Lam-Son Phan Tran · Sikander Pal
Editors

Phytohormones: A Window to Metabolism, Signaling and Biotechnological Applications

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Preface

The phytohormones regulate various biological processes in plants. In the last few decades, comprehensive research efforts have displayed the existence of phytohormonal signals and their transduction in plants. Intensive molecular studies have elucidated various plant hormonal pathways, each of which consists of many signaling members, linking a specific hormone perception to the regulation of downstream genes. Among phytohormones, signal transduction pathways of auxin (Aux), abscisic acid (ABA), cytokinins (CKs), gibberellins (GAs), and ethylene (ET) have been thoroughly investigated. In the last decade, extensive research efforts have recognized brassinosteroids (BRs) as a new class of plant hormones with multiple roles in plant physiological processes. The signal transduction pathway and crucial implication of BR signaling components in execution of BR responses in plants have been recently established. Emerging evidence also supports specific signal perception and transduction pathways for salicylic acid (SA) and jasmonates (JAs). Latest research findings also support strigolactones as plant hormones.

The advanced molecular studies have demonstrated crucial implication of phytohormonