998).

Surprisingly little is known about if and in what way bacteria on the one hand and fungi or yeasts on the other interact in the phyllosphere. Most of what is known comes from biocontrol studies (Section 11.9.3), which leaves many questions on the biology and ecology of bacteria/fungi interactions in the phyllosphere still unanswered.

11.6 **Surviving (or not) in the phyllosphere**

11.6.1 *Concept of epiphitness*

Epiphytic fitness, or 'epiphitness' as it shall be referred to here, can be defined as the ability of a microorganism to survive and thrive on plant cuticular surfaces. Typically, this ability is demonstrated experimentally in the laboratory by inoculation of plants and subsequent monitoring of changes in population sizes. Epiphitness is therefore often expressed as a population size after a defined period of time after inoculation, or as an x -fold increase compared to population sizes immediately after inoculation. Generally, epiphitness is used as a relative measure: if under the same experimental circumstances the population size of one strain is lower than that of another, it is

said that the former has a lower epiphytiness than the latter. An example is the comparison of near-isogenic strains that differ in one gene or more – often a mutant versus the wildtype – to assess the role of that gene in phyllosphere competence (Section 11.6.3).

11.6.2 Adaptive strategies

The cuticular surface is considered a harsh environment not only because of the relative extremes to which epiphytic microorganisms are exposed (e.g. water stress, nutrient availability, UV radiation), but perhaps even more so because of the rapidity with which such conditions change, for example, even within the time it takes a bacterium to double. Beattie and Lindow (1999) proposed two not mutually exclusive strategies that allow epiphytic bacteria to survive and thrive on plant cuticular surfaces. The first is a strategy of tolerance, which is based on traits that protect microorganisms against and help them to deal with the harsh environment of the phyllosphere. A good example is UV-protective pigmentation which is quite common among phyllosphere bacteria (Goodfellow *et al.*, 1976; Dickinson, 1986; Lindow and Brandl, 2003; Jacobs *et al.*, 2005), as are repair mechanisms for UV-induced damage to the DNA (Kim and Sundin, 2000; Sundin *et al.*, 2000; Zhang and Sundin, 2004).

In response to nutrient limitation in the phyllosphere, the bacterium *P. syringae* reduces its size (Björklöf *et al.*, 2000; Monier and Lindow, 2003b), which is an active process, probably to optimise surface-to-volume ratio and nutrient uptake capacity (Monier and Lindow, 2003a). The phyllosphere fungus *Epicoccum nigrum* accumulates solutes such as glycerol and arabitol in response to water stress, which may assist in its survival and establishment on leaf surfaces (Pascual *et al.*, 2003).

The ever-changing conditions in the phyllosphere demand a high degree of plasticity from microbial epiphytic colonisers. There are several lines of evidence that suggest such plasticity in phyllosphere bacteria. For example, cells of *P. syringae* that were recovered from bean leaf surfaces and re-applied to uninoculated leaves showed a higher epiphytiness than cells that were applied to leaves from a plate or broth culture (Wilson and Lindow, 1993). It has been proposed that nutrient limitation, in particular starvation for carbon, makes bacterial cells more adapted to the stressful conditions of the phyllosphere (Monier and Lindow, 2003a). Bacterial plasticity is probably correlated to the ability to go from a solitary lifestyle to life in an aggregate (see later), and vice versa (Boureau *et al.*, 2004). Inability to tolerate the phyllosphere probably results in death or transition into a VBNC state. This may be the fate of many new microbial immigrants to the leaf or to those that are not part of a protective environment such as described later. It is unknown whether VBNCs can recover from their state, and under what conditions such recovery would occur.

The second strategy proposed by Beattie and Lindow (1999) is a strategy of avoidance, which is based on so-called protective sites on the leaf surface. Some foliar pathogens avoid many of the leaf surface stresses by escaping to the interior of the leaf. This niche poses its own challenges for survival (Manceau and

Kasempour, 2002), but many bacteria have learned to cope with these. The abaxial side of a leaf may protect better against the damaging effects of UV than the adaxial side (Sundin and Jacobs, 1999). Trichomes or other leaf structures may also represent protective sites as they offer shading from UV radiation, or retain water. Bacteria living in aggregates or biofilms in fact also employ a strategy of avoidance, as they create a local environment that is more conducive to growth and survival (see Section 11.8.1).

11.6.3 *Epiphitness genes*

The common definition of epiphitness genes is that they confer a measurable advantage to a bacterium, yeast or fungus in colonising a leaf surface. Most epiphitness genes have been identified in knock-out studies: the underlying hypothesis is that mutations in epiphytic genes would reduce the epiphitness of its carrier. Lindow (1993) identified 82 transposon mutants of *P. syringae* with altered behaviour on leaf surfaces. Several of these were not able to withstand desiccation stress (Lindow *et al.*, 1993a). None of the mutants were disrupted in their utilisation of 31 different carbon compounds, suggesting that *P. syringae* has a broad spectrum of carbon sources it can use or has redundant genes for utilisation of the most important carbon sources. Several mutants showed decreased motility, osmotolerance and extracellular polysaccharide production suggesting that these are important traits for establishment in the phyllosphere. Mutants of *P. syringae* lacking the *gac* regulon were less fit on bean plants in the field (Hirano *et al.*, 1997). The *gac* regulon consists of the two-component regulatory genes *gacA* and *gacS*, which in *P. syringae* regulate, among other things, swarming, the synthesis of quorum sensing signal molecules called *N*-acyl homoserine lactones (AHLs; see Section 11.8.4) and production of the extracellular polysaccharide alginate, all of which have been considered to play a role in the epiphytic lifestyle of this and other epiphytic bacteria (Yu *et al.*, 1999). Genes of the *hrp* regulon also have been shown to contribute significantly to epiphitness in field situations (Hirano *et al.*, 1999). Both *gac* and *hrp* genes were initially identified as being involved in pathogenicity, suggesting a close link between epiphitness and being a successful pathogen (Section 11.9.2). Several other examples of epiphytic genes that have been identified through mutation analysis are available (e.g. Andersen *et al.*, 1998; Brandl and Lindow, 1998; Roine *et al.*, 1998; Sundin, 1999); they code for such traits as pili formation, alginate synthesis, production of the plant hormone indole-3-acetic acid and AHL production.

A well-recognised problem with knock-out approaches is their limited effectiveness in identifying genes with incremental contribution to leaf surface fitness (Marco *et al.*, 2003). An alternative approach is the use of reporter gene technology, which aims to answer what genes are specifically expressed during colonisation of the phyllosphere. Cirvilleri and Lindow (1994) used random insertion of a promoterless *lux* gene into the genome of *P. syringae* to identify transposon mutants that were bioluminescent on leaf surfaces. This analysis revealed that about 3% of all *P. syringae* genes are expressed on the leaf surface. Independently,

Marco *et al.* (2003) came to a similar estimate using *in vitro* expression technology by which random fragments of the *P. syringae* genome were screened for their ability to complement a conditionally lethal phenotype on bean leaves. Using this strategy, called HIRS for habitat-inducible rescue of survival, Marco *et al.* (2005) were then able to describe a number of phyllosphere-induced loci with anticipated or presumed epiphytic functions such as water stress tolerance or utilisation of organic sulfur. Interestingly, several virulence-associated genes were also identified by the HIRS method, indicating that *P. syringae* expresses virulence factors during leaf colonisation. To some loci, no function could be assigned on the basis of sequence homology of DNA or protein, suggesting that yet-to-identify epiphitness traits may exist.

11.7 Microbial growth in the phyllosphere

11.7.1 Growth requirements

Individual epiphytic microorganisms have different nutritional requirements. For example, it does not take much for a bacterium to duplicate: it was calculated that the epiphytic bacterium *E. herbicola* needs 0.3 pg of sugar to double (Leveau and Lindow, 2001). But growth yield, that is, the efficiency with which food is converted into biomass, is not the only factor that determines the success of a leaf coloniser. There are three additional factors to be considered, namely versatility, affinity and growth rate. Microorganisms differ in the range of nutrients, for example, carbon sources that they can use for growth (Ji and Wilson, 2002). Comparison of the nutrient utilisation spectrum of microbial species with the presence or absence of specific nutrients on leaf surfaces could, in theory, predict whether a bacterium or fungus has the potential to grow in the phyllosphere. By comparing nutrient utilisation spectra of two or more different microbial species, it is possible to derive a so-called nutritional niche overlap index (Wilson and Lindow, 1994a; Ji and Wilson, 2002). It may be used as a predictor of co-existence or competition. A low index value, for instance, indicates that species vary greatly in their nutritional spectrum, so that there is a low probability for competition for the same nutrients and a high probability for co-existence. This has also been referred to as nutritional resource partitioning (Wilson and Lindow, 1994c). It should be noted that niche overlap indices may be influenced by external factors including water availability and temperature (Lee and Magan, 1999). Another factor determining growth in the phyllosphere is affinity, which divides microorganisms into those that can use nutrients at very low concentrations and those that cannot. This dichotomy probably overlaps for a large part with the separation of microbial epiphytes into so-called K- and r-strategists (Andrews, 1984). The K-strategists reproduce more slowly and tend to be successful in resource-limited situations, whereas r-strategists are characterised by a high growth rate and dominate in situations where nutrient resources are abundant.

11.7.2 *Types and sources of nutrients*

One of the most obvious sources of nutrients on the plant leaf and fruit surface is the cuticle. It consists of the cutin polymer and associated waxes with high carbon and energy contents, and so would seem a logical growth substrate. However, there is no evidence to support the theory that cuticle components are used by microorganisms for growth (Beattie, 2002). Exogenous nutrients may be available fortuitously in the form of pollen, honeydew, dust, air pollution or microbial debris (Stadler and Müller, 2000; Leveau, 2004). However, the major source of nutrients to microbial colonisers is represented by plant metabolites that leach from the leaf's interior to the surface. Leaching is a passive process, and is stimulated by the presence of water on the leaf, for example, in the form of rain drops or fog. Leaf leachates contain a variety of compounds (Tukey, 1970), but the most abundant are photosynthates such as glucose, fructose and sucrose (Fiala *et al.*, 1990; Mercier and Lindow, 2000; Leveau, 2004). Uninoculated bean plants in the greenhouse carry 0.2–10 micrograms of sugars per leaf (Mercier and Lindow, 2000), enough to support bacterial populations of 10^7 per leaf. The carrying capacity, that is, the maximum microbial population which a given leaf can support, is often correlated to the availability of sugars. This is in agreement with the observation that the availability of carbon, not nitrogen or phosphate, is generally limiting the sizes of bacterial populations in the phyllosphere (Wilson and Lindow, 1994a; Wilson *et al.*, 1995; Mercier and Lindow, 2000). Other perhaps less known examples of carbon sources in the phyllosphere are methanol and methylamine which are plant waste products that are preferentially used by PPFMs (Holland and Polacco, 1994), and the plant hormone indole-3-acetic acid which also has been shown to be a substrate for growth by phyllosphere bacteria (Leveau and Lindow, 2005).

11.7.3 *Nutrient bioavailability*

There are several lines of evidence to suggest that nutrients are not equally available on different leaves from the same plant or field, nor that they are evenly distributed across the surface of a single leaf. Uninoculated bean leaves contain on average 2.5 μg of surface sugar per gram of leaf (Mercier and Lindow, 2000), but this may vary by about 25-fold from leaf to leaf. The variation has been explained due to differences between leaves in, for example, cuticle leakiness (Mercier and Lindow, 2000), photosynthesis rates, for example, as a result of positional effects (Fiala *et al.*, 1990), leaf age or plant nutrition. After inoculation and incubation of bean plants with the bacterium *P. fluorescens*, original sugar abundances were decimated, suggesting (1) that bacteria utilise sugars and (2) that any new immigrants would face nutritional conditions different from those the first immigrants did. Interestingly, a residual amount of sugars remained on the leaf surface, even after the bacterial populations reached carrying capacity (Mercier and Lindow, 2000). This has been explained by the heterogeneous distribution of sugars, that is, some sugars remain unavailable to the bacteria. By using the size of individual cells as an

indicator for trophic status, it has been shown that *P. syringae* bacteria on a single bean leaf experience very different nutrient bioavailabilities (Monier and Lindow, 2003b). Bacteria near glandular trichomes or veins were larger than those located elsewhere, suggesting that such sites offer more nutrients. Glandular trichomes have been shown to secrete a number of plant compounds such as sugars, proteins, oils, secondary metabolites and mucilage, all of which may contribute to microbial growth. In addition, their ability to retain water would favour local leaching of nutrients.

A pattern of high heterogeneity in sugar availability has been demonstrated with bacterial bioreporters for fructose. It was estimated that newly arrived *E. herbicola* bacteria on a bean leaf were exposed to local initial fructose abundances ranging from less than 0.15 pg to more than 4.6 pg fructose (Leveau and Lindow, 2001). Uneven leaf surface distribution applies not only to fructose but also to other nutrients such as for example sucrose (Miller *et al.*, 2001) and iron (Joyner and Lindow, 2000). One of the ways that epiphytic bacteria deal with low iron availability is by the production of siderophores (Loper and Buyer, 1991). These low-molecular-weight molecules chelate ferric ion and after recognition by specialised receptors are taken up by the bacteria. The molecular biology of siderophore production and recognition has been exploited to construct iron-responsive bioreporter strains of *Pseudomonas* species (Loper and Lindow, 1994; Joyner and Lindow, 2000). Using the *inaZ* reporter gene, it was possible to estimate that the average bacterial cell in the phyllosphere experienced low-iron conditions (Loper and Lindow, 1994). A similar result was obtained with a *gfp*-based iron bioreporter (Joyner and Lindow, 2000), but because GFP allows for the interpretation of reporter activity in individual cells, it was possible to show that actually there existed substantial microscale heterogeneity in iron availability in the phyllosphere.

11.8 Microbial interactivities in the phyllosphere

11.8.1 Niche modification

Niche modification represents one of the microbial strategies to change local conditions away from the harsh environment that characterises the phyllosphere. The production of the plant hormone indole-3-acetic acid (Brandl *et al.*, 2001) or the phytotoxin syringomycin (Lindow and Brandl, 2003) by bacterial leaf colonisers is thought to stimulate the localised release of nutrients by the plant. Another strategy to increase nutrient availability is by altering cuticular surface permeability (Schreiber *et al.*, 2005), or by decreasing the contact angle of sessile droplets on the cuticular surface (Knoll and Schreiber, 2000). The latter stimulates leaf wetting, for example, by the production of surfactants (Bunster *et al.*, 1989), thereby facilitating the leaching of nutrients (Knoll and Schreiber, 2000; Chapters 7 and 8).

Extracellular polysaccharides are quite commonly used by phyllosphere bacteria to firmly attach to the cuticular surface. They may in addition serve to improve

living conditions, for example, to protect from desiccation, trap nutrients, as a barrier against chemical, biological or environmental stresses or as a matrix for the communication via small diffusible molecules such as AHLs (see Section 11.8.4).

11.8.2 Competition

Competition for nutrients is thought to be most fierce when nutrients are scarce. Evidence for nutrient competition in the phyllosphere comes from studies that demonstrate the principle of pre-emptive competitive exclusion (Lindow and Leveau, 2002), that is, the ability of an established microbial epiphyte to inhibit the development of a population of a second strain on leaves (Kinkel and Lindow, 1993). This principle is based on nutrient depletion and has been applied as a biocontrol strategy (Section 11.9.3). So-called r-strategists (Section 11.7.1) are more affected by competition than K-strategists, and strategies based on pre-emptive competitive exclusion are thought to be more effective in controlling the former than the latter (Marois and Coleman, 1995).

11.8.3 Antibiosis

Several epiphytic yeasts have been shown to produce antibacterial compounds (McCormack *et al.*, 1994), and antifungal activities have been attributed to epiphytic bacteria (Giesler and Yuen, 1998; Nair *et al.*, 2002; Collins *et al.*, 2003; Daayf *et al.*, 2003). Antibiosis, however, has not been explicitly demonstrated to be a major mechanism in the interaction between bacteria on leaf surfaces, despite observations that antibiosis of bacterial epiphytes can be demonstrated in the laboratory (Lindow, 1988). It could well be that under such conditions production of and sensitivity towards antibiotics is different than under phyllosphere conditions.

11.8.4 Communication

Many plant-associated bacteria produce quorum sensing signals, such as AHLs (Cha *et al.*, 1998). These allow for the indirect sensing of population density and density-dependent control of gene expression (Juhás *et al.*, 2005). In the epiphytic bacterium *P. syringae*, the production of AHLs is regulated in a complex and hierarchical manner (Quiñones *et al.*, 2004), involving the GacS/GacA two-component system (Section 11.6.3). In the model proposed by Quiñones *et al.* (2004), the *ahlI* gene, which codes for the synthesis of 3-oxo-C6-homoserine lactone, is positively regulated by the AhIR protein in combination with 3-oxo-C6-homoserine lactone, resulting in a typical positive feedback (auto-induction). The enhanced survival of bacterial cells in densely packed aggregates on leaf surfaces seems to suggest that many epiphitness traits may be controlled in a cell-density-dependent manner. An *ahlI*⁻ *ahlR*⁻ double mutant of *P. syringae* had a reduced epiphitness on dry leaves compared to the wildtype (Quiñones *et al.*, 2004), suggesting a role for quorum sensing in withstanding desiccation stress in the phyllosphere.

11.8.5 Gene exchange

Many bacterial epiphytes have been shown to carry plasmids (Kobayashi and Bailey, 1994; Sundin *et al.*, 2004). Together with transposons, these constitute the so-called horizontal gene pool (Bailey *et al.*, 2002). This gene pool often confers traits that promote survival in the phyllosphere, including virulence factors (Sundin *et al.*, 2004) and resistance to antibiotics such as tetracycline which are sprayed in apple orchards (Schnabel and Jones, 1999). Cuticular surfaces are hotspots for gene exchange and have been called ‘breeding grounds for microbial diversity’ (Lindow and Leveau, 2002). The aggregated nature of bacterial cells on the cuticular surface is believed to play a key role in the efficiency of plasmid transfer. Rates of plasmid transfer on bean leaf surfaces were 30-fold higher than on membrane surfaces (Normander *et al.*, 1998). Using a reporter gene system that is based on de-repression of GFP expression in plasmid recipients, plasmid transfer has been observed in situ on leaf surfaces (Normander *et al.*, 1998). With this bioreporter, it became clear that plasmid exchange occurs not randomly, but primarily in junctures between epidermal cells and in substomatal cavities. Apparently, plasmid exchange did not require the bacteria to be metabolically active (Normander *et al.*, 1998), although other environmental factors such as water availability do seem to matter (Björklöf *et al.*, 2000).

11.9 Biocontrol in the phyllosphere

11.9.1 Phyllosphere diseases

Examples of phyllosphere microorganisms that harm plants are plenty. Some, such as *Pseudomonas* and *Erwinia* species, cause frost injury through biological ice nucleation (Lindow, 1983). Bacterial ice nucleation has been shown to be mediated by bacterially produced proteins that serve as nucleators for ice formation at subzero temperatures. Genes encoding ice nucleation activity (*ina*) have been isolated from *P. syringae*, *P. fluorescens*, *E. herbicola*, *E. ananas* and *X. campestris* among others. Sequence analyses suggest that *ina* genes have a common ancestor, but the selective advantage of ice formation to bacteria is still unknown; perhaps they benefit for some unidentified reason from frost injury to plants (Hirano and Upper, 2000). Several bacteria and fungi can produce phytohormones which may disrupt normal plant functioning and cause growth deformation, such as leafy gall on sweet pea caused by *Rhodococcus fascians* (Vandeputte *et al.*, 2005). Other bacteria affect plant productivity by the formation of leaf spots or lesions, or by inducing leaf blight or curling (Agrios, 1997). Some examples of foliar fungal diseases (and their causative agents) are rice blast disease (*Magnaporthe grisea*), downy mildew of grape (*Plasmopara viticola*) and powdery mildew affecting all kinds of plants (different fungal species). Interestingly, no yeasts are known to cause foliar disease (Agrios, 1997). Most common bacterial pathogens of the phyllosphere include members of the genera *Erwinia*, *Pseudomonas* and *Xanthomonas*. Different pathovars of

P. syringae cause leaf spots and blights on tobacco, cucumber, bean, lilac or tomato, while *X. campestris* pathovars affect bean, cotton, rice cereals, tomato or pepper.

A growing concern is the occurrence of microorganisms such as *Salmonella* and *Campylobacter* species in the phyllosphere of produce. By definition, these bacterial residents of cuticular surfaces are not epiphytic, as they are usually not capable of multiplying, yet they are apparently quite able to survive for prolonged periods of time on leafy vegetables (Brandl and Mandrell, 2002), posing a health danger to human consumers.

11.9.2 What makes a plant pathogen?

There are many different ways in which pathogenic microorganisms attack plants. Common themes, however, are invasion of the plant tissue, nutrient acquisition and counteracting plant defence reactions (Agrios, 1997). The mechanisms that underlie pathogenicity involve, among others, degradation of plant structural components (e.g. cuticle, cell walls) by enzymatic activity (e.g. cutinase, pectinase, cellulase), the production of phytotoxins (e.g. tabtoxin, syringomycin), injection of effector molecules (e.g. type III secretion in certain bacteria) and modulation of phytohormone levels (e.g. indole-3-acetic acid) to exploit plant physiology. In bacteria, pathogenicity factors such as production of plant growth hormones, phytotoxins and other virulence genes are often plasmid-borne (Bailey *et al.*, 2002), suggesting a role of the horizontal gene pool in the evolution and spreading of disease.

Many bacterial plant pathogens differ from non-pathogenic epiphytes in having the ability to colonise also the interior of leaves, thereby avoiding the stresses associated with the cuticular surface. These internal populations are generally believed to be responsible for disease induction, and the larger the internal population size, the more likely it is that disease symptoms occur (Beattie and Lindow, 1999). The bacterium *Xanthomonas campestris* avoids the leaf surface and actively seeks the interior of the leaf (Hugouvieux *et al.*, 1998) where it reaches high population sizes that may egress to the leaf surface. Other bacterial pathogens, such as *P. syringae* generally establish large surface populations before they ingress into the leaf's interior and proliferate there (Hirano and Upper, 2000). For the latter type of pathogens, leaf surface population sizes are predictive of the probability of disease occurrence (Beattie and Lindow, 1999). For both types of pathogens, these external populations are probably important sources of inoculum for emigration to other plant leaf surfaces (Upper and Hirano, 2002).

11.9.3 Strategies for biocontrol

Strategies for biocontrol of foliar diseases are often based on the prevention of establishment of the pathogen in the phyllosphere. Spraying leaves with spores of common phyllosphere fungi such as *Alternaria*, *Cochliobolus*, *Septoria* and *Phoma* has been shown to reduce fungal foliar diseases (Agrios, 1997). Yeasts have been successfully used as antagonists of pathogenic fungi (Fiss *et al.*, 2000; Avis and

Bélanger, 2002; Buck, 2002; Urquhart and Punja, 2002) and bacteria (Assis *et al.*, 1999), while bacteria have been shown to control disease symptoms caused by fungi (Kucheryava *et al.*, 1999; Zhang and Yuen, 1999; Nair *et al.*, 2002; Collins *et al.*, 2003) and bacteria (Volksch and May, 2001; Stromberg *et al.*, 2004). Some mixtures of biocontrol agents show more anti-fungal activity than the single strains alone (Guetsky *et al.*, 2002). The mechanisms underlying some of these strategies are not always clear. Mycoparasitism, that is, fungal attack on fungi, is an effective biocontrol mechanism which is best described for *Trichoderma* species (Bélanger and Avis, 2002). Some biocontrol agents have been shown to induce systemic resistance in plants (Bargabus *et al.*, 2002). Others produce antibiotics (Giddens *et al.*, 2003), but antibiosis in the laboratory is not necessarily a guarantee for success in the field (Lindow, 1988).

Another strategy for biocontrol is based on the principle of pre-emptive competitive exclusion which assumes that growth in the phyllosphere is limited by the availability of nutrients. When plants are deliberately inoculated with a non-pathogen (the biocontrol agent) which will use up most of the nutrients, any immigrating pathogen will find itself unable to grow and form sufficiently large population sizes to cause damage. Naturally occurring non-ice-nucleating (Ice^-) strains of *P. syringae* and *E. herbicola* applied pre-emptively to plants prevented successful colonisation by Ice^+ *P. syringae* strains and reduced the severity of frost injury (Wilson and Lindow, 1994a). Crucial to success of this strategy is the fact that the nutrient overlap indices of the pathogen and the biocontrol agent are quite similar. However, nutrient-overlap indices are not always predictive for the ability of a non-pathogen to control disease in the laboratory or in the field (Ji and Wilson, 2002).

11.10 Future directions of phyllosphere microbiology

Historically, much of the research on microbial communities in the phyllosphere has been driven by the desire to understand the biology and ecology of microorganisms that are harmful to aerial plant parts. This trend is obvious from the bias, even in this chapter, towards our knowledge and understanding about plant pathogens. Clearly, the attention is slowly shifting in favour of non-pathogenic inhabitants of primary plant surfaces, not only because they have the potential to affect the function of phyllosphere pathogens, but also because they hold many undiscovered traits of adaptation to life on cuticular surfaces. The use of reporter genes such as *gfp* for the first time has opened up the possibility to study microbial epiphytes as individuals, and has fuelled the realisation that microorganisms operate at micrometre scales, and that the cuticular surface at that level of magnification is a highly heterogeneous environment. The ability of studying epiphytic individuality needs to be exploited much more than it has been already: it may help to explain phyllosphere-related phenomena for which there are currently no good explanations if one continues to stick to the traditional, that is, macroscale, view of single leaves as operational units.

Furthermore, as more and more microbial interactivities in the phyllosphere are revealed and in ever more detail, their interconnected complexity will soon reach (or has already reached) a level where the interpretation of experimental results through linear thinking is no longer realistic. Instead, phyllosphere microbiologists will need to rely more on predictive and interpretive modelling, for which at this point much more quantitative data is needed, both on leaf surface structures and properties and on microbial (inter)activities in the phyllosphere. Also, the cautious embrace by phyllosphere microbiologists of culture-independent methods will need to mature into a full-scale exploitation of the molecular toolbox, including the application of such exciting new technologies as metagenomics analysis.

As a final point, phyllosphere microbiology has entered the genomic era with the sequencing of whole-genomes of several plant-pathogenic, epiphytic lifestyle microorganisms. Many more genomes of phyllosphere microorganisms will become available in the next decade, and it will be a huge and incredibly exciting challenge to explain this enormous wealth of sequence information in the light of the experimental data from the 50 years of pre-genomic phyllosphere microbiology research.

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12 Filamentous fungi on plant surfaces

Tim L.W. Carver and Sarah J. Gurr

12.1 Introduction

Huge numbers of filamentous fungi spend at least part of their lives on the subterranean or aerial surfaces of the myriad plant species in existence. Almost nothing is known about most of these relationships, and little is known about the majority of the remainder. Here, therefore, we shall focus on relatively few of the better-studied interactions and restrict our consideration to relationships between leaf surface characteristics and pathogenic leaf-infecting fungi, concentrating on obligate biotrophic and hemi-biotrophic fungi. Further, we shall restrict our thoughts to those fungi which have evolved relatively sophisticated relationships with their hosts, infecting them via specialised infection structures known as appressoria.

Key features of mature germlings developed from the unicellular spheroidal conidia of *Blumeria graminis* (the cereal and grass powdery mildew fungus), *Erysiphe pisi* (the pea powdery mildew fungus) and the multicellular, pyriform conidia of *Magnaporthe grisea* (the rice blast fungus) are illustrated in Figure 12.1. We shall draw both on our own experiences researching early interactions involving *B. graminis* and on the wealth of information from additional systems including pea powdery mildew (*E. pisi*), grape black rot (*Phyllosticta ampellicida*), rusts of mono- and dicots (*Puccinia* and *Uromyces* spp.), rice blast (*M. grisea*) and diseases caused by *Colletotrichum* species.

Compared to the relatively consistent subterranean environment, fungi that infect leaves are likely to have to survive rapid and dramatic fluctuations in temperature, humidity and light as well as having to avoid being dislodged or damaged by direct and indirect physical forces imposed by wind and rain. Plant surface characteristics are vitally important during the earliest stages of pathogen development before disease becomes established, and although they may have some influence thereafter, in most cases this is likely to be relatively small and has been little studied. We shall concentrate, therefore, on the influence on leaf surface features during the phase from spore deposition up to the time when the fungus enters the leaf.

The resources carried by fungal spores are finite and must be used effectively and efficiently during the time taken to germinate, differentiate infection structures, invade the host and gain access to host-derived nutrient sources. The fungal germling/leaf surface interaction presents something of a paradox. On one hand, the cuticle and underlying epidermal cell wall present an immediate and formidable barrier to infection. On the other hand, many fungi have apparently evolved dependence upon physical, topographical and chemical information contained within their

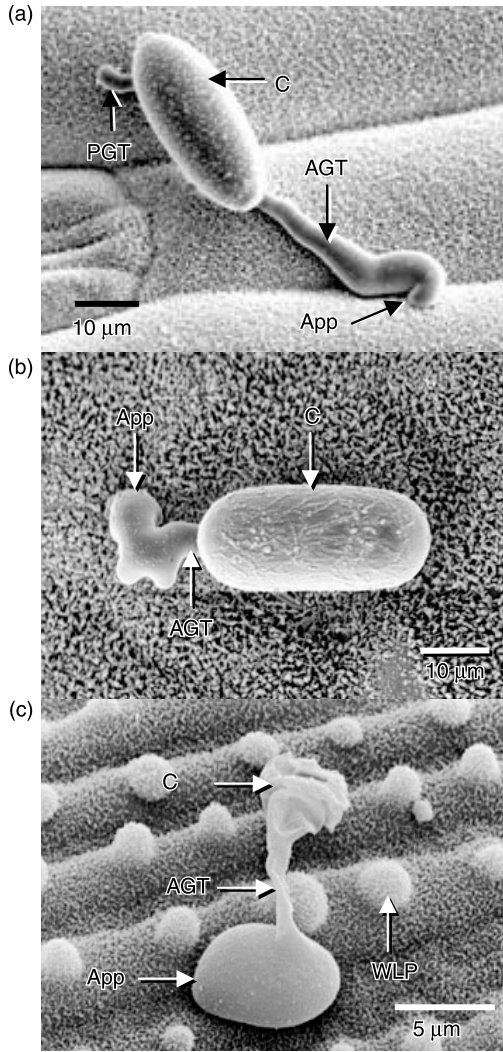


Figure 12.1 Cryo-SEM images of mature appressoria formed by the single-celled conidia of *Blumeria graminis* (on oat) and *Erysiphe pisi* (on pea) and the multicelled conidium of *Magnaporthe grisea* (on rice). Note, conidia of *B. graminis* and *E. pisi* show domed and ribbon-like surface projections, respectively. (a) *B. graminis* conidia (C) uniquely form a short primary germ tube (PGT) at around 1 h after inoculation before the appressorial germ tube (AGT) emerges and elongates to ca 40 μm before differentiating a simple, hooked appressorium (App) by 8–10 h. The PGT and App frequently form on different epidermal cells. (b) The AGT of *E. pisi* grows little before differentiating a multi-lobed appressorium by around 4 h. (c) The conidium and AGT of *M. grisea* collapse as the cytoplasm migrates into the domed appressorium. Micrograph kindly supplied by N.J. Talbot and G. Wakely (Exeter University). Note, the rice leaf has many wart like protuberances on which conidia are often deposited.

host plant surface to stimulate activities and provide the signals regulating fungal responses driving the complex developmental processes which are pre-requisite to infection.

12.2 Adhesion prior to germination

12.2.1 *The environment, leaf surface characteristics and spore shape*

Spores are not only dispersed by wind and rain but they also may be displaced by their action after delivery to potential infection sites. Water can wash away deposited spores and rain drop impact causes radial air movements that may briefly exceed 150 km h^{-1} over a short distance (Hirst and Stedman, 1963). Due to the boundary layer, direct wind effects are likely to be small close to leaf surfaces but wind shake can generate considerable force (Bainbridge and Legg, 1976; Wright *et al.*, 2002a). It is imperative for survival, therefore, that ungerminated spores become rapidly attached to their host, and plenty of evidence indicates that this occurs.

Gross effects of plant architecture (erect or prostrate habit) and canopy density can obviously affect spore deposition, but minute topographic leaf surface features may also influence spore adhesion and retention considerably. At a relatively crude level, leaf surfaces vary considerably in the abundance and form of trichomes and other epidermal protuberances, and becoming trapped on these structures can prevent spore deposition on the epidermal surface proper. Further, the outer epidermal cell wall is generally not flat but is convex in at least one dimension while anticlinal cell wall junctions form troughs. At the ultrastructural level, leaves are generally covered in hydrophobic epicuticular waxes that are frequently crystals which present minute outer tips or edges (see Chapter 1). Thus, a relatively large spore may be supported on the extremity of very few wax crystals so that the actual interface between surfaces is miniscule (Figure 12.2a). The geometry of the spore-surface interface is also important. Spore shape varies enormously from single-celled spheroids (e.g. *B. graminis* conidia) to more complex multicelled structures (e.g. *M. grisea* conidia). Some, e.g. *Colletotrichum graminicola* conidia, are sickle-shaped (falcate), and this can increase potential interfacial contact with plant cell surfaces. The concave profile of these conidia would 'fit' better to the convex plant cell surface, its convex profile would fit best when deposited across the trough between adjacent epidermal cells and its more linear, lateral profile would fit a flatter surface such as offered by the long axis of a graminaceous epidermal cell. In any case, axial rotation of a falcate spore settling from a dispersing water film would maximise its contact area with the plant surface. Although some spores appear smooth (e.g. *C. graminicola*), many show pronounced surface ornamentation such as the surface projections of *B. graminis* conidia or the spines of rust urediniospores. In these cases the interface may be restricted to a few minute points of contact between spore surface ornaments and leaf wax crystals (Figure 12.2a). In such cases, extremely efficient means of adhesion must come into play.

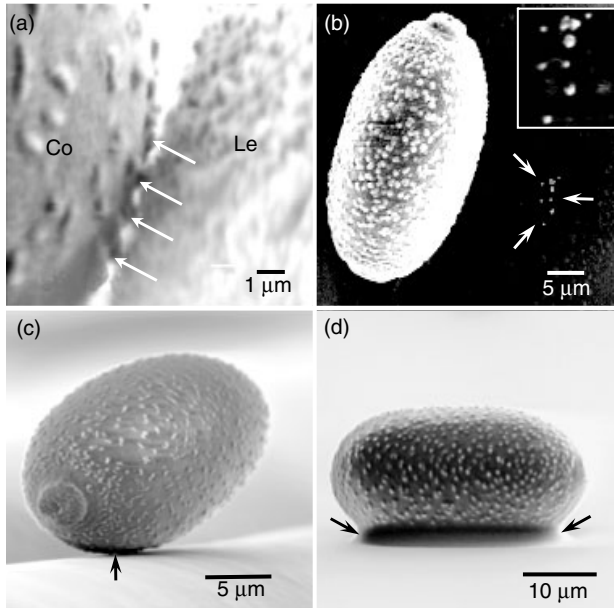


Figure 12.2 Cryo-SEM images of *Blumeria graminis* conidia on host leaves and an artificial substratum. (a) After 60 min on a barley leaf with epicuticular wax in place. Conidium (Co) to left and leaf (Le) to right. Contact is limited to the tips of a few conidial surface projections touching the raised edges of leaf wax crystals (white arrows) and intervening spaces are clear (from Carver *et al.*, 1999). (b) After 1 min incubation on a de-waxed leaf the cryofixed spore was displaced by micromanipulation to reveal the original contact site (white arrows) where minute droplets of extracellular material (ECM, inset) were released from conidial surface projections that made leaf surface contact (from Wright *et al.*, 2002b). (c) If conidia are not displaced, the minute droplets of ECM cannot be discerned at the contact site (black arrow) even after 15 min incubation on a de-waxed leaf. (d) On planar, hydrophobic artificial substrata (here silanized plastic), copious ECM is released and within 15 min fills the entire conidium/substratum interface.

12.2.2 *The challenges and nature of spore adhesion*

Foliar pathogens must adhere to the hydrophobic, waxy leaf surface, and many studies using artificial substrata show a strong relationship between the occurrence and/or strength of adhesion and hydrophobicity, while spores of many species fail, or show much reduced capacity, to stick to hydrophilic surfaces (e.g. Hamer *et al.*, 1988; Clement *et al.*, 1993; Terhune and Hoch, 1993; Kuo and Hoch, 1996). As Jones (1994) noted, and discussed in some detail by Kuo and Hoch (1996), both fungal spores and plant surfaces carry a negative charge and electrostatic repulsion must result. This may be counteracted by various means including hydrophobic interaction, van der Waal’s attraction forces and hydrogen bonding. These forces might simply help spores remain in place over the short term. After a brief period in contact with a leaf, however, spores of many phytopathogenic fungi release adhesive extracellular materials (ECM) and relevant work has been reviewed in

detail (Nicholson, 1996; Epstein and Nicholson, 1997; Kunoh *et al.*, 2001, 2004; Tucker and Talbot, 2001). Nicholson (1996) points out that with the exception of the powdery mildew fungi most spores commence their host association in the presence of free-water in which case adhesives must be water-insoluble or rapidly become so after release. The precise chemistry of spore adhesives varies greatly between fungi although they commonly contain glyco-proteins, lipids and polysaccharides and in some cases enzymes such as cutinase. Jones (1994) considered there to be two basic strategies for spore adhesion, namely 'passive' and 'active'. Most workers have adopted this distinction, which regards passive adhesion as resulting from the presence of pre-formed material on or within the spore, while active adhesion requires the *de novo* production of adhesive.

12.2.3 Examples of passive and active adhesion and combinations of the two

The timing of adhesion merits attention because it conveys information about the passive or active nature of underpinning adhesion mechanisms. An example of extremely rapid attachment by passive means is shown by *P. ampellicida* pycnidiospores which, in an acidified aqueous environment, adhere firmly to hydrophobic but not hydrophilic artificial substrata within milliseconds, even if they are killed before inoculation (Kuo and Hoch, 1996). In this case, the role of spore ECM is unclear although the extracellular conidial sheath appears to mediate initial attachment and probably plays a role in selection for substratum specificity (Kuo and Hoch, 1995; Shaw and Hoch, 1999). However, a classic case of passive adhesion due to the release of ECM is shown by conidia of *M. grisea* (Hamer *et al.*, 1988; Howard, 1997). In dry conidia, a large periplasmic deposit lies within the cell wall at the tip of the apical cell of the pyriform conidium, and this is released instantly upon hydration as 'spore tip mucilage'. It contains α -linked-mannosyl and glucosyl residues, protein and lipids, and adheres immediately and robustly to Teflon (hydrophobic) though less strongly to glass (relatively hydrophilic). This requires no post-deposition energy expense, and if (see later) the material makes plant surface contact, it may be expected to convey fast and effective spore retention.

Scanning electron microscopy of *C. graminicola* conidia shows that these also release ECM where their tip contacts maize leaves and along their entire region of contact with artificial hydrophobic substrata (Mercure *et al.*, 1995). This ECM contains glycoprotein (Sugui *et al.*, 1998) that was shown to play a role in active adhesion since it was abolished by addition of pronase and impeded by inhibitors of glycoprotein transport and protein synthesis (Mercure *et al.*, 1994). Although reasonably rapid, maximum adhesion was not attained until around 30 min after contact, presumably reflecting the time taken for *de novo* synthesis and accumulation of the adhesive.

Urediniospores of the rust fungi probably depend on a sequence of passive and then active mechanisms to effect increasingly strong adhesion as time passes. Thus, evidence from Clement *et al.* (1994) suggests that the spine-like ornaments of *U. viciae-fabae* urediniospores are invested in a hydrophobic lipid-containing

sheath that promotes immediate, passive adhesion to hydrophobic substrata. This hydrophobic interaction is sufficient for spore retention during the more lengthy processes involved in imbibition of water that is a prerequisite for rust urediniospore germination. Furthermore, Clement *et al.* (1997) showed that although imbibition by spores is almost instant in the presence of free water, in its absence, even at 100% relative humidity, spore hydration awaits accumulation, by capillary condensation, of water that forms a 'condensation pad' at the spore-substratum interface, and this may take many hours. In the early stages, this condensate can be removed entirely by freeze drying, but capillary force generated by the developing condensation pad may itself confer adhesive force to support the hydrophobic interaction. However, as the spore hydrates the pad cannot be removed by freeze-drying, indicating the accumulation of non-volatile components in a structure termed the 'adhesion pad'. Deising *et al.* (1992) had shown previously that while pads form beneath living and autoclaved *U. viciae-fabae* spores, only living spores adhere strongly to a leaf surface. They provided good evidence that this was due to the presence of a cutinase and two non-specific serine esterases present on the surface of living spores. Evidently, these enzymes were released into the adhesion pad following spore hydration to interact with the host surface and cause adhesion. This, they considered, represents an active process.

The simple single-celled conidia of the powdery mildew fungi favour humid conditions but unlike the cases discussed so far, the spores can be damaged by free water. Amongst the powdery mildews, adhesion by *B. graminis* has been relatively well studied but it remains unclear whether this is solely passive or also incorporates active processes. The native spore surface is hydrophobic (Nicholson *et al.*, 1993) and so hydrophobic interactions may be expected to lead to immediate passive adhesion. However, when on a true leaf surface, the conidia almost instantaneously release minute droplets of ECM that appears to emanate from the few surface projections (ornamentations that cover the spore surface) making leaf surface contact (Figure 12.2b; Wright *et al.*, 2002b). Centrifugation studies (Wright *et al.*, 2002a) show that within 10 min of deposition (the quickest it was possible to make a test) about 80% of conidia remained attached to leaves even when subject to about 4.2×10^{-9} N force, which is far greater than likely to be generated by wind-shake of crop leaves. Such strong adhesion was attributed to effects of the conidial ECM since it is known to contain non-specific esterases and a cutinase (Nicholson *et al.*, 1988; Pascholati *et al.*, 1992), isolated ECM is able to degrade host surface features (Kunoh *et al.*, 1990), and similar enzymes are involved in adhesion by *U. viciae-fabae* (earlier). The uncertainty of whether this enzyme-associated adhesion is simply a passive process involving the release of pre-formed ECM components stems from data (Nicholson *et al.*, 1988) showing that non-specific esterase release on artificial substrata is a two-phase process. The first occurs within 2 min of contact and is unaffected by metabolic inhibitors but the second, occurring after about 15 min, is inhibited by cycloheximide indicating *de novo* synthesis. It now seems possible that this second phase is not induced on true plant leaves. First, Wright *et al.* (2002b) obtained no evidence for increase in the number or size of

ECM deposits from the first minute up to 12 h after deposition. Second, Wright *et al.* (2002a) found little evidence for increased adhesion to barley leaves between 10 and 30 min after spore deposition, whereas, by contrast, on hydrophilic glass there was a substantial increase in adhesion during this time and the quantity of deposited ECM continued to increase between 1 min and at least 3 h (Wright *et al.*, 2002b). These observations indicate, therefore, that whereas contact with an artificial substratum may induce the second phase of ECM release, only the first phase, involving release of preformed ECM, occurs on the host leaf surface. This would indicate passive adhesion to leaves.

12.2.4 *Plant surface versus artificial substrata*

While artificial substrata offer a valuable means of simplifying systems for experimental studies, the previously mentioned experiences using *B. graminis* indicate the potential danger of extrapolating from observation of spore behaviour on artificial substrata to the natural situation. In fact, previous studies of *B. graminis* had already indicated a problem of using artificial substrata to study conidial ECM release. Thus, while it was impossible even by low-angle, low-temperature SEM to resolve ECM deposits beneath in-place conidia on leaves (Figure 12.2a), even with epicuticular wax removed (Figure 12.2c), extraordinarily large accumulations were evident after as little as 15-min incubation on the silanized surface (hydrophobic) of planar glass or plastic (Figure 12.2d; Carver *et al.*, 1999). In part this might be explained by the geometry of the interface: on the planar surface a larger number of ECM-releasing conidial surface projections would make contact with the substratum than when the ellipsoidal spore is in contact with the convex epidermal cell surface (Wright *et al.*, 2002b). The additional capillary forces brought into play on the planar surface might then draw the spore closer to the surface, bringing more conidial projections into contact, causing more ECM to be released. The idea that interfacial geometry is important is supported by subsequent observations showing copious accumulation of ECM on flat, isolated leaf cuticle (Fujita *et al.*, 2004a).

A further case indicating the necessity of considering the nature of the true leaf surface came from recent observations of *M. grisea* on rice leaves (Koga and Nakayachi, 2004). As described previously, earlier studies (Hamer *et al.*, 1988) indicated the potential of *M. grisea* spore tip mucilage to adhere to hydrophobic surfaces and this is seen as a classic example of passive spore adhesion. This fungus has a wide host range and it may be true that on many host species this mucilage acts as a spore adhesive. However, Koga and Nakayachi (2004) pointed out that the leaf surface of rice (*Oryza sativa* L., the most economically important host of *M. grisea*) is densely covered by 'wart-like' protuberances of the epidermis (Figure 12.1c). They found that spores were generally deposited on these protuberances and so had no contact with the underlying surface while their tip cell often had no contact with any plant structure. Thus, when spore tip mucilage was released upon hydration, it failed to make plant surface contact, conidia failed to adhere and for at least 1 h after deposition virtually all could be washed away simply by dipping

into water. In this circumstance, adhesion of ungerminated spores to the rice leaf is at best very weak and certainly trivial compared to that conferred when spore tip mucilage is released onto an artificial planar surface. Strong adhesion to the rice leaf apparently depends on later contact by the fungal germ tube (Koga and Nakayachi, 2004).

12.2.5 *Effects of ungerminated conidia on underlying host cells*

Few studies of spore ECM have considered its potential effects on underlying host cells although several early reports from the powdery mildew/barley system have suggested this possibility (see Fujita *et al.*, 2004b). Direct evidence for an effect was provided recently by Fujita *et al.* (2004a) who allowed barley epidermal cells to have contact for less than 1 h with conidia of the non-pathogenic powdery mildew species *E. pisi* (the pea mildew pathogen) or *B. graminis* f.sp. *tritici* (a pathogen of wheat but not barley). During this time, conidia released ECM onto epidermal cells but they were removed before germinating. Epidermal cells that received ECM showed 'induced inaccessibility' being far more resistant to later attack by the pathogenic barley mildew fungus (*B. graminis* f.sp. *hordei*). Importantly, however, ECM from pathogenic conidia (*B. graminis* f.sp. *hordei*), had no detectable effect. Thus, this study showed perception and response of plant cells to component(s) of ECM released by the non-pathogenic fungi, but also indicated either the absence of inductive component(s) in the true pathogen's ECM or the presence of a suppressive factor. It also implied that fungal ECM component(s) move into the plant cell. This could be facilitated by modification of the plant cell surface not only by the action of cuticle-degrading enzymes (mentioned earlier) but also by cell-wall-degrading enzymes known to be present in *B. graminis* conidial ECM (reviewed by Kunoh *et al.*, 2004). We now have evidence for such movement.

As mentioned by Kunoh *et al.* (2004), Thomas and colleagues (unpublished) used immunogold labelling to trace distribution of a monoclonal antibody (MAb AF-BBG1) raised against surface washings of *Aspergillus flavus* (by M.F. Dewey, Oxford) and applied to sections of *B. graminis* conidia incubated for 30 min on oat leaves. The exact chemistry of the target antigen remains to be determined, but MAb AF-BBG1 has been shown to recognise commercially available cutinase and component(s) of *B. graminis* conidial cytoplasm, cell wall and ECM; importantly, it recognises no constituent of healthy cereal leaves (T. Gjetting, personal communication). Figure 12.3 shows that within 30 min of inoculation the antigen was present not only in the spore and its ECM released at the site of leaf contact, but also within the wall and cytoplasm of the plant epidermal cell. It is unknown whether this particular component directly affects plant cell activity, but the observation demonstrates the principle that a spore component may pass into plant cells prior to germination. It would be surprising if this had no effect on those cells. Clearly, consideration of spore ECMs should include not only their role in adhesion but also their possible influences on plant cells which may express enhanced resistance (or perhaps susceptibility?) to subsequent attempted infection.

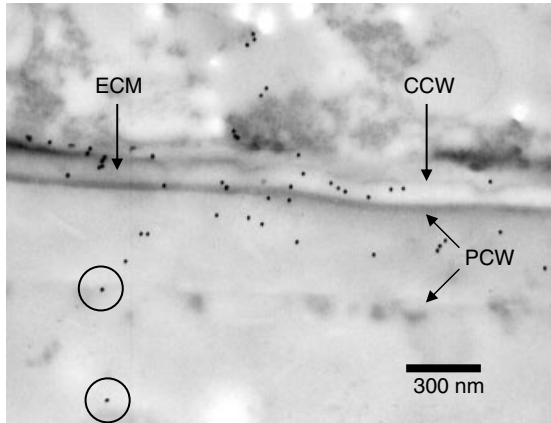


Figure 12.3 Transmission electron micrograph of the interaction site between a *Blumeria graminis* conidium and an underlying oat leaf epidermal cell 30 min after inoculation and following immunogold labelling with MAb AF-BBG1. The antigen is present not only within the conidium, its cell wall (CCW) and ECM, but gold label indicates that it has moved into the plant cell wall (PCW) and underlying cytoplasm (circled gold particles).

12.3 Influence of leaf surface characteristics on spore germination

There is little evidence of whether leaf surface characteristics directly influence germination by spores of obligate biotrophic and hemi-biotrophic fungi. For *P. ampellicida*, whose spores germinate in an aqueous environment, Kuo and Hoch (1996) showed that spore adhesion to a substratum was a mandatory prerequisite for germination. However, this requirement has not been clearly demonstrated for any other fungus and it is unknown whether leaf surface features may impede adhesion by *P. ampellicida* spores and hence influence germination. It has also been shown that germination by *C. gloeosporoides* is stimulated by contact with surface wax of avocado (Podila *et al.*, 1993) but the specificity and basis of this effect is unclear.

For the cereal powdery mildew fungi that favour a water-free environment, a number of early studies (e.g. Carver and Adaiqbe, 1990 and work cited therein) suggested that leaf age, position and host genotype may all affect germination by *B. graminis*, but these relatively small effects remain unexplained. However, leaves of oat, barley and wheat are all covered by crystalline plate-like waxes and it is possible that the nature of these waxes changes as leaves age (e.g. Riederer and Markstädter, 1996) and varies between genotypes, and it is possible that these factors influence *B. graminis* germination. Thus, Carver and Thomas (1990) showed that removal of these waxes prior to inoculation can significantly reduce germination frequency. Although this relatively crude approach suggested an influence of epicuticular wax, removal of the crystalline plates would have altered the fine details of the contact interface between spore and leaf surface (see Figure 12.2a–c), and the physical consequences of this are unknown.

A far more subtle evaluation of epicuticular wax influence was recently undertaken by Gniwotta *et al.* (2005) in the pea (*Pisum sativum* L.)–powdery mildew (*E. pisi*) system. Under identical environmental conditions, germination by *E. pisi* conidia was significantly higher on the adaxial (80%) than abaxial (57%) leaf surface although even here germination was higher than on hydrophilic or hydrophobic glass (49% germinated). This indicates not only that germination is specifically promoted by plant surface signals, but also that the efficacy of these signals is greater on the adaxial surface. Furthermore, Gniwotta *et al.* showed differences in both the structure and chemistry of waxes from the different surfaces. On the adaxial surface they formed plate-like crystals and contained mainly primary alcohols (71% of total content), being dominated by 1-hexacosanol. By contrast, the abaxial waxes formed ribbon-like crystals that consisted mainly of alkanes (73%) with little primary alcohol content.

From many studies it is clear that, as for *E. pisi*, conidia of *B. graminis* germinate on a range of artificial substrata (reviewed by Green *et al.*, 2002), and in this case germination may be a non-specific response to substratum contact, stimulated perhaps by ECM release. Nevertheless, Gniwotta's observations of *E. pisi* (mentioned earlier) indicate that plant surface components can promote germination, although the details of how this may be mediated are unknown. However, Nielsen *et al.* (2000) showed hydrolytic enzyme activity in the region of *B. graminis* conidial ECM within 3 min of spore deposition. Further, they showed that prior to germination, anionic low-molecular weight compounds with physical properties similar to cutin monomers could be taken up by conidia incubated on host leaves. This suggests that fungal enzymes present in the conidial ECM (Nicholson *et al.*, 1988; Pascholati *et al.*, 1992) may degrade components of the plant surface/cuticle (Kunoh *et al.*, 1990) to release breakdown products that can then be taken up by the spore as part of a signalling system involved in germination. What evidence is there for this? Transcript abundance assays (Perfect, 2005) for *B. graminis* cutinase 1 gene from conidia deposited on host leaf or on glass or plastic overlaid with cutin, revealed elevated transcription of the gene within 30 min of deposition. By contrast, no equivalent transcript induction was detectable for conidia deposited on clean glass or plastic surfaces on which normal germling development is not induced. Clearly, further experimentation is needed to resolve the details of this system in the powdery mildews and to search for analogous systems in other fungal/plant associations.

12.4 Directional emergence of fungal germ tubes

Germ tubes must make contact with the host leaf in order to function and since spores are three-dimensional bodies lying on an undulating leaf surface, the likelihood of germ tube contact will be greatly increased if they emerge from the spore at a point close to the leaf surface contact site. Although this idea is obvious, it received little attention until Wright *et al.* (2000) considered the primary germ tube (PGT) of *B. graminis*. As noted earlier, the PGT is invariably short (it ceases growth at

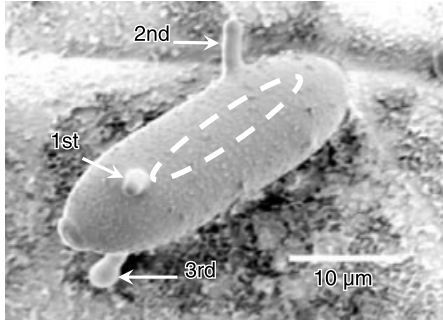


Figure 12.4 Cryo-SEM image showing a *Blumeria graminis* conidium on a barley leaf epidermal strip where it was incubated for 15 min before it was rolled over so that its original site of contact with the leaf (region marked by dashed line) faced away from the leaf. Repeated light microscope observation of the living spore showed that the first and second (1st, 2nd) formed germ tubes then emerged close to the original contact site, demonstrating that initial contact had programmed these sites of emergence. Eventually, the conidium responded to the new site of contact so that its third (3rd) germ tube made leaf contact (from Wright *et al.*, 2000).

around 5–10 μm) and this clearly restricts its chances of fortuitous contact with a substratum. Wright *et al.*, (2000) developed a geometric model taking account of the relationships between the ellipsoidal conidium and curved host cell surface. From this they calculated that according to circumstances (spore orientation, epidermal cell size), only between 8 and 22% of PGTs would make contact if they emerged at a random point on the spore surface. However, in reality, observation shows that at least 80% of PGTs make host surface contact. This clearly indicates existence of a precise system controlling PGT emergence very close to the contact site. The accuracy of this control becomes even more remarkable when one remembers that the actual area of spore/leaf surface contact consists of the interface between very few conidial surface projections with the fine edges of epicuticular wax crystals (Figure 12.2a). The extraordinary sensitivity of the perception system is underlined by the ability of many conidia to recognise even the minute contact made with a 0.5 μm diameter spider's suspension thread (Wright *et al.*, 2000).

Wright *et al.* (2000) also showed that recognition of contact is extremely fast. By micromanipulation, deposited *B. graminis* conidia were rolled over before they germinated so that their original site of contact then faced away from the leaf. Repeated observation of these living spores showed that even when they were rolled within 1 min of deposition, the majority of tubes eventually emerged very close to the original site of leaf contact and so grew upwards, away from the leaf (Figure 12.4).

How could such a rapid response to contact be engaged? Present knowledge, from *B. graminis* and more recently from *E. pisi*, implicates conidial ECM release which, as discussed earlier, is known to occur within seconds of spore deposition onto artificial and plant surfaces. Details of the signalling system(s) are not understood, but in *B. graminis* this must apparently involve some form of non-specific response since most PGTs emerge very close to contact sites with

all artificial substrata tested although the proportion is significantly higher if they are hydrophobic than hydrophilic (Carver *et al.*, 1999; Wright *et al.*, 2000). Furthermore, for *E. pisi*, substratum hydrophobicity promotes not only the frequency of emergence close to the contact site but also the speed of conidial ECM release and the speed of germ tube emergence (Fujita *et al.*, 2004b). This suggests that it may be the simple act of localised ECM release that mediates non-specific response to contact. However, in *E. pisi* the response to surface contact can be overcome by positive phototropic effects. Thus, even when incubated on hydrophobic substrata or barley leaf epidermis, if light is supplied from directly above conidia, most germ tubes emerge from the surface facing away from the underlying surface and grow away from it (Fujita *et al.*, 2004b). This indicates not only the existence of a photoreceptor system within ungerminated *E. pisi* spores, but also that light can have a more powerful influence on germ tube emergence than surface contact. In *B. graminis*, however, no such photoreceptor system appears to exist (Fujita *et al.*, 2004b). Moreover, the frequency of *B. graminis* PGT contact is greater on leaf cells than on any artificial substrata even though geometric relationships predict a lower frequency than on planar substrata (Wright *et al.*, 2000). This implies that specific characteristics of the host surface augment the non-specific response to surface contact. This may involve the release of leaf surface components by ECM enzymes and their subsequent uptake by conidia (Nielsen *et al.*, 2000). If so, it is possible that differences in plant surface chemistry (due to genotype or species) may affect the signalling system and impair control of directed germ tube emergence although this possibility has never yet been tested.

12.5 The special case of the PGT of *B. graminis*

Although the multicellular spores of some fungi (e.g. *M. grisea* and *Colletotrichum* spp.) may produce a germ tube from more than one of their cells, this appears to be an unregulated phenomenon. Even among the powdery mildew fungi, *B. graminis* is to our knowledge unique in invariably producing a PGT before forming an appressorial germ tube (AGT). Why *B. graminis* should have evolved dependence on its PGT is unknown, but the vital functions it plays in pathogenesis illustrate the intimacy of relations between a fungus and its host surface.

On contacting the leaf surface, the PGT secretes ECM that adheres rapidly and tenaciously to the leaf (Wright *et al.*, 2002). Unlike conidial ECM, the ECM secreted by PGTs is not water soluble although it does contain protein, and its adhesion is so strong that if the germ tube is displaced the region of underlying cuticle is often torn away from the leaf cell wall (Carver *et al.*, 1995; Figure 12.5). After attaching, the PGT forms a short penetration hypha, the 'cuticular peg', which penetrates the plant cuticle but not the cell wall (Edwards, 2002), probably aided by secretion of cutinase (Francis *et al.*, 1996). Through its PGT the germling can absorb host water (Carver and Bushnell, 1983) which allows survival and continued germling growth under arid conditions that would otherwise desiccate and kill it. From the host plant's

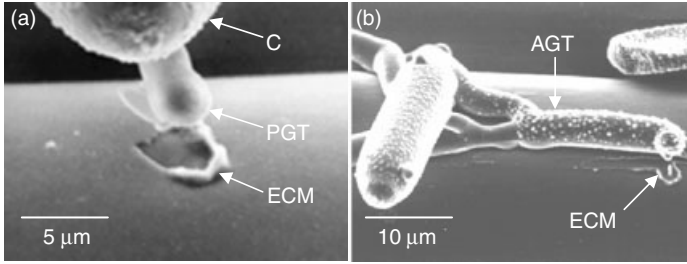


Figure 12.5 Cryo-SEM images showing *Blumeria graminis* ECM and strength of adhesion revealed by stress displacement of the fungus during cryo-fixation. Leaves were de-waxed before inoculation so as to reveal ECM. (a) During fixation, the conidium and its PGT lifted away from the leaf surface, but strong adhesion at the PGT tip tore away the cuticle leaving traces of peripheral ECM around the wound site. (b) During fixation the appressorial germ tube of this young colony rolled back from the leaf surface exposing a track of ECM that is particularly thick around the appressorium contact site. Note: the fungal penetration peg is evident in the centre of this site (from Carver *et al.*, 1995).

point of view, PGT contact triggers concomitant responses that include up-regulated transcription of many genes (Eckey *et al.*, 2004; Hein *et al.*, 2004), hydrogen and calcium ion efflux from the apoplast (Felle *et al.*, 2004) and a series of cytological responses culminating in the deposition of a small apoplastic papilla (cell wall apposition) beneath the PGT tip (Zeyen *et al.*, 2002). This early passage of signals through the plant cell wall apparently primes the capacity of cells to defend against later attack. Thus, a number of host genes are differentially transcribed according to the single gene-controlled resistance the plant carries (Hein *et al.*, 2004), and, even where they possess no such resistance, cells contacted by a PGT show enhanced penetration resistance to later attack from the appressorium (Woolacott and Archer, 1984).

Perhaps the most intriguing function of the PGT lies in its ability to recognise host surface features. Indeed this recognition is an absolute requirement since it drives elongation of the AGT that subsequently emerges from the conidium, and elongation of this germ tube is in turn a pre-requisite for appressorium differentiation. Why should the fungus have this requirement for energy- and time-consuming elongation of the AGT? One clear benefit is that growth away from the spore increases the likelihood that the appressorium will eventually form on an epidermal cell that has not been primed towards resistance by direct prior contact with either the conidial ECM or the PGT. The PGT's function of recognising host surface features was first demonstrated (Carver and Ingerson, 1987) by the observation that although conidia suspended on spider's thread or deposited on agar will germinate, they form only a series of short germ tubes none of which elongates and differentiates an appressorium. However, if germlings with a single short germ tube are transferred from spider's thread to lie with their spore on agar but the germ tube tip placed in contact with a host epidermal strip, then the next germ tube to emerge is most likely to elongate.

Subsequent studies have identified certain host surface characteristics that are recognised by the PGT. The presence of epicuticular wax crystals is not important because their physical removal does not reduce inductivity (Carver and Thomas, 1990). However, even after removal of epicuticular wax, leaves remain highly hydrophobic (Wright *et al.*, 2000), presumably because of intracuticular wax and cutin, and substratum hydrophobicity is a key factor recognised by PGTs. Thus, although contact with clean, hydrophilic glass or agar does not induce AGT elongation, contact with biologically inert hydrophobic substrata is inductive, albeit less so than contact with the host leaf surface (Carver *et al.*, 1996; Wright *et al.*, 2000). Nevertheless, although leaf waxes are highly hydrophobic, not all components of leaf wax are recognised by the PGT and quite subtle differences in chemistry may have a relatively large effect. This was shown by Tsuba *et al.* (2002) who found that C₂₆ chain length aldehydes (hexacosanal) present in barley leaf wax induce elongation of the AGT while C₃₀ chain length aldehydes (triacontanal) in cabbage leaf wax (a non-host plant) are far less effective and the alcohol forms of these molecules are even less inductive. Furthermore, it has been known for some time that PGTs recognise various cellulose-containing membranes, since they stimulate AGT elongation, even though their surface is highly hydrophilic (Kobayashi *et al.*, 1991; Carver *et al.*, 1996). It is now established, however, that various cellulose degrading enzymes are produced by young germlings (Suzuki *et al.*, 1998) and cellobiohydrolase I is released specifically at the PGT tip (Pryce-Jones *et al.*, 1999) suggesting that enzymatic activity may generate cellulose breakdown products that act as signalling molecules perceived *via* the PGT. Similarly, *B. graminis* cutinase may release cutin monomers that can act as additional signalling molecules to fungi (Kolattukudy, 1996). Thus, Francis *et al.* (1996) showed that although glass coated with cutin monomers remained relatively hydrophilic, this treatment substantially enhanced induction of AGT formation. Conversely, treating leaves with cutinase inhibitor greatly reduced AGT formation. The current evidence therefore suggests that the plant leaf offers multiple factors recognised by the PGT. First it encounters leaf waxes and a combination of their hydrophobicity and chemical constitution can stimulate response. Then, as the peg emerges to penetrate the cuticle and make contact with the underlying cellulose matrix of the cell wall, fungal enzymes may release products that act as supplementary signal molecules. The multiplicity of these factors makes the plant surface more inductive than any artificial substratum possessing a single inductive characteristic (Carver *et al.*, 1996).

Molecular plant pathologists have recently started attempting to identify the intracellular signalling processes involved in germling morphogenesis, and in *B. graminis* it is becoming clear that signal transduction relay is complex. A simple signal is not sufficient to trigger germling morphogenesis. Indeed, multiple signals are needed to promote true differentiation and these are likely relayed by several signal transduction cascades, notably cAMP/PKA (protein kinase A) (Hall *et al.*, 1999; Hall and Gurr 2000; Kinane *et al.*, 2000), PKC (protein kinase C) (Zhang *et al.*, 2001) and MAPK (mitogen activated protein kinase) (Bindslev *et al.*, 2001; Zhang and Gurr, 2001; Kinane and Oliver, 2003). Of these, most is

known about the cAMP/PKA pathway. Here, endogenous concentrations of cAMP, monitored in populations of conidia and germlings differentiating on host leaves or artificial surfaces (non-inductive glass or partially inductive cellulose membrane), revealed peaks in cAMP levels prior to both PGT and AGT emergence. Furthermore, this biphasic flux pattern was mirrored by changes in PKA activity (Kinane *et al.*, 2000). Collectively, the model emerging from these studies is that upon landing, PGT emergence is mediated by a transient release of cAMP and a burst of PKA activity. Thereafter, surface perception by the PGT drives a second release of cAMP activity engaging MAPK cascade(s) (Kinane and Oliver 2003) and invoking AGT emergence. However, recent evidence points to appressorium formation being driven by the MAPK pathway alone (Kinane and Oliver 2003). Much remains to be unmasked, not least the precise link between host perception and signal transduction relay.

Interest in understanding PGT/plant surface interactions arises from the possibility of plant breeding to alter the leaf surface so as to interfere with germling growth, and there is reason to believe this may be possible. For example, failure of PGTs to recognise leaf surface characteristics is not only a feature of interactions with certain non-hosts (Tsuba *et al.*, 2002) but also occurs in species of the grass genus *Lolium* which are true hosts of the fungus (Carver *et al.*, 1990). Here, PGTs recognise properties of the adaxial leaf surface on which the fungus forms functional appressoria and establishes thriving infection. In complete contrast, however, on the abaxial surface of *Lolium* leaves no infection develops and this is largely because most conidia fail to form an AGT. The reason for this is unknown although it obviously relates to the nature of the abaxial epicuticular waxes since their removal by chloroform washing restores inductivity. The physical appearance of the ab- and adaxial epicuticular waxes is quite different. On the abaxial surface they form amorphous overlapping sheets whereas those on the adaxial surface form crystalline plates similar in appearance to those seen on both surfaces of host cereal leaves. As would be predicted, some preliminary evidence (Carver *et al.*, 1996) indicates differences in the chemistry of the waxes found on the two surfaces, but precise analyses and tests of individual components have not yet been performed. It seems possible, however, that some component(s) of the abaxial wax actively inhibit signal perception by the PGT because the intact surface is highly hydrophobic and the hydrophobicity of inert artificial substrata is sufficient to cause response (Carver *et al.*, 1996, 1999; Wright *et al.*, 2000). Understanding the basis of this effect may reveal means to develop novel forms of resistance that impede the pathogen's early stages of development.

12.6 AGT growth, appressorium differentiation and penetration

According to their infection strategy, germ tubes of some fungi differentiate an apical appressorium relatively rapidly after emerging from the spore, while for others the germ tube may elongate considerably over a relatively long period before doing so. All available data indicate that appressorium differentiation is driven by perception of host-derived signals, that this depends upon intimate contact between elongating

germ tube and substratum surface and that this in turn requires adhesion of the germ tube to the surface (Staples and Hoch, 1997; Tucker and Talbot, 2001; Apoga *et al.*, 2004).

12.6.1 Adhesives associated with AGTs

Adhesion to the hydrophobic leaf surface is pivotal to the disease process, both in anchoring the germling and as a prerequisite for full appressorium differentiation. In many cases, adhesion has been attributed to ECM released by the germ tube, although, as with the ECM associated with ungerminated spores (Section 12.2), the abundance of ECM varies greatly between species and for most there is little information on its chemical constitution or mode of action. For example, abundant ECM is visible by cryo-EM beneath the AGT of *B. graminis* (Figure 12.5b) and its release coincides with extremely strong adhesion (Wright *et al.*, 2002a), but other than the fact that some components are water insoluble and that it contains protein, little is known of its chemistry (Carver *et al.*, 1999). By contrast, the ECM associated with germ tubes of *P. ampellicida* is barely discernible by cryo-SEM although it is revealed by staining (Kuo and Hoch, 1995). Several attempts to characterise fungal components involved in adhesion have used lectins which bind to specific glycoproteins, and antibodies which recognise carbohydrate epitopes. Application of these lectins and antibodies blocks adhesion of numerous fungi e.g. *C. graminicola* (Mercure *et al.*, 1994; Sugui *et al.*, 1998), *C. lindemuthianum* (Hughes *et al.*, 1999) and *P. ampellicida* (Kuo and Hoch, 1995) indicating the role of glycoproteins in adhesion.

One class of proteins capable of mediating germ tube attachment are the hydrophobins, and these are key in *M. grisea*. Hydrophobins comprise a family of small cysteine-rich peptides which self-assemble into an amphipathic film at hydrophilic–hydrophobic interfaces, with the hydrophobic nature of the assembly aiding binding to the hydrophobic cuticle (Tucker and Talbot, 2001). Furthermore, hydrophobins may also play a role in sensing contact with a hydrophobic surface (Talbot *et al.*, 1993; Beckerman and Ebbole, 1996). Thus, in *M. grisea*, deletion of the small, secreted hydrophobin gene *mpg1* gave mutants showing reduced adhesion which in turn led to impaired appressorium formation and reduced pathogenicity (Talbot *et al.*, 1996).

More recently, attention has turned to the so-called ‘adhesins’, a family of ECM proteins carrying the RGD (arginine-glycine-aspartate) tripeptide motif, which are recognised by integrins on the host cell surface (Mellersh and Heath, 2001). This protein family is known to play key roles in cell adhesion, cytoskeleton scaffolding organisation and in intracellular signalling in a range of animal and microbial systems (Hostetter, 2000). Appressorium induction of *Uromyces appendiculatus* is blocked by exogenous application of RGD peptides, suggesting the presence of integrin-like molecules on the fungus or its host (*Phaseolus vulgaris*) leaves (Corrêa *et al.*, 1996). Furthermore, in *Oidium neolycopersici*, the tomato powdery mildew fungus, application of RGDS (arginine-glycine-aspartate-serine) peptides

leads to aberrant appressorial differentiation (Jones *et al.*, 2001). However, various data cast doubt on a universal adhesion mechanism involving integrin-like proteins. Thus, RGD-peptide treatment does not affect germination or germ tube differentiation in *B. graminis* and whilst exogenous application of RGD peptides leads to enhanced adhesion this is directly attributable to the sticky surface residue left by the peptides (Perfect, 2005). Moreover, adhesion and morphology of the Oomycete *Phytophthora megasperma* f.sp. *glycinea* was unaffected by RGD-peptide treatment (Ding *et al.*, 1994). Clearly, though vital to germling/host surface interaction, the mechanisms of germ tube adhesion are various but remain poorly understood.

12.6.1.1 Germ tube growth and appressorium differentiation by fungi that penetrate the host surface directly

The germ tubes of many hemibiotrophic fungi differentiate appressoria after growing for only a short distance so that the appressorium forms very close to the mother spore. This implies rapid perception/response to features of the underlying surface. Speedy response not only maximises the likelihood that finite resources carried within the propagule will suffice to support appressorium formation and attempted penetration, but also minimises the time that the fungus is directly exposed to potentially adverse environmental influences (e.g. the presence or absence of free water, temperature extremes) before it penetrates the host to access the more stable internal environment of the leaf and establishes parasitism to support its nutritional requirements. For example, the germ tubes of *C. graminicola* differentiate appressoria within 3 h of emergence and differentiation is triggered by only 4.5 μm of continuous contact with a hard, hydrophobic artificial surface (Apoga *et al.*, 2004). A comparable time-course of germination and appressorium formation is evident in other *Colletotrichum* spp. (*C. coccodes*, *C. dematium* and *C. lagenarium*; pathogens of red pepper) although here appressoria differentiate simply as a response to contact with a hard surface irrespective of its hydrophobicity (Ahn *et al.*, 2003). In all these cases, therefore, the observations imply that a relatively simple perception system controls rapid differentiation. It is not possible to generalise on 'simplicity' within this fungal genus, however, because a much more complex perception system appears to operate in *C. gloeosporoides*. Here, contact with non-host waxes apparently inhibits appressorium formation whereas particular components of the host (avocado fruit) surface wax strongly stimulate appressorium differentiation (Kolattukudy *et al.*, 2000). Relatively complex recognition systems also operate in *M. grisea* and *P. ampellicida*. The fully differentiated germ tubes of *M. grisea* formed on rice leaves and Teflon (hydrophobic surfaces) are generally very short, but differentiation is also stimulated on cellophane and glass (hydrophilic) although on these substrate germ tubes elongate far more before appressoria form (Jellito *et al.*, 1994). Similarly, on its host grape leaves, *P. ampellicida* germ tubes also remain short, growing only about 5 μm before appressoria begin differentiating to become fully formed by 3–6 h after spore deposition (Kuo and Hoch, 1995). Here, differentiation also appears to involve hydrophobicity of the leaf surface since appressoria are induced

on hydrophobic artificial substrata (Kuo and Hoch, 1996) although even on these substrata the germ tubes grow considerably longer (up to 40 μm) before differentiating. Thus, data from both *M. grisea* and *P. ampellicida* suggest that various signals can cause appressorium differentiation and that the true host may provide multiple cues that drive more rapid response.

Among the obligate biotrophic fungi, AGT development and differentiation has been studied most intensively in *E. pisi* and *B. graminis*. The need for substratum contact as a driver of appressorial differentiation by *E. pisi* is easily demonstrated from the observation that the single germ tube formed by conidia suspended on spider's suspension elongates to great length through the air (>100 μm) but never differentiates an appressorium (Carver *et al.*, 1996), whereas, when incubated in darkness on pea leaves, the AGT swells and differentiates within a few microns (Ayres, 1983). As with *M. grisea*, a relatively simple surface perception system is indicated by published evidence. Apparently, substratum contact leads to a high frequency of appressorium differentiation irrespective of whether the surface is hydrophilic or hydrophobic (Ayres, 1983; Fujita *et al.*, 2004b) although, like *M. grisea*, on hydrophilic surfaces far longer germ tubes are formed before appressoria differentiate (Carver *et al.*, 1996). Nevertheless, because germ tube emergence close to the spore/substratum contact site is promoted on hydrophobic substrata (Section 12.4) frequencies of germ tube contact are higher on a hydrophobic artificial surface and here response is induced rapidly so that most appressoria differentiate within 4 h of spore deposition. A greater level of complexity is indicated, however, by very recent evidence that pea leaf wax constitution can also influence recognition by *E. pisi* germ tubes (Gniwotta *et al.*, 2005). Thus, Gniwotta *et al.* (2005) found a far higher rate of appressorium differentiation on the adaxial (70%) than abaxial (49%) leaf surface, and this correlated to difference in the chemical constitution of waxes present on the different surfaces (see Section 12.3). Even further complexity in the *E. pisi* response system is demonstrated by the fact that lighting environment also has a marked effect: when incubated in lateral light, germ tubes differentiate appressoria only after growing to approximately three times the length of those formed in darkness (Ayres, 1983; Fujita *et al.*, 2004b). Apparently, light superimposes a regulatory effect over germ tube response to contact stimuli. To some extent this is also true in *M. grisea*, although here germ tubes grow longer in darkness than in light (Jelitto *et al.*, 1994).

Although no such effect of light is seen in *B. graminis* (Fujita *et al.*, 2004b), appressorial differentiation by the fungus can be driven by a variety of surface features implying that it possesses a number of recognition systems. These, however, do not induce rapid appressorial differentiation because, compared to e.g. *Colletotrichum* spp., *M. grisea*, *P. ampellicida* and *E. pisi*, on its host leaf *B. graminis* AGTs always elongate considerably (to around 40 μm) before differentiating appressoria. Evolution of this characteristic suggests an advantage to *B. graminis* pathogenesis despite the fact that growth must have the disadvantages of expending resources and taking time (around 6–8 h after germ tube emergence). It is likely that the benefit arises from the fact that elongation increases the probability of

appressoria eventually forming on epidermal cells that have not been primed for defence by response to prior contact with PGTs of the fungus (Section 12.5).

Studies (reviewed by Carver *et al.*, 1996; Green *et al.*, 2002) using artificial substrata indicate that the surface factors recognised by the *B. graminis* PGT (Section 12.5) are also recognised by elongating AGTs and induce their differentiation. Thus, surface hydrophobicity is inductive while simple contact with a hard surface is not. Breakdown products of leaf cutin and cellulose, released by fungal enzyme activities, are also implicated in different signalling systems. Together, these provide cues that act additively or interactively to stimulate appressorium differentiation with far greater efficiency on host leaf surfaces than on any artificial surface with a single inductive characteristic. Again, however, studies of artificial substrata do not reveal the full subtlety of interactions occurring with leaves, and recent studies indicate the influence that relatively small variations in plant surface chemistry may have on appressorial differentiation. It is well established that for *B. graminis* the physical presence of epicuticular leaf wax crystals is not required because appressoria differentiate normally even if the crystals are removed to expose the underlying cuticle proper before inoculation (Carver and Thomas, 1990). However, displacement of powdery mildew germ tubes from intact leaves reveals a distinct track where epicuticular waxes are missing, suggesting the possibilities that they either adhere to (and are lost with) the detached germ tube, or that they are 'dissolved' or degraded by components of the germ tube ECM (Staub *et al.*, 1974). Support for the latter possibility comes from observations that *B. graminis* conidial ECM is capable of modifying the appearance of barley leaf surface waxes and rendering the surface more hydrophilic (Kunoh *et al.*, 1990; Nicholson *et al.*, 1993). If they are indeed degraded by fungal activity, their breakdown products may act as signals to the fungus. The ability of different wax components either to promote or to inhibit appressorium formation by *C. gloeosporoides* is well established (reviewed by Kolattukudy, 1996; Kolattukudy *et al.*, 2000) and for *B. graminis* the importance of wax chemistry is indicated by a somewhat reduced frequency of appressorial differentiation on barley genotypes carrying certain *eceriferum* (wax) mutations (Yang and Ellingboe, 1972; Rubiales *et al.*, 2001). The significance of relatively small differences in chemistry is further suggested from the finding that synthetic long-chain aldehydes are more inductive of *B. graminis* appressorium differentiation than equivalent alcohols, and that the C₂₆ chain length is more inductive than C₃₀ chain length (Tsuba *et al.*, 2002). The significance of wax chemistry in driving appressorial differentiation by plant pathogens clearly deserves further study; manipulation of leaf wax chemistry through plant breeding may offer a form of resistance that can disrupt the early stages of pathogenesis.

12.6.1.2 *Signal transduction in fungi that penetrate the host surface directly*

The nature of signal transduction pathways engaged during appressorium formation has received a little attention. Recent evidence suggests that cAMP dependent (PKA) and MAPK pathways play a central and intertwined role driving differentiation of

the appressorium in phytopathogenic fungi (reviewed by Lee *et al.*, 2003). Most comprehensive amongst these studies are the data from *M. grisea* concerning the importance of cAMP signalling during appressorium differentiation in mutants lacking *mac1* adenylate cyclase (Choi and Dean 1997; Adachi and Hamer 1998), *mpg1* hydrophobin (Talbot *et al.*, 1993), heterotrimeric G proteins components $G\alpha$, *magB* (Liu and Dean 1997), $G\beta$ subunit, *mgb1* (Nishimura *et al.*, 2003) and cPKA (Xu and Hamer, 1996): all can be 'rescued' or 'defect suppressed' to varying degrees, by addition of cAMP. There is also growing evidence of the conservation of core elements of the MAPK signal pathway in pathogenic fungi and of interplay with the cAMP signal transduction cascade. For example, exogenous cAMP restores hooking and tip flattening in *M. grisea* Δ *pmk1* mutants but not appressorial function. Far less is known, however, about signal transduction via PKC in phytopathogenic fungi. The signalling cascades mentioned may be linked to perception. However, it is extremely difficult to separate such signalling cascades from those involved in other aspects of fungal development. Thus, cAMP is known to influence growth and morphogenesis (Bencina *et al.*, 1997) and infection structure differentiation, including appressorium formation in *M. grisea* (Lee and Dean, 1993). Indeed, in only one documented case is there a direct link between surface perception and signalling. This involves a lipid induced protein kinase (LIPK) from *Colletotrichum trifolii*, which is specifically and rapidly induced by purified plant cutin or long-chain fatty acids that are monomeric constituents of cutin (Dickman *et al.*, 2003). Gene replacement studies indicate that LIPK also plays a central role in triggering infection structure formation – deficient mutants were unable to form appressoria on hard surfaces and could not penetrate intact tissues, whereas over-expressors formed multiple abnormal appressoria. LIPK shares catalytic domain identity with PKCs and merits more study.

Calcium/calmodulin-dependent signalling systems are involved in many biological systems and, amongst its functions within the fungi, Ca^{2+} is known to be required for appressorium formation by both *P. ampellicida* and *C. trifolii* (Warwar and Dickman, 1996; Shaw and Hoch, 2000). In *M. grisea*, exogenous application of calcium modulators, EGTA (a chelator) and calmodulin antagonists all inhibit appressorium formation although they do not affect germination. Indeed, EGTA-induced inhibition of appressorium formation is reversed by addition of $CaCl_2$ (Lee and Lee, 1998). Similarly, in *B. graminis* calcium channel blockers, chelators and calmodulin inhibitors impeded appressorium differentiation (Hall, 1999). Collectively, these data attest to a broad role for Ca/calmodulin dependent signalling in appressorium formation.

12.6.1.3 Germ tube growth and appressorium differentiation by fungi that enter via stomata

An immediate problem for fungi that enter the host through its stomata is that spore deposition occurs randomly and stomatal pores occupy a very small percentage of the leaf area: the probability of a chance encounter is extremely small if germ

tube growth is unregulated. Much early work (reviewed by Staples and Macko, 1984; Hoch and Staples, 1991) focusing on this problem revealed that urediniospore germ tubes of many rust fungi show thigmotropic response to junctions between plant anticlinal cell walls. It became clear that germ tube growth orientates at right angles to these junctions and this maximises the likelihood of encountering a stoma. Even so, the germ tube may have to elongate for a great distance (up to several hundred micrometres) before either a stoma is located or reserves are exhausted. In some cases, leaf wax characteristics influence the ability of germ tubes to locate or recognise stomata. Thus, *Puccinia hordei* rarely forms appressoria over stomata of *Hordeum chilense* leaves, and this is thought due to their heavy encrustation with wax (Rubiales and Niks, 1996). In a more subtle way, certain *eceriferum* mutations in barley also affect *P. hordei* development, and this has been ascribed to influence surface wax properties and the ability of germ tubes to recognise anticlinal cell wall junctions and orient their growth (Rubiales *et al.*, 2001). Strong evidence indicates that this thigmotropic sensing is contingent upon firm adhesion to the leaf surface and for *Uromyces appendiculatus* substrate hydrophobicity favours adhesion (Terhune and Hoch, 1993). On host leaves, the presence of epicuticular wax structures appears important for germ tube adhesion by *Puccinia sorghi* because on 'waxless' mutants of maize the germ tubes fail to adhere or orientate and therefore to locate or respond to stomata (Wynn and Staples, 1981). Germ tube ECM has been implicated in adhesion for a number of rust species (Beckett, 1990; Chaubal *et al.*, 1991) and in *P. sorghi* the ECM consists of glycoproteins rich in acidic amino acids and β -1,3-glucan polymers, although there is likely to be variability in ECM constitution between rust species (Chaubal *et al.*, 1991).

When urediniospore germ tubes of most rust fungi encounter a stoma, the tip of the germ tube continues growth until it lies over the stomatal guard cells. Providing the germ tube is adhered, elongation ceases and the cytoplasm accumulates in the tip which differentiates into a swollen appressorium separated from the germ tube by a septal wall. This differentiation is known to be a further contact-mediated response that is shown by many different rust fungi (Allen *et al.*, 1991). It has been best characterised in *U. appendiculatus* where studies using artificial substrata showed that germ tubes differentiate appressoria in response to encountering a surface ridge. Furthermore, maximal response is obtained following contact with a single ridge of height 0.5 μm ; this correlates closely to the mean height of the lip of its host (*P. vulgaris*) guard cells (Hoch *et al.*, 1987; Terhune *et al.*, 1993). This extraordinary ability to recognise minute topographic details is arguably even more remarkable in certain *Puccinia* spp. There had been some controversy regarding the signals perceived by *P. graminis* and other cereal rusts because they respond poorly to single ridges or grooves, leading to suggestions that other signals associated with stomata may be more important stimulants of appressorium formation. However, although a range of chemical and environmental factors can stimulate differentiation (Hoch and Staples, 1991), Read *et al.* (1997) showed that while germ tubes of *P. graminis tritici* (wheat stem rust) and *P. hordei* (barley brown rust) did not respond significantly to single ridges, a very high proportion (83–86%) of germ tubes

differentiated appressoria after encountering multiple ridges and grooves mimicking the topographic conformation of cereal stomatal complexes.

Studies of *U. appendiculatus* by Hoch and co-workers provide the best insight into signal control of appressorium differentiation by a rust fungus (reviewed by Tucker and Talbot, 2001). Good evidence suggests that membrane stress imposed within the germ tube tip as it encounters a stomatal ridge engages a mechanosensitive ion channel capable of transporting various cations including Ca^{2+} . The fact that provision of Ca^{2+} *in vitro* can induce appressorium formation by *U. appendiculatus* is consistent with the proposition that Ca^{2+} flux plays a part in the differentiation process. Within 4 min of signal perception the cytoskeleton and cytoplasmic vesicles become reorganized along the cell wall of the germ tube tip and it seems likely that microtubule organisation is also involved in appressorium differentiation. That protease-treated germlings fail to respond to topographic signals supports the idea that trans-membrane proteins may link extracellular sensing proteins associated with the ECM to cytoplasmic proteins as part of the regulatory process. The involvement of integrins in this system is indicated from findings showing the reversible inhibitory effects of a number of RGD peptides on appressorium differentiation by *U. appendiculatus* (Corrêa *et al.*, 1996).

12.7 Entry into the host leaf

12.7.1 Direct penetration of the host surface from appressoria

Fungi may penetrate their host surface by physical force, by enzymatic degradation or by a combination of the two strategies. *M. grisea* offers the classic example of forceful entry into the host epidermal cell. This relatively unsubtle strategy can be extremely effective because host cell responses that involve minor modifications to strengthen the underlying plant surface/cell wall structures are unlikely to impede penetration. The sophistication of the strategy lies, however, in the complex processes of appressorium development that allow the generation of sufficient force to effect penetration. Cell collapse assays reveal that turgor within *M. grisea* appressoria can rise up to 8 MPa immediately preceding emergence of a specialised hypha, the penetration peg, that can breach not only the plant cuticle and outer cell wall to enter the epidermal cell lumen, but also has the capacity to penetrate extremely hard artificial substrata (Howard *et al.*, 1991). To counteract the penetrative force, ECM glues the appressorium tenaciously to the surface. This enormous turgor is due to the accumulation of large quantities of glycerol, attributed to the action of triacylglycerolipase on lipids imported from the spore, which causes rapid water influx and the generation of hydrostatic pressure (reviewed by Tucker and Talbot, 2001). From an architectural perspective, the appressorium is able to contain the pressure by synthesis of a veneer of melanin that lines and strengthens the chitin-rich appressorial wall. This effectively seals the wall and allows turgor pressure to build up in the appressorium. Melanization is seen in appressoria of many other fungi

including *P. ampellicida* and *Colletotrichum* spp., and in these too physical force is likely to be key to penetration. Thus the penetration peg of *C. graminicola* generates a force of around 16.8 μN , which is certainly sufficient to breach most plant cuticles (Bechinger *et al.*, 1999). Nevertheless, the ability to generate turgor is not dependent on melanization because the hyaline appressoria of *B. graminis* are also capable of generating 2–4 MPa turgor pressure (Pryce-Jones *et al.*, 1999). In this case, however, the evidence indicates that penetration is achieved by a combination of physical force and the secretion of cutin and cell wall degrading enzymes (Suzuki *et al.*, 1998; Pryce-Jones *et al.*, 1999).

As appressoria mature and penetration pegs emerge and attempt to breach the host surface, host cells often respond by strengthening their wall and in many cases by depositing apoplastic wall appositions (papillae) beneath appressoria (Zeyen *et al.*, 2002). These defensive responses clearly depend on passage of information through the plant cell wall and it is obviously important that plant cell walls have evolved to allow this transmission of information. One of the most rapid responses to pathogen attack is an oxidative burst focused in the plant cell wall/cytoplasm directly beneath the fungal appressorium. Thus, a fungal penetration peg entering the cell wall will be confronted with an oxidising environment which may disrupt further fungal development. It seems, however, that pathogens have developed anti-oxidative systems to cope with this problem. In *B. graminis* up-regulated expression of a catalase gene coincides with the timing of penetration and the CATB protein is secreted at the host–pathogen interface (Zhang *et al.*, 2004). This is a closely controlled phenomenon that is dependent upon plant characteristics because no equivalent up-regulation of gene expression or focus of secreted protein occurs on an artificial surface. This data taken collectively with assays designed to detect fungal antioxidant activity, provoked speculation that this detoxification may play a role in *B. graminis* pathogenicity. Alternatively, it could be that antioxidants are needed to strengthen the fungal cell wall as it penetrates. However, neither hypothesis could be confirmed due to the lack of a robust transformation system in *B. graminis*. The idea was pursued, therefore, using *M. grisea*, and here a catalase B knockout mutant showed slightly attenuated pathogenicity and a slight reduction in cell wall strength (Henderson 2005). However, in *M. grisea* a knockout (MMT1) of a metallothionein (with high affinity for zinc) which acts as a powerful antioxidant, produced mutants unable to penetrate intact cuticle (Tucker *et al.*, 2004) but able to cause disease if the cuticle was removed before inoculation. Cuticle removal obviously causes wound responses that are likely to include the rapid accumulation of plant reactive oxygen species. Thus, in *M. grisea*, MMT1 is more likely associated with differentiation of a fully functional cell wall, since MMT1 mutants are supersensitive to cell wall degrading enzymes, than in detoxifying plant-generated active oxygen species.

With successful penetration of the cell wall and any defensive barriers erected in response to attempted entry, plant resistance to many pathogenic fungi probably depends upon factors that lie beyond the influence of the leaf surface. This may not be entirely true of the powdery mildew fungi. For these, the feeding structure

(haustorium) develops as the tip of the penetration peg swells and differentiates within invaded epidermal cells, but the fungal hyphae are epiphytic. As they grow, these hyphae secrete ECM and hyphal adhesion is presumably as important to continued colony development as germ tube attachment is to the germling. However, to our knowledge, no studies have yet considered the effect that leaf surface features may have on development of established powdery mildew colonies.

12.7.2 *Entry via stomata*

Successful penetration through the stomatal pore takes a fungus beyond the influence of leaf surface features and any defence these may offer. Although the need for an elaborate strategy to support entry through the stomatal pore may seem unnecessary, penetration is often achieved in darkness when stomata are closed. Unlike many fungi that directly penetrate epidermal cells, however, it is questionable whether those that enter via the stomatal pore employ hydrostatic force. Turgor pressure within *U. appendiculatus* appressoria is only 0.35 MPa, but it is possible that concentration of cytoskeletal elements in the penetration hypha (Mendgen *et al.*, 1996) contributes to physical force which is capable of distorting the lip of artificial guard cells (Terhune *et al.*, 1993). Nevertheless, entry is likely to be facilitated by enzymatic activity. In *U. viciae-fabae*, differentiation of appressoria and penetration hyphae is accompanied by secretion of a number of proteases and cellulolytic enzymes (Mendgen *et al.*, 1996). Given that penetration is successful, the penetration hyphae swell within the substomatal cavity to form a vesicle from which infection hyphae emerge that grow endophytically until they contact a plant cell where they differentiate a haustorial mother cell and penetrate the host cell to form a haustorium. In the absence of resistance, endophytic hyphae ramify, many additional haustoria are formed, sporulation commences and the pustule ruptures the plant surface to release its spores.

12.8 Conclusions

Recent years have seen rapid progress in studies of interaction between leaf surfaces and certain pathogenic fungi (Gniwotta *et al.*, 2005). In revealing the complexity of these interactions, however, we have exposed how little we truly understand of the basis of even the few favoured 'model' systems that have received attention. Coupled with conventional approaches, the advent of stable transformation systems applicable to both hosts and fungi and associated -omics technology, the continuing development of sophisticated techniques for molecular cell biological studies and advancement in methodologies for chemical analyses of small quantities, the coming decades promise deeper understanding. This will inevitably help towards unravelling the basis of host resistance mechanisms operating at the leaf surface to prevent or limit disease development. It is already clear that in compatible relationships, vital host-pathogen interactions commence within seconds of an encounter.

The challenge is to identify phases of interaction where intervention to prevent or control loss due to disease is possible through, e.g. plant breeding or the development of novel targets for fungicides.

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13 Plant–Insect interactions on cuticular surfaces

Caroline Müller

13.1 Introduction

Insects explore the surface of a plant for many different purposes: they may search for an ideal food source or a spot to oviposit, for prey present on a plant or for a shelter to hide from predators, parasitoids or unfavourable abiotic conditions. The outermost surface of the primary parts of higher plants, the cuticle, is composed of the cuticular layer and the cuticle proper which is covered by epicuticular waxes. The certain chemical and physical properties of the cuticle (Chapter 4) influence the abilities of insects to move on the substrate and offer information about the characteristics and suitability of a plant (Eigenbrode and Espelie, 1995; Jenks and Ashworth, 1999; Müller and Riederer, 2005). The ability to attach to the surface is relevant on all trophic levels: plants show different cuticle properties that impede attachment of herbivores for tissue protection but also for catching insects in case of carnivorous plants. Herbivorous insects need a surface on which their legs and also their eggs will adhere and to which their mouthparts will have access. For predators and parasitoids the attachment on the plant surface will influence their predation and parasitism efficiency on herbivores.

After successful contact with the plant cuticle, the chemical characteristics of the epicuticular waxes affect the acceptance or rejection behaviour the insect will show towards the plant. The chemical recognition of the suitability of a plant is particularly important for monophagous or oligophagous herbivores that can feed and develop only on one or a few plant species within one plant family. But also for generalists, the cuticular surface will offer valuable cues about acceptability of a plant. Classically, scientists have focused on secondary metabolites that are characteristic for certain plant species or families to link plant–insect relationships. However, it is becoming more and more clear that these compounds often will only play a role secondarily once an insect has damaged the plant cuticle and comes into contact with epidermal and mesophyll tissue. Only some secondary metabolites are indeed located in or on the cuticle. This chapter summarises the multifaceted aspects of the role of cuticular surfaces in plant–insect interactions.

13.2 Access to the plant surface

13.2.1 Impeding attachment from the plant perspective

Plants can be protected against herbivory by properties that hinder attachment of legs and eggs. The reproductive tissue of several plants is elevated on a greasy pole, a stem densely covered by epicuticular wax crystals, that prevents access of crawling insects (Juniper, 1995). Many buds, fruits and seeds are sticky, glaucous or coated with secreted mucilage which averts attack by insects (Juniper, 1995). Supracellular structures such as trichomes or thorns on the surface of leaves and stems can hinder crawling and walking of insects physically (Kerner, 1879; Jeffree, 1986; Romeis *et al.*, 1999), hamper access of mouthparts to the nutritious tissue and negatively influence larval survival and oviposition behaviour of females (Städler, 2002). In several plant species a higher trichome density is induced by feeding of specific herbivores (Agrawal, 2000; Traw and Dawson, 2002) which increases morphological resistance when needed.

The epicuticular waxes can impede locomotion due to physical and chemical characteristics (Müller and Riederer, 2005) and thereby avert access to stems, leaves, flowers and fruits for feeding, oviposition and nymphoposition (Kerner, 1879; Stork, 1980; Eigenbrode and Espelie, 1995; Ni and Quisenberry, 1997; Ni *et al.*, 1998; Powell *et al.*, 1999). Prominent waxblooms as well as bloomless glossiness, dependent on the presence and crystalline properties of the epicuticular wax layer, have been demonstrated to provide resistance to insects by influencing attachment (Stoner, 1990; Bodnaryk, 1992; Eigenbrode and Pillai, 1998; Eigenbrode, 2004). Loose crystalline material on the surface or an often observed epicuticular exfoliation are also discussed to reduce insect adhesion (Juniper, 1995; Eigenbrode, 1996).

Carnivorous plants may derive some portion of their nutrients from insect prey. Highly adapted features of the cuticular surface are needed to trap and retain the insects. Downward pointing hairs are but one characteristic. The rim (peristome) of *Nepenthes bicalcarata* pitcher leaves causes an 'aquaplaning' by disrupting the attachment of the soft adhesive pads of visiting insects. This happens when the radial ridges of the smooth overlapping epidermal cells are completely covered by a liquid film from nectar and rainwater. Furthermore the surface topography provokes anisotropic friction for claws of the insect legs (Bohn and Federle, 2004). The slippery zone of *Nepenthes alata* pitchers is lined by platelet-shaped crystals, consisting mainly of triacontanol. These polymeric crystals are probably rather stable, resisting erosion by insect feet and thereby impeding attachment of approaching insects and of caught prey trying to escape (Riedel *et al.*, 2003). In the bromeliad species *Brocchinia reducta* and *Catopsis berteroniana* (Bromeliaceae) the surface waxes consist of thread-shaped crystalloids that form a dense homogenous network. The crystals easily break off from the epidermis, thereby interacting with adhesive fluids secreted by the pads of the legs of visiting flies. The attachment of the fly is thereby hindered and insects will be trapped (Gaume *et al.*, 2004).

Some myrmecophilous plants are protected against natural enemies by living in symbiosis with ants. The epicuticular wax covers of different species of the genus *Macaranga* (Euphorbiaceae) form an efficient physical barrier against all 'foreign' non-specialised ant species, allowing only the associated ant species to climb and inhabit the stems (Federle *et al.*, 1997).

13.2.2 Attachment from the insect perspective

The attachment of herbivorous and carnivorous insects is in general higher on surfaces with reduced wax layers (Eigenbrode and Pillai, 1998; Eigenbrode and Kabalo, 1999; Eigenbrode and Jetter, 2002; Gorb and Gorb, 2002; Eigenbrode, 2004), but the reverse is also commonly seen (Eigenbrode and Espelie, 1995; Brennan *et al.*, 2001; Eigenbrode and Jetter, 2002; Eigenbrode, 2004).

Specialised morphological structures or behaviour might allow insects to overcome mechanical impediments of the plant cuticular surface that usually hamper attachment. *Chrysoperla carnea* (Stephen) (Neuroptera: Chrysopidae) produces mucilaginous secretions from an anal adhesive organ that mediate suction on waxy blooms (Eigenbrode, 1996). The tarsae of the chrysomelid beetle *Hemisphaerota cyanea* (Say) (Coleoptera: Chrysomelidae) are oversized and collectively bear some 60 000 adhesive bristles, each with two terminal pads. By touching ground with an increased number of these bristles, the beetle can improve a secure hold on the substrate (Eisner and Aneshansley, 2000). Each tarsus of the bug *Coreus marginatus* L. (Heteroptera: Coreidae) is provided with a pair of smooth flexible pulvilli adapted for attachment to the relatively smooth surface of its host plant *Rumex crispus* L. (Polygonaceae) (Gorb and Gorb, 2004). Weaver ants [*Oecophylla smaragdina* (Fab.), Hymenoptera: Formicidae] possess a flexible adhesion pad between the claws – the arolium – that allows, in combination with a wet adhesive secretion, strong attachment forces to smooth surfaces (Federle *et al.*, 2002). The particular proportion between length of tibia and femur may enable the aphid *Brevicoryne brassicae* (L.) (Homoptera: Aphididae) to apply its tarsae perpendicular to the plant surface, thereby reducing lateral vectors that could hamper adhesion (Southwood, 1986). Walking on the edge of a leaf and thereby opposing the tarsae can have a similar effect, whereby more force is applied perpendicularly to the surface than by gravity (Eigenbrode, 1996). Hessian flies [*Mayetiola destructor* (Say), Diptera: Cecidomyiidae] prefer adaxial over abaxial leaf sides for oviposition due to more pronounced physical features such as grooves and ridges (Kanno and Harris, 2000a); possibly, the eggs attach better to this side compared to the relatively smooth abaxial side.

13.3 Recognition cues for insects

The cuticular surface of the plant is the primary contact zone for an approaching insect. Therefore, it is highly adaptive for a herbivore to recognise a suitable plant

by the epicuticular waxes or secondary compounds present on the surface, without wasting energy and time on a non-host or even running the risk of poisoning by biting into unsuitable leaf tissue. Table 13.1 lists studies where effects of plant surfaces on herbivores are investigated. The references provided are those that were published after the thorough review by Eigenbrode and Espelie (1995) ten years ago on the effects of epicuticular lipids on insect herbivores or those that were not cited by these authors. Most studies (more than two-thirds) were done on crop plants, particularly on cabbages and cereals. A quarter of the studies include tree species (Table 13.1). Among the insect species studied in this context, one-third belong to Lepidoptera. Within the beetles, most is known on behaviour of chrysomelids on plant surfaces, within the Hymenoptera mainly ants were studied. From these data it becomes obvious that many further investigations using different plant as well as insect taxa are needed to gain a more general picture on effects of the cuticle on insects. The various behaviours of the insects that have been shown to be evoked by epicuticular waxes or surface extracts of plants are discussed in the following sections.

13.3.1 Deterrent properties

By impeding locomotion or attachment through trichomes or various wax bloom properties on the plant surface (see earlier), access of insects to the plants is forestalled (Stoner, 1990; Bodnaryk, 1992; Eigenbrode and Pillai, 1998; Eigenbrode, 2004). However, insects able to walk on the plant cuticle can be hindered by a 'secondary strategy' from feeding or ovipositing. Sesquiterpenes are located in trichomes, but are easily released on the plant surface when the cuticular sac of the trichomes matures (Talley *et al.*, 2002). These sesquiterpenes can be toxic to neonate larvae of *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Carter *et al.*, 1989; see Table 13.1). Feeding by several herbivores can induce amounts of secondary compounds such as alkaloids and flavonoids in trichomes, thereby altering the chemical composition of the plant surface to which these compounds are exuded. Such trichome exudates are known to act toxically or as a deterrent towards many insect species (Roda *et al.*, 2003). Chemical compounds of the cuticle are known to evoke rejection behaviour, as e.g. 1-hexacosanol and short-chain fatty acids (C₃–C₁₃) that prevent aphid settling (Phelan and Miller, 1982; Powell *et al.*, 1999) or carboxylic acids and wax esters that have antixenotic properties, deterring feeding (Shepherd *et al.*, 1995). Long-chain alcohols and amyriins reduce biting and increase walking time of neonate caterpillars [*Plutella xylostella* (L.), Lepidoptera: Plutellidae] on leaves of a cruciferous host (Eigenbrode and Pillai, 1998; see Table 13.1). Insect damage, such as punctures by aphids, has been shown to induce wax production (Bystrom *et al.*, 1968).

Deterrent compounds on the leaf are not always produced by the plant itself: destruxins derived from the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin accumulate on the plant surface where they show

Table 13.1 Studies on attractive and deterrent effects of plant surface characteristics on herbivores. In general, leaves were tested, except where noted otherwise. Only references that were published after the review by Eigenbrode and Espelie (1995) or that were not cited by these authors are given

Plant species (Family)	Insect species (Order: Family)	Solvents for extraction/wax removal or source of surface compounds	Involved chemistry	Evoked behaviour	Reference
<i>Daucus carota</i> L. (Apiaceae)	<i>Papilio polyxenes</i> F. (Lepidoptera: Papilionidae)	Chloroform (10 s) and subsequently near-boiling water (1 s)	Flavonoid glycosides, chlorogenic acid	Oviposition stimulants	Brooks <i>et al.</i> (1996)
<i>Lycopersicon hirsutum</i> f. <i>hirsutum</i> (Solanaceae)	<i>Lepinotarsa decemlineata</i> Say (Coleoptera: Chrysomelidae)	Compound in trichomes located on the leaf surface	Zingiberene (sesquiterpene)	Toxic to neonate larvae	Carter <i>et al.</i> (1989)
<i>Triticum aestivum</i> cultivars (Poaceae) with different surface wax composition	<i>Mayetiola destructor</i> (Say) (Diptera: Cecidomyiidae)	Hexane (20 s)	Fifty different wax compounds	Different acceptance for oviposition (13 components potential stimulants, 19 potential deterrents for oviposition)	Cervantes <i>et al.</i> (2002)
<i>Populus</i> clones (Salicaceae)	<i>Chrysomela scripta</i> F. (Coleoptera: Chrysomelidae)	Hexane	Long-chain primary alcohols and α -tocopherylquinone in varying concentrations and ratios	Feeding stimulants	Coyle <i>et al.</i> (2003)
<i>Allium ampeloprasum</i> L. (Alliaceae), <i>Zea mays</i> L. (Poaceae)	<i>Ostrinia nubilalis</i> Hbn. (Lepidoptera: Pyralidae)	Leachates	Free amino acids, soluble carbohydrates (sugars)	Sugars involved in oviposition preference	Derridj <i>et al.</i> (1996)

<i>Brassica oleracea</i> varieties (Brassicaceae)	Neonate <i>Plutella xylostella</i> (L.) (Lepidoptera: Plutellidae)	Primary alcohols; a mixture of α - and β -amyrins; C14-alkanoic acid	Reduce biting; reduce the time of biting and increase the time of walking; increase the time of palpatting More cuttings on leaves with crystalline adaxial surface wax than on glabrous	Eigenbrode and Pillai (1998)
<i>Cercis canadensis</i> var. <i>mexicana</i> (Rose) M. Hopk. (Fabaceae), glossy and glaucous leaves <i>Cajanus cajan</i> (L.) Millsp. (Fabaceae), pods	Leaf-cutter bees <i>Megachile sidalceae</i> Cockerell (Hymenoptera: Megachilidae) Fifth-instar <i>Helicoverpa armigera</i> Hbn. (Lepidoptera: Noctuidae)	Quercetin-3-methyl ether, 3-hydroxy-4-prenyl-5-methoxystilbene-2-carboxylic acid (stilbene)	Feeding stimulant	Eigenbrode <i>et al.</i> (1999) Green <i>et al.</i> (2003)
<i>Mimulus aurantiacus</i> Curtis (Scrophularia-ceae)	<i>Euphydryas chalcedona</i> (Doubleday) (Lepidoptera: Nymphalidae)	Seven resins: ortho dihydroxy resins and methoxylated flavonoids	No correlation between different levels of resins and feeding	Hare (2002)
<i>Brassica</i> genotypes (Brassicaceae)	<i>Delia floralis</i> Fallen (Diptera: Anthomyiidae)	Glucosinolates and other compounds	Other compounds than glucosinolates as oviposition stimulants	Hopkins <i>et al.</i> (1997)
<i>Eucalyptus regnans</i> F. Muell. (Myrtaceae), tip versus centre of leaves	<i>Chrysophtharta bimaculata</i> Oliver (Coleoptera: Chrysomelidae)	Thirty-six wax components in varying proportions on tip and centre	Oviposition preference for leaf tips probably not correlated with leaf chemistry	Howlett and Clarke (2003)

Continued

Table 13.1 Continued

Plant species (Family)	Insect species (Order: Family)	Solvents for extraction/wax removal or source of surface compounds	Involved chemistry	Evoked behaviour	Reference
Forty-two tropical species	<i>Atta cephalotes</i> L. (Hymenoptera: Formicidae)	Soxhlet extracts in chloroform of whole leaves	Terpenoids, steroids and waxes	Repelled fungus-growing ants	Hubbell <i>et al.</i> (1984)
<i>Brassica oleracea</i> cv. <i>botrytis</i> CC Cross (Brassicaceae)	<i>Delia radicum</i> (L.) (Diptera: Anthomyiidae)	Dichloromethane and subsequently two times methanol (5 s per solvent)	1,2-Dihydro-3-thia-4,10,10b-triazacyclo-penta[<i>a</i>]fluorene-1-carboxylic acid (CIF) ¹	Oviposition stimulants	Hurter <i>et al.</i> (1999)
<i>Brassica napus</i> L. (Brassicaceae), glossy and waxy strains and herbicide-treated	<i>Plutella xylostella</i> (L.) (Lepidoptera: Plutellidae)			Oviposition preference for glossy and herbicide-treated leaves	Justus <i>et al.</i> (2000)
Six grasses (Poaceae)	<i>Mayetiola destructor</i> (Say) (Diptera: Cecidomyiidae)	Cool (10°C) dichloromethane (50 s)		Oviposition preferences on surface extracts similar to those on real plants	Kanno and Harris (2000b)
<i>Populus deltoides</i> x <i>P. nigra</i> (Salicaceae)	<i>Chrysomela scripta</i> F. (Coleoptera: Chrysomelidae)	Hexane (30 s)	<i>n</i> -Docosanol, <i>n</i> -tetracosanol, <i>n</i> -hexacosanol (C ₂₆), <i>n</i> -octacosanol (C ₂₈), <i>n</i> -triacontanol (C ₃₀), α -tocopherylquinone	Feeding stimulants	Lin <i>et al.</i> (1998)

<i>Malus domestica</i> var. golden delicious (Rosaceae), fruits and leaves Continued	<i>Cydia pomonella</i> L. (Lepidoptera: Tortricidae)	Ultra-pure water (spraying)	Sugars and sugar alcohols (in particular fructose, sorbitol, myo-inositol)	Oviposition stimulants	Lombarkia and Derridj (2002)
<i>Vitis vinifera</i> cv. Chasselas (Vitaceae), grapes	<i>Lobesia botrana</i> Den. & Schiff. (Lepidoptera: Tortricidae)	Water or methanol; chloroform or hexane (5 min)		Oviposition stimulants; not stimulating	Maher and Thiery (2004)
<i>Brassica napus</i> cv. Express (Brassicaceae)	<i>Delia radicum</i> L. (Diptera: Anthomyiidae)	Chloroform and subsequently two times in methanol (5 s per solvent)	Glucosinolates and CIF ¹	Oviposition stimulants	Marazzi <i>et al.</i> (2004a)
<i>Brassica napus</i> cv. Express (Brassicaceae)	<i>Plutella xylostella</i> L. (Lepidoptera: Plutellidae)	Chloroform and subsequently two times in methanol (5 s per solvent)	Glucosinolates	Oviposition stimulants	Marazzi <i>et al.</i> (2004b)
Different <i>Macaranga</i> ant-plants (Euphorbiaceae), stems	Several ant-species (Hymenoptera: Formicidae)			Slippery for ants	Marksstädtter <i>et al.</i> (2000)
<i>Eucalyptus maculata</i> Hook. (Myrtaceae)	<i>Atta sexdens rubropilosa</i> Forel (Hymenoptera: Formicidae)	Dichloromethane (3 × 30 s)	Large amounts of triterpenoids form crystalline wax blooms	No bioactivity	Marsaro <i>et al.</i> (2004)
<i>Triticum aestivum</i> L. (Poaceae)	<i>Mayetiola destructor</i> (Say) (Diptera: Cecidomyiidae)	Dichloromethane	1-Octacosanal and 6-methoxy-2-benzoxazolinone	Oviposition stimulants	Morris <i>et al.</i> (2000)
<i>Tanacetum vulgare</i> L. (Asteraceae)	<i>Cassida stigmatica</i> Suffr. (Coleoptera: Chrysomelidae)	Hexane/cellulose acetate		Arrestants to females	Müller and Hilker (2001)

Continued

Table 13.1 Continued

Plant species (Family)	Insect species (Order: Family)	Solvents for extraction/wax removal or source of surface compounds	Involved chemistry	Evoked behaviour	Reference
Five genotypes of <i>Triticum aestivum</i> L. (Poaceae)	<i>Diuraphis noxia</i> (Mordvilko) (Homoptera: Aphididae)		Differences in density of trichomes and wax flakes	More or less antixenotic	Ni and Quisenberry (1997)
<i>Triticum aestivum</i> L. c.v. 'Arapahoe' and 'Hall', <i>Hordeum vulgare</i> L. c.v. 'Morex', <i>Avena sativa</i> L. c.v. 'Border' (Poaceae)	<i>Diuraphis noxia</i> (Mordvilko) (Homoptera: Aphididae)	Ethyl ether		Only limited influence of wax removal on probing and nymphosition preferences	Ni <i>et al.</i> (1998)
<i>Vicia faba</i> L. (Fabaceae; host); <i>Avena sativa</i> L. (Poaceae, non-host)	<i>Aphis fabae</i> Scopoli, <i>Sitobion avenae</i> F. (Homoptera: Aphididae)	Chloroform (10 s)/cellulose acetate	1-Hexacosanol predominant on <i>A. sativa</i>	Triggered stylet penetration; non-host only accepted after wax removal	Powell <i>et al.</i> (1999)
<i>Brassica napus</i> L. var. 'Martina', <i>Nasturtium officinale</i> R. Br. (Brassicaceae)	<i>Phaedon cochleariae</i> (F.) (Coleoptera: Chrysomelidae)	Chloroform : methanol: water (2 : 1 : 1) (3 × 20 s); gum arabic as adhesive	Minor amounts of glucosinolates in solvent extracts	No stimulatory activity by wax extracts and/or glucosinolates; feeding preference for de-waxed leaves	Reifenrath <i>et al.</i> (2005)
<i>Nicotiana attenuata</i> Torr. Ex Wats. (Solanaceae)	<i>Tupiocoris notatus</i> Distant (Heteroptera: Miridae)	Glandular trichomes, content excreted onto the leaf surface	Flavonols: quercetin and seven methylated derivatives	Quercetin as feeding attractant	Roda <i>et al.</i> (2003)

<i>Didymopanax vinosum</i> Marchal (Araliaceae)	<i>Atta sexdens rubropilosa</i> Forel (Hymenoptera: Formicidae)	Chloroform (three times for 30 s)	Lupcol, primary <i>n</i> -alcohols	Deterrent for some colonies	Salatino <i>et al.</i> (1998)
<i>Cajanus scarabaeoides</i> (L.) Thouars (Fabaceae)	<i>Helicoverpa armigera</i> Hbn. (Lepidoptera: Noctuidae)	Water		Feeding deterrents	Shanower <i>et al.</i> (1997)
<i>Cajanus cajan</i> (L.) Millsp. and <i>C. platycarpus</i> (Fabaceae)	<i>Helicoverpa armigera</i> Hbn. (Lepidoptera: Noctuidae)	Acetone		Feeding stimulants	Shanower <i>et al.</i> (1997)
<i>Rubus idaeus</i> L. cultivars (Rosaceae)	<i>Amphorophora idaei</i> Börner (Homoptera: Aphididae)	Dichloromethane (10 s)	Sterols, particularly cycloartenol, branched alkanes, presence or absence of C-29 ketones and symmetrical C-29 secondary alcohol, and other compounds	Settling preference on leaves with greater wax coverage and higher levels of the compounds of shorter chain length	Shepherd <i>et al.</i> (1999a)
<i>Rubus idaeus</i> L. cultivars (Rosaceae)	<i>Amphorophora idaei</i> Börner (Homoptera: Aphididae)	Dichloromethane (10 s)	Higher levels of cycloartenyl esters, α -ameryl esters and other compounds in wax from the resistant cultivar	Different resistant properties to aphids	Shepherd <i>et al.</i> (1999b)
<i>Brassica oleracea</i> L. (Brassicaceae)	<i>Plutella xylostella</i> L. (Lepidoptera: Plutellidae)	Artificially mixed	<i>n</i> -Alkane mixture in combination with glucosinolate	Oviposition stimulants	Spencer (1996), Spencer <i>et al.</i> (1999)

Continued

Table 13.1 Continued

Plant species (Family)	Insect species (Order: Family)	Solvents for extraction/wax removal or source of surface compounds	Involved chemistry	Evoked behaviour	Reference
<i>Eucalyptus globulus</i> subsp. <i>pseudoglobulus</i> (Naudin ex Maiden) Kirkpatr. and putative hybrid (Myrtaceae)	<i>Mnesampela privata</i> (Guenée) (Lepidoptera: Geometridae)	Hexane (50 min)	Differences in several wax esters between waxy and glossy leaves	Oviposition preference for waxy leaves and young leaves; reduced performance of offspring on such leaves	Steinbauer <i>et al.</i> (2004)
Nine woody species	<i>Atta sexdens rubropilosa</i> Forel (Hymenoptera: Formicidae)	Chloroform (three times for 30 s)		Different wax extracts caused different responses (no negative influence, slight and strong deterrence)	Sugayama and Salatino (1995)
<i>Zea mays</i> L. (Poaceae)	<i>Ostrinia nubilalis</i> Hbn. (Lepidoptera: Pyralidae)	Pentane (20 s)	<i>n</i> -Alkanes (hexacosane, heptacosane, octacosane, nonacosane, tritriacontane)	Oviposition stimulants	Udayagiri and Mason (1997)
<i>Brassica oleracea</i> L. (Brassicaceae)	<i>Pieris brassicae</i> (L.) (Lepidoptera: Pieridae)	Dichloromethane and subsequently in methanol (3 s per solvent)	Glucobrassicin (3-indolyl-methyl-glucosinolate)	Oviposition stimulant	van Loon <i>et al.</i> (1992)
<i>Solanum berthaultii</i> Hawkes (Solanaceae)	<i>Leptinotarsa decemlineata</i> Say (Coleoptera: Chrysomelidae)	Ice-chilled methylene chloride (10 + 10 + 5 s); further fractionated		Feeding deterrents, mainly in non-volatile fraction	Yencho <i>et al.</i> (1994)

¹ CIF, cabbage identification factor.

antifeedant properties (Amiri *et al.*, 1999). Compounds derived from yeasts, possibly polyamines, can prevent oviposition (Städler, 2002).

Generalist herbivores might use surface chemicals as cues in associative learning of aversion. The polyphagous grasshopper *Schistocerca americana* (Drury) (Orthoptera: Acrididae) first has to bite into leaf tissue to recognise an unpalatable plant but afterwards, palpation of the surface is sufficient to evoke rejection (Chapman and Sword, 1993). Larvae and adults of the tansy leaf beetle *Galeruca tanacetii* (L.) (Coleoptera: Chrysomelidae) prefer to feed on leaves of *Tanacetum vulgare* L. (Asteraceae) where waxes are removed with cellulose acetate treatment (Müller and Hilker, 2001) over intact leaves, while they do not discriminate between waxy and de-waxed leaves of *Brassica pekinensis* (Lour.) Rupr. (Brassicaceae) (Figure 13.1). This generalist has a rather poor performance with long

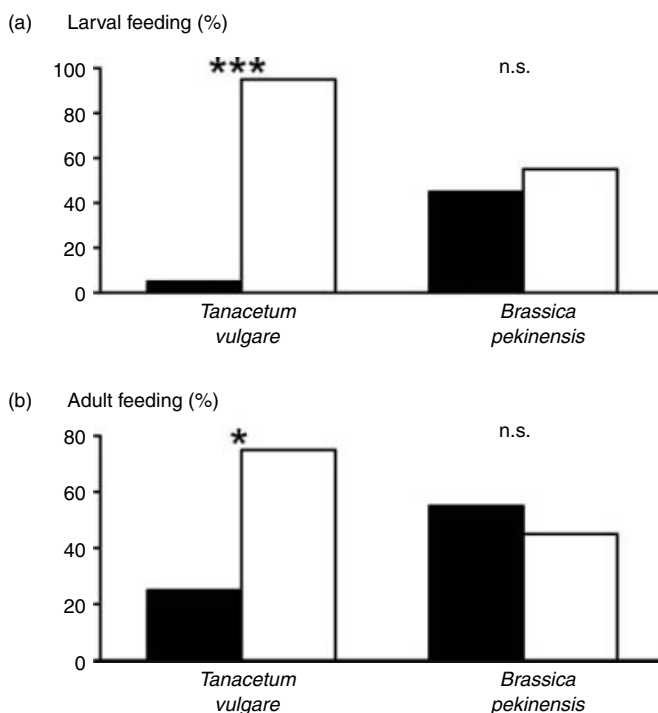


Figure 13.1 Percentage of individual larvae (a) and adults (b) of *Galeruca tanacetii*, feeding on intact (black bars) or de-waxed (white bars) leaf parts of the host plants *Tanacetum vulgare* and *Brassica pekinensis*. Leaflets (*T. vulgare*) or leaf discs (*B. pekinensis*) were dipped half in cellulose acetate dissolved in acetone (5–10% w/v). After evaporation of acetone, a white film that appeared on the surface of the treated side was carefully removed by forceps. Leaves of one species were offered to individual second-instar larvae or adults ($N = 20$ per host plant and per developmental stage). * $P < 0.05$; *** $p < 0.001$; n.s., not significant; Wilcoxon signed-rank test for paired differences, two-sided). While *G. tanacetii* avoids waxes of the poor host plant *T. vulgare*, waxes of the highly suitable host plant *B. pekinensis* (cultivar without trichomes) do not influence feeding.

developmental times and a low pupal weight when reared on *T. vulgare* compared to *B. pekinensis* (Müller, 1999). The surface waxes of *T. vulgare* presumably imply negative information for this generalist, either chemically or mechanically. The specialist beetle *Phaedon cochleariae* (F.) (Coleoptera: Chrysomelidae) prefers de-waxed over intact leaf parts of its host plants *Nasturtium officinale* R. Br. and *Brassica napus* L. var. *Martina* (Brassicaceae) (Reifenrath *et al.*, 2005). In this case, epicuticular waxes might cover access to or 'hide' feeding stimulants present in leaf cells of the interior. This might be an adaptive trait for the protection of the plant against herbivorous specialists.

Leaf-cutting, fungus-growing ants have been shown to be repelled or deterred to different degrees by terpenoids, steroids and waxes extracted from various potential host plants (Hubbell *et al.*, 1984; Sugayama and Salatino, 1995). Certain compounds of epicuticular waxes of *Didymopanax vinosum* Marchal (Araliaceae) were only deterrent for some but not all colonies of *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae) (Salatino *et al.*, 1998; Table 13.1).

13.3.2 Attractive properties

Many studies focus on secondary plant compounds that are characteristic for certain plant species or families and should therefore be used by insect specialists for host recognition (Harborne, 1995). Within the last two decades several species-specific wax compounds of the cuticle have been determined (Chapter 4) that could mediate the host-finding process. Waxy compounds such as long-chain alkanes, alcohols, carboxylic acids, as well as secondary metabolites such as quinones and flavonoids located at the plant surface have been shown to be involved in feeding stimulation (Adati and Matsuda, 1993; Lin *et al.*, 1998; Coyle *et al.*, 2003; Green *et al.*, 2003). More studies dealt with oviposition stimulants and revealed various alkanes, aldehydes, flavonoids, chlorogenic acids, glucosinolates, the 'cabbage identification factor' (1,2-dihydro-3-thia-4,10,10b-triaza-cyclo-penta[.a.]fluorene-1-carboxylic acid) (Brooks *et al.*, 1996; Udayagiri and Mason, 1997; Hurter *et al.*, 1999; Spencer *et al.*, 1999; Marazzi *et al.*, 2004a,b) as well as sugars that leak on the plant surface (among others released from extrafloral nectaries) (Derridj *et al.*, 1996; Lombarkia and Derridj, 2002; Leveau, 2004) as active principles in host recognition (see Table 13.1). In several insect species, epicuticular wax compounds act in synergism with secondary metabolites as oviposition stimulants (Roessingh *et al.*, 1992; Spencer, 1996; Spencer *et al.*, 1999; Morris *et al.*, 2000; Marazzi *et al.*, 2004a).

A given herbivorous species, such as for example the Colorado potato beetle (*Leptinotarsa decemlineata*) or the Cotton bollworm *Helicoverpa armigera* Hbn. (Lepidoptera: Noctuidae), can distinguish between closely related plant species primarily by infochemicals perceived at or near the leaf surface (Harrison, 1987; Shanower *et al.*, 1997). Waxes of a given host plant, on the other hand, can comprise opposing information for herbivores with different degrees of specialisation. While the polyphagous generalist *G. tanacetii* is deterred by waxes of leaves of the less

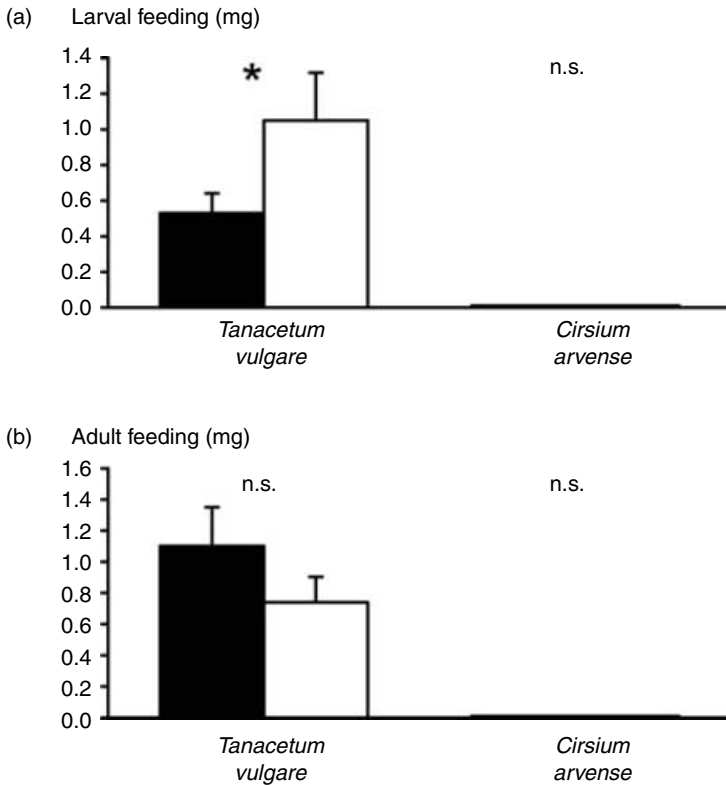


Figure 13.2 Mean amount (\pm se) of individual third-instar larvae (a) and adults (b) of *Cassida denticollis*, feeding on intact (black bars) or de-waxed (white bars) leaf parts of the host plant *Tanacetum vulgare* and the non-host *Cirsium arvense*. Leaflets were dipped half in cellulose acetate dissolved in acetone (5–10% w/v). After evaporation of acetone, a white film that appeared on the surface of the treated side was carefully removed by forceps. Leaves were offered to individual third-instar larvae or adults ($N = 20$ per host plant and per developmental stage). * $P < 0.05$; n.s., not significant; Wilcoxon signed-rank test for paired differences, two-sided).

suitable host *T. vulgare* (see earlier and Figure 13.1) the oligophagous specialist *Cassida denticollis* Suffr. (Coleoptera: Chrysomelidae) that feeds on a few species of Asteraceae only, seems to be not influenced by waxes. Third-instar larvae and adults of *C. denticollis* do not discriminate between intact or de-waxed leaflets of *T. vulgare* and accept both in a comparable manner. Leaflets of the non-host *Cirsium arvense* (L.) Scop. are not accepted for feeding, neither with nor without cuticular waxes, by *C. denticollis* (Figure 13.2). For the strictly monophagous specialist *Cassida stigmatica* (Coleoptera: Chrysomelidae), epicuticular waxes of *T. vulgare* constitute an important recognition cue in oviposition stimulation. Females of *C. stigmatica* prefer to lay eggs on the abaxial leaf side of the plant. While they do not discriminate between leaves with and without waxes when being offered the adaxial side only,

they clearly prefer to oviposit on intact leaves offered with the abaxial side over de-waxed parts (Müller and Hilker, 2001).

As the chemistry of the epicuticular waxes also influences the fine structure of the cuticular surface (Jeffree, 1986; Markstädter *et al.*, 2000), it cannot easily be differentiated if the insect might use mainly the chemical and/or mechanical information for its host plant selection process (Eigenbrode and Espelie, 1995; Kanno and Harris, 2000a,b). Due to the chemical composition and micromorphology, optical characteristics of the cuticular surface are highly affected (Chapter 6). Visual cues, such as UV-reflectance, might be important recognition properties for pollinators, dispersers and non-pollinating herbivores (Juniper, 1995). However, a causal link between visual cues of the cuticle and attractiveness to herbivores has rarely been addressed (Eigenbrode and Espelie, 1995; Justus *et al.*, 2000; Steinbauer *et al.*, 2004). Preferences of insects can be significantly influenced by leaf colour which is a result of wavelength reflectance of different intensities (due to surface properties). This has been shown for example for the leafhopper *Empoasca fabae* (Harris) (Homoptera: Cicadellidae) (Bullas-Appleton *et al.*, 2004). The colour perception of an insect can be further affected by reflection polarisation, again determined by wax structure (Grant *et al.*, 1993), if it has a polarisation-dependent colour vision system. *Papilio* butterflies are sensitive to such interferences (Kelber, 1999; Kelber *et al.*, 2001). Perception of polarisation reflection might enable them to discriminate shiny from matt surfaces (Horváth *et al.*, 2002) which are in turn determined by the presence of a waxy layer or other microstructures. It is likely that visual and chemical cues of the surface act in concert in the host recognition process of an insect (Prokopy and Owens, 1983; Degen and Städler, 1997).

For predators, an infestation of a plant with potential prey might be recognised by changes in the visual appearance caused by the herbivore feeding. Sawfly damage induces amongst others an increase of surface flavonoids in the leaves of mountain birch (*Betula pubescens*, Ehrh., Betulaceae) which affects the UV spectral maxima. Predators such as birds could well respond to these cues to detect their prey (Mäntylä *et al.*, 2004). The pupal parasitoid *Pimpla turionellae* (L.) (Hymenoptera: Ichneumonidae) has been demonstrated to perceive changes in the chromatic and achromatic plant surface appearance that are due to an infestation with its endophytically living host (Fischer *et al.*, 2004).

13.4 Mimicry

Mimicry systems are characterised by an organism (mimic) simulating signal properties of a second organism (model) which are in turn recognised by a third organism (operator). The organism that mimics a model has an advantage due to the fact that the operator falsely identifies it as a model (Vane-Wright, 1980). In several cases mentioned later it is not entirely unambiguous to determine the model or operator organism. Nevertheless, similarities of two organisms in complex traits like hydrocarbon patterns imply an ecological meaning.

13.4.1 *Mimicry from the plant perspective*

Plants have evolved intriguing mimicry systems to attract insects as pollinators or dispersers by false cues. They might be either mimicking food or brood resources by odours or contact stimuli that visually and/or chemically resemble e.g. fresh or rotten fruit or by odours that actually mimic sex, brood or alarm pheromones of the cheated insect (Dettner and Liepert, 1994). When volatiles are attractive, they need to be emitted from the plant surface, and are either produced there directly by trichomes or in the interior of the plant and subsequently transported to the outer wax layer (Chapter 7). In many of the mimicry systems, hydrocarbons are involved that mediate signals in the short range as contact cues. The plant family of Orchidaceae is probably best known to include a high number of plants that need very specific insect species as pollinators and therefore have evolved diverse strategies to attract them, often without giving them an actual reward (Wiens, 1978). Compounds in orchid species resembling female sex pheromones of the respective pollinators have been identified as C₁₁–C₁₉ unsaturated and saturated hydrocarbons, primary and secondary alcohols, aliphatic short and long-chain esters and mono- and sesquiterpenes and their esters (Dettner and Liepert, 1994). The relative proportions of the hydrocarbon compounds can however differ between the orchid and the pollinating insect (Schiestl *et al.*, 2000). Also, some unique compounds such as trace amounts of (ω -1)-hydroxy and (ω -1)-oxo acids, especially 9-hydroxydecanoic acid, were identified to be released from an orchid whose flowers seem to be even more attractive to pollinating male wasps than their females (Ayasse *et al.*, 2003).

Seeds of several tropic epiphytes are dispersed by ants. In extract of these seeds commonly 6-methyl salicylic acid methyl ester was detected which triggers a dose-dependent response in *Camponotus femoratus* (F.) (Hymenoptera: Formicidae). This compound also occurs in the mandibular glands of *Camponotus* ants and acts as a brood pheromone (Davidson *et al.*, 1990). To stimulate ants to carry the seeds, 6-methyl salicylic acid methyl ester must be detectable at the surface.

The sesquiterpene hydrocarbon (*E*)- β -farnesene is present in glandular hairs and is continuously released from intact wild potato plants. As this sesquiterpene is also the alarm pheromone of several dozen aphid species it is an efficient way of the plant to keep aphids away (Gibson and Pickett, 1983).

13.4.2 *Mimicry from the insect perspective*

Espelie (Espelie and Bernays, 1989; Espelie and Brown, 1990; Espelie *et al.*, 1991) noted that plant surface chemicals could be transferred to the insect surface either by direct contact or metabolically. These plant chemicals could either be derived from trichomes as for example found in herbivores feeding on Solanaceae, or might be a powder consisting of long-chain alcohols of the plant epicuticular waxes deposited on the insect cuticle. Chemicals mimicking the plant surface might be also dietary (Espelie and Bernays, 1989) or even synthesised *de novo* by an insect (Espelie and Brown, 1990). It is suggested that such plant chemicals on the insect surface might

help the herbivore to be cryptic to predators and parasitoids (Espelie *et al.*, 1991). A social wasp that nests in trees of *Acacia collinsii* Saff. (Fabaceae) is probably protected against aggression by an ant species occupying these *Acacia* trees due to a mimicry system. The hydrocarbon pattern, characterised by certain alkene–alkane pairs, is not only similar between the surface wax of the wasp and the ant, but also *n*-alkanes of the surface wax of the wasp nest resemble those detected in the surface wax of acacia thorns in which the ants live (Espelie and Hermann, 1988).

13.5 Methods of investigation

To investigate the role of plant epicuticular waxes on the behaviour of visiting insects, various approaches have been employed. On the one hand, mutants or genotypes of plant species differing in wax morphology and chemistry are used to test influences of the surface characteristics on attachment and acceptability by herbivores and carnivores (e.g. Bodnaryk, 1992; Ni *et al.*, 1998; Eigenbrode and Kabalo, 1999; Kanno and Harris, 2000b; Cervantes *et al.*, 2002; Chang *et al.*, 2004). Even within a given plant, the epicuticular waxes of leaves can strongly vary with developmental stage and thus offer a genetically identical substrate for elegant comparative work (Brennan *et al.*, 2001; Steinbauer *et al.*, 2004).

On the other hand, or additionally, waxes are extracted from plant material (leaves, stems, fruits) in various ways (see later). These extracts can be applied on neutral substrates and offered in bioassays to test for the activity of wax compounds (e.g. Sugayama and Salatino, 1995; Hurter *et al.*, 1999; Kanno and Harris, 2000b; Cervantes *et al.*, 2002; Lombarkia and Derridj, 2002). Such extracts are also used to test for electrophysiological responses (e.g. Hurter *et al.*, 1999; Justus *et al.*, 2000; Steinbauer *et al.*, 2004). Bioassay-guided fractionation might help to isolate the active principles within the plethora of surface compounds (Malony *et al.*, 1988; Foster and Harris, 1992; Yencho *et al.*, 1994) that can be analysed by chemical analysis (see Table 13.1). Extracts are often gained by dipping plant material shortly in solvents of different polarity (see Table 13.1). However, a drawback of this extraction procedure is the fact that compounds might be derived from locations other than the actual outermost epicuticular leaf surface (Roessingh *et al.*, 1992; Riederer and Markstädter, 1996; Jetter *et al.*, 2000) and mostly not sampled from one specific leaf side only. If leaves are kept in darkness for a few hours before extraction, stomata will be at maximal closure. This lowers the risk of washing out compounds from the leaf interior through treatment with solvents (Reifenrath *et al.*, 2005). Wax load as well as chemical composition can vary on abaxial and adaxial leaf sides (Premachandra *et al.*, 1993; Yang *et al.*, 1993a; Eigenbrode and Pillai, 1998; Eigenbrode *et al.*, 1999; Reifenrath *et al.*, 2005). Accordingly, many insects show oviposition preferences for one leaf side that might be determined by differences in chemistry and structure (Kanno and Harris, 2000a; Müller and Hilker, 2001). To probe leaf sides separately, intact leaves can be placed on a flexible rubber mat, and a glass cylinder gently pressed onto the exposed surface. In these cylinders

solvents are shortly agitated and then removed for testing (Premachandra *et al.*, 1993; Jetter *et al.*, 2000; Reifenrath *et al.*, 2005).

A third way to investigate how insects are influenced by the cuticle is the removal of epicuticular waxes and test of the remaining tissue. Such de-waxed plant material can then be offered against intact material to test for behavioural answers (Powell *et al.*, 1999; Müller and Hilker, 2001; Reifenrath *et al.*, 2005; Figures 13.1 and 13.2). When waxes are removed by dipping plant material in solvents, the chemistry of the surface and the interior can be drastically changed. Therefore it is more suitable to remove waxes mechanically. A (side-)specific mechanical removal of epicuticular waxes without damaging underlying tissue could be achieved by treatment with cellulose acetate (Powell *et al.*, 1999; Müller and Hilker, 2001) or gum arabic (Jetter *et al.*, 2000; Jetter and Schäffer, 2001; Riedel *et al.*, 2003; Reifenrath *et al.*, 2005), respectively. After evaporation of solvents (acetone or water, respectively), both adhesives form a film that can be removed. In case of gum arabic, the polymeric film can be dissolved in a solvent for further chemical analysis (Riedel *et al.*, 2003; Reifenrath *et al.*, 2005). Frozen liquids can be used as a cryo-adhesive (Ensatat *et al.*, 2000; Riedel *et al.*, 2003). For this purpose, cylinders are gently pressed on a leaf surface, filled with glycerol or water and frozen in liquid nitrogen. The epicuticular waxes will stick to the frozen liquid and allow to test the wax film (after evaporation of the liquid) or the de-waxed leaf in a bioassay, and to analyse the chemical composition of the waxes.

13.6 Application in biological pest management

In many crop plants varieties exist that differ in wax cover, wax composition or trichome density and therefore are more or less suitable for a herbivorous pest. For example, glossy plant mutants of *Brassica oleracea* L. (Brassicaceae) are more prone to water stress. This can cause an accumulation of deterrent compounds which in turn reduces feeding attacks by aphids (Cole and Riggall, 1992). Trichomes have been shown to be efficient physical barriers against herbivorous pests for a number of important crop plant species within the Brassicaceae, Poaceae, Malvaceae (cotton) and other plant families (Peter and Shanower, 2001). However, altering just one of the plant surface features, through traditional breeding or genetic engineering, might not have the desired economic benefit: for herbivorous insects a variety of cues are important in the host acceptance process, detected by chemo- as well as mechanoreceptors and playing in concert together. Therefore, reliable predictions about the feasibility of breeding antixenotic resistance are difficult (Kanno and Harris, 2000b).

Furthermore, the selection of a certain genotype that is less suitable for one insect pest species, might impact suitability to other organisms. Other insect pests or fungi could be even more attracted. Wax characteristics also influence visiting predators or parasitoids (McAuslane *et al.*, 2000; Eigenbrode *et al.*, 2000; Eigenbrode, 2004) whose presence would be advantageous for the plant. Therefore, interactions on

all trophic levels have to be considered in choosing the suitable crop cultivar for planting.

Using pesticides can change the surface characteristics drastically, for example by affecting the fine structure of the epicuticular wax layer (Chapter 7) or wettability. This in turn influences the acceptability by herbivores. Treatment with a carbamate herbicide (S-ethyl dipropylthiocarbamate) of canola (*Brassica napus* L.) has been shown to increase oviposition by the diamond back moth (*P. xylostella* L., Lepidoptera: Plutellidae) (Justus *et al.*, 2000). Stress hormones such as jasmonic acid and analogues are involved as signal transducers in plant defence against pathogens and herbivores. However, treatment of celery leaves (*Apium graveolens* cv. *secalinum*, Apiaceae) with these substances increases the acceptability by the carrot fly *Psila rosae* (F.) (Diptera: Psilidae). Due to the jasmonic acid treatment, furanocoumarins accumulate in the leaf surface, which in turn stimulate oviposition by the insect pest (Stanjek *et al.*, 1997). Induced changes of the surface chemistry of crop plants can thus have strong impacts on suitability to herbivores.

Also, changes in abiotic conditions can influence plant–insect interactions. Caution is advised when insight from studies of plants grown in the greenhouse or growth chambers is transferred to the field. The epicuticular lipid composition can vary considerably between both environmental conditions and impact herbivore responses to various degrees (Woodhead, 1981; Yang *et al.*, 1993b).

13.7 Conclusion

Since the last thorough review by Eigenbrode and Espelie (1995) on effects of plant surface lipids on insects, much progress has been made. Main new findings have been reported in this chapter. Several more studies have elucidated the important role of leaf surface characteristics and even of particular lipid compounds for host acceptance and deterrence of herbivores. Elegant research approaches shed more light on influences of cuticle properties on the third trophic level, mainly by investigations of tarsal attachment, as well as on the underlying biomechanical characteristics. The same is true for knowledge about carnivorous plants.

The development of highly selective probing methods for epicuticular waxes now allows the clear separation of surface cues from cues that are actually only active once the insect has damaged the leaves with its mouthparts or by scratching with its legs or ovipositor to get access to inner plant material. Still very little is known about visual effects of waxes on host plant selection by insects, probably due to experimental issues. Also, in the area of changes of surface characteristics due to induction an augmentation of knowledge can be expected, as in general, research on plant responses after induction increased within the last years.

It would be desirable to link studies on different levels, plant physiology, molecular biology, biomechanics, behaviour and ecology, in order to get a broader picture of the impact of the plant cuticle in biotic interactions and even the ecosystem. For example, the physiological role of the plant cuticle, primarily to prevent water loss,

might be linked with the preference behaviour of an insect (Müller and Riederer, 2005). The insect might be able to determine plant quality by leaf toughness, chemistry and transpiration properties acting together with some factors yet to be elucidated.

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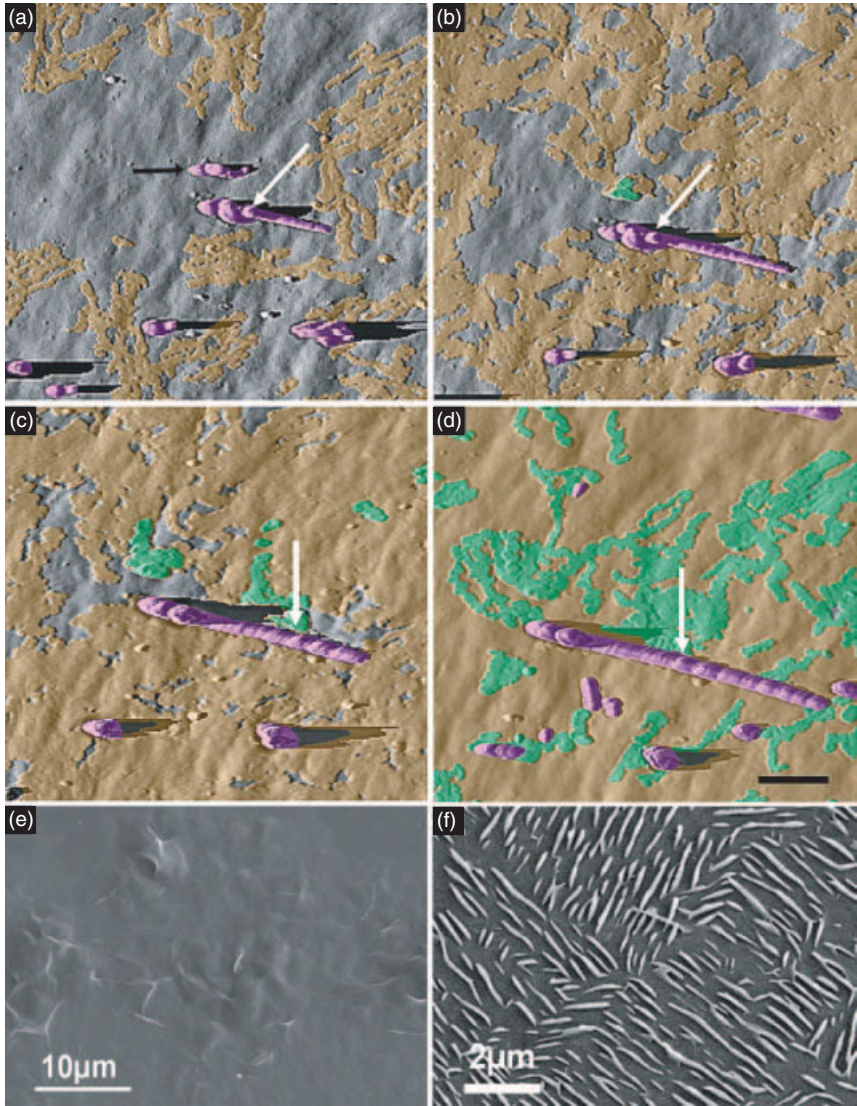


Plate 1 Atomic force microscopy (AFM) images of epicuticular wax regenerating on a living *Galanthus nivalis* leaf adaxial surface, following removal of the earlier-secreted wax with epoxy resin glue. The images show changes in a $6 \times 6 \mu\text{m}$ area during an 80-min time frame. Flat, lobed plates extend over the whole surface to form first a monolayer (light grey) (a,b) and then bilayered structures (dark grey) (c,d). Independently, rod-like crystals arise directly from the cleaned cuticle proper (CP) surface, extending by growth at their distal ends, not from the base. Diagonal arrows (a,b) and vertical arrows (c,d) mark reference points that remain in fixed positions during rod extension growth. The black arrow in (a) marks a crystal that in (b) has been removed by the AFM tip. (a–d) bars = $1 \mu\text{m}$; reproduced with permission from Koch *et al.* (2004), *Journal of Experimental Botany*, **55**, 711–718. (e,f) SEM images of wheat leaf wax recrystallised from chloroform on glass forming an amorphous film (e) and on freshly cleaved highly ordered pyrolytic graphite (HOPG; PLANO GmbH, Wetzlar, Germany) (f), producing highly ordered wax crystals; reproduced with permission from Kerstin Koch (see also Koch, K., Barthlott, W., Koch, S., Hommes, A., Wandelt, K., Mamdouh, H., De-Feyter, S. and Broekmann, P. Structural analysis of wheat wax (*Triticum aestivum*, c.v. 'Naturastar' L.): from the molecular level to three dimensional crystals, *Planta*, in press).

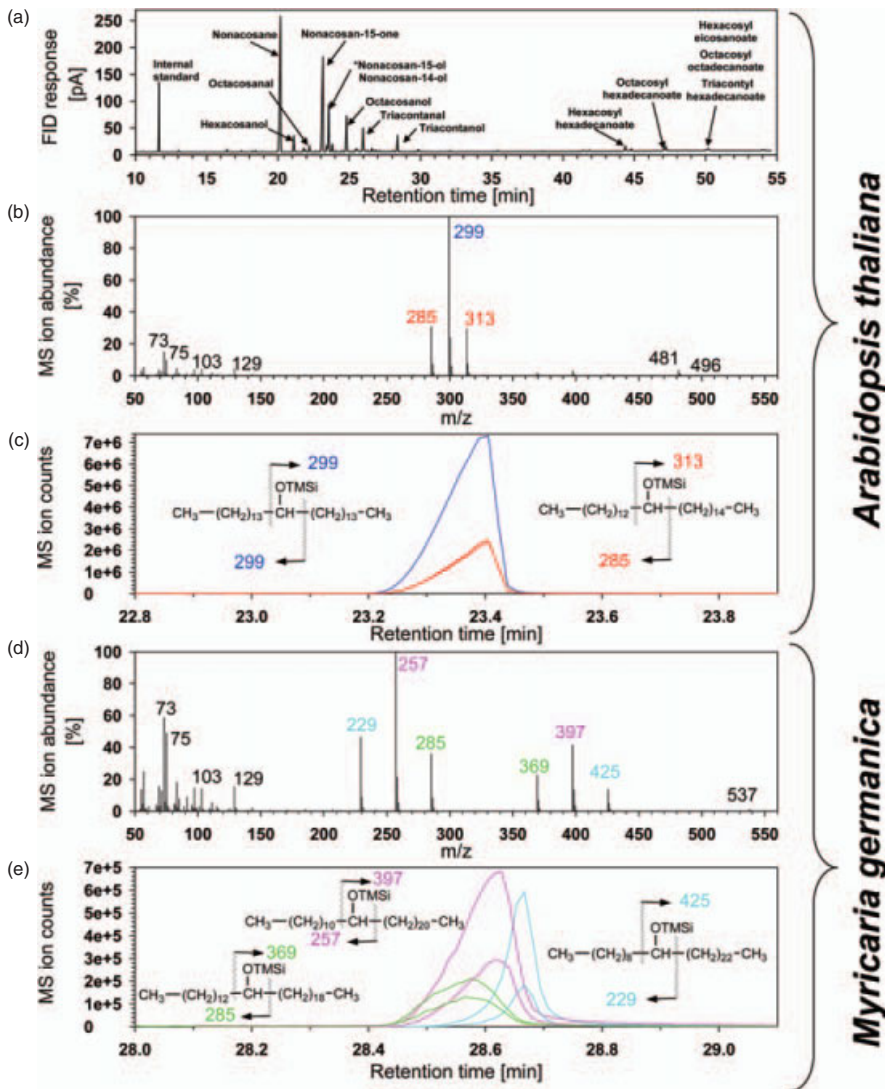


Plate 2 Gas chromatographic (GC) separation and mass spectrometric (MS) identification of plant cuticular wax components. (a) GC-FID trace of the wax mixture extracted from inflorescence stems of *Arabidopsis thaliana* ecotype Columbia. (b) Mixed mass spectrum of trimethyl silyl (TMSi) derivatives of nonacosan-14-ol and nonacosan-15-ol [corresponding to the GC peak at 23.5 min in (a)] showing characteristic α -fragments at $m/z = 285, 299, 313$. (c) GC-MS traces of the α -ions, demonstrating that the isomeric 14- and 15-alcohols cannot be separated under the GC conditions used. (d) Mixed mass spectrum of TMSi derivatives of tritriacontan-10-ol, tritriacontan-12-ol and tritriacontan-14-ol (from leaf wax of *Myricaria germanica*) showing characteristic α -fragments at $m/z = 229, 257, 285$ and $m/z = 369, 397, 425$, respectively. (e) GC-MS traces of the α -ions, demonstrating that the isomeric 10-, 12- and 14-alcohols can be partially separated under the GC conditions used.

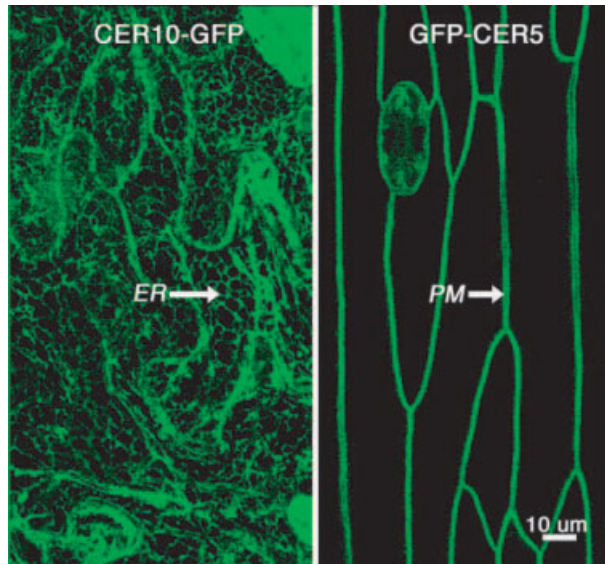


Plate 3 Green fluorescent protein (GFP) experiments demonstrate the location of the proteins involved in wax production. These images represent a projection of optic sections collected with the laser scanning confocal microscope. The enoyl-CoA reductase (CER10-GFP) component of the elongase was found in the endoplasmic reticulum of pavement cells in the leaf of *Arabidopsis thaliana*. ABC transporter CER5 was localised to the plasma membrane in *A. thaliana* stems (GFP-CER5). Magnification bar = 10 μm .

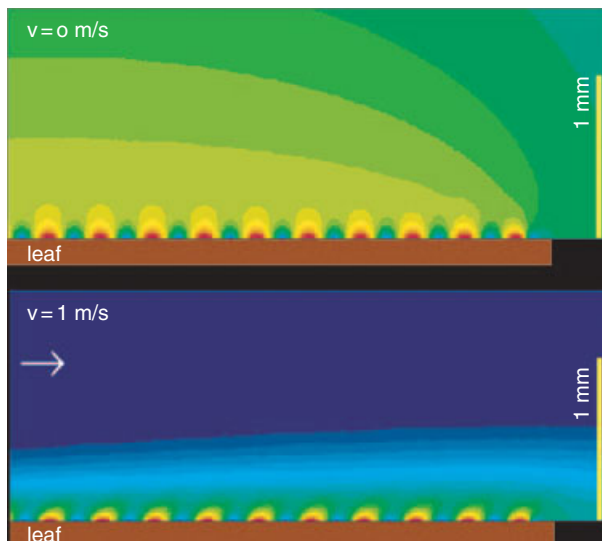


Plate 4 Isolines of the relative concentration of a semi-volatile compound in the unstirred layer in the vicinity of the leaf surface. Relative concentrations are colour-coded from red (highest) to dark blue (lowest). In the finite-element model leading to this picture a regular distribution on the leaf surface of solid deposits of the organic compound (red spots) and stomata was assumed. The concentration distributions at two wind speeds (0 and 1 m s^{-1}) are shown. Figure from Riederer *et al.* (2002) by kind permission by Oxford University Press.

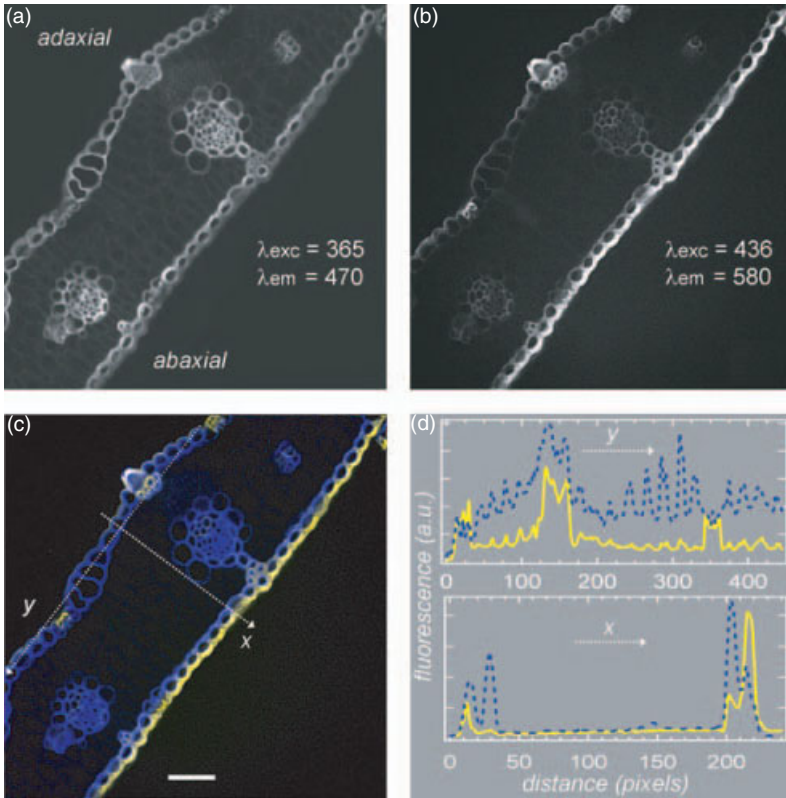


Plate 5 Multispectral autofluorescence microimaging of a 100- μm -thick cross-section from a *Triticum aestivum* L. leaf in phosphate buffer. Autofluorescence images in panel (a) were excited at 365 nm and detected in the blue range at 470 nm, and in panel (b) were excited at 436 nm and detected in the yellow range at 580 nm. Combination of monochrome images, (a) and (b), with blue and yellow colours assigned to the 470 and 580 emission bands, respectively, is shown in panel C (bar = 50 μm). Panel (d) depicts fluorescence intensity profiles for the 470 and 580 nm bands along the two, x and y, directions indicated by dotted arrows in (c). Panels (c) and (d) clearly reveal inhomogeneous fluorescence characteristics and, thus, quite large variations in spatial localisation of different fluorescing compounds: the UV-induced blue fluorescence emanates from cell walls, while the blue-induced yellow signal appears to be confined to cuticles, guard cells and sclerenchyma bands (G. Agati, Corrado Tani and Z.G. Cerovic, unpublished data).

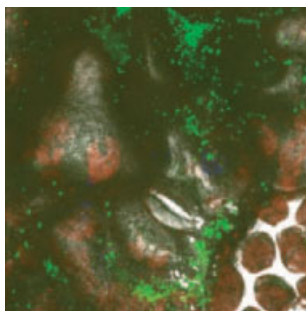


Plate 6 View of a colonised bean leaf using confocal laser scanning microscopy. Individual bacterial colonisers producing GFP are visible as green fluorescent dots. The centre shows a stoma; the cuticular top of an epidermal plant cell is visible on the left top corner while at the bottom right the picture slices into the leaf's palisade parenchyma. Chlorophyll is coloured red in this picture.

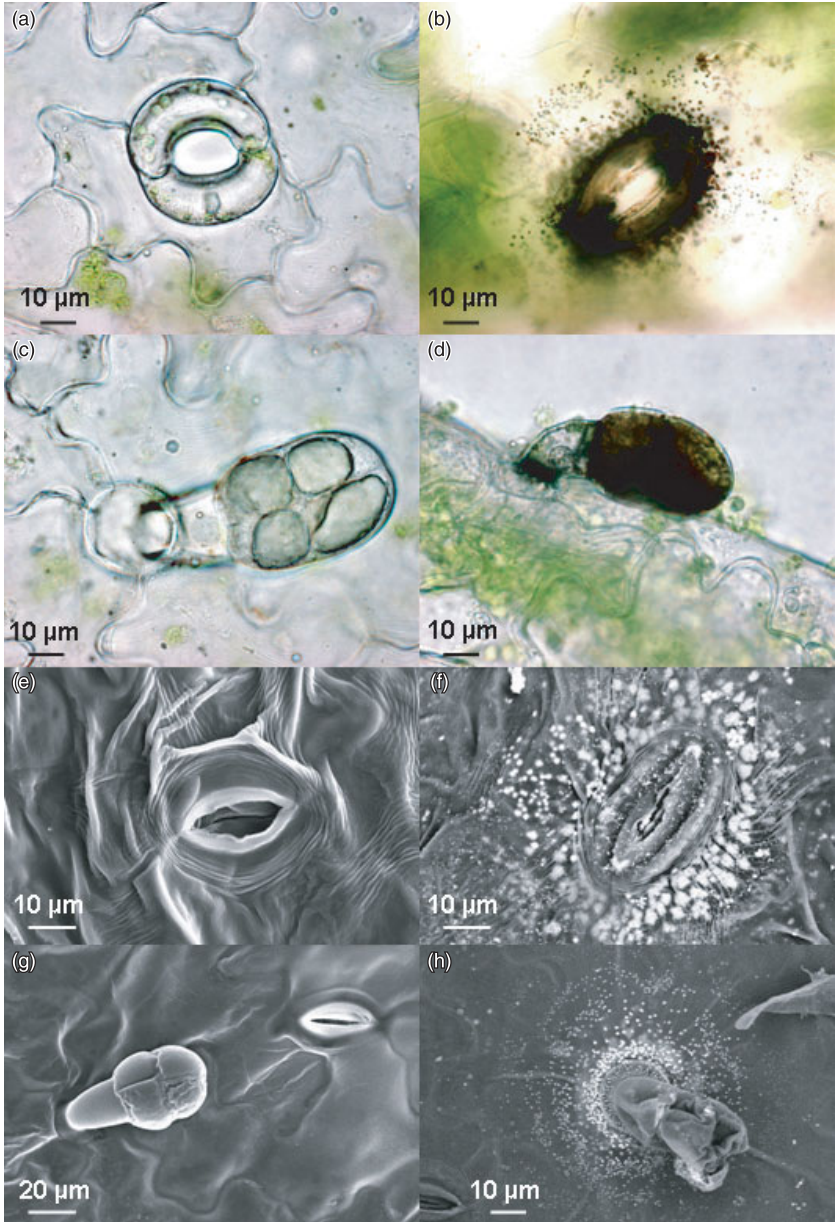


Plate 7 (a–d) Light (LM) and (e–f) scanning electron microscopic (SEM) investigation of silver (Ag) deposits around stomata and in trichomes of *V. faba* leaves after treatment with AgNO_3 . (a and e) Stomata of untreated leaf surfaces serving as control. (b and f) Stomata of AgNO_3 -treated leaf surfaces with characteristic silver deposits surrounding the stomatal pore. (c and g) Trichomes of untreated leaf surfaces serving as control. (f and h) Trichomes of AgNO_3 -treated leaf surfaces with characteristic silver deposits in the base and head of the trichome. Data from Schlegel *et al.* (2005).

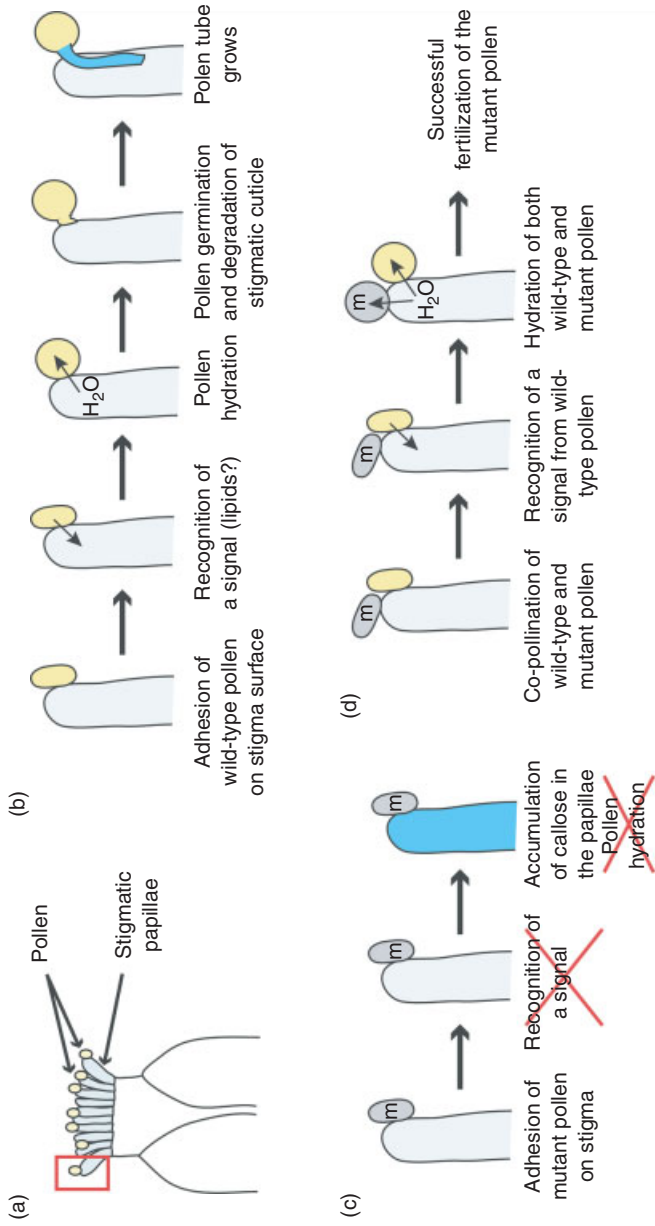


Plate 8 Schematic diagram of successive events in compatible pollination. (a) Diagram of stigma of *Arabidopsis thaliana*. (b) Sequential events following compatible pollination. (c) Events following pollination by mutant pollen defective in pollen-stigma recognition. (d) Restoration of mutant pollen fertility by co-pollination with wild-type pollen.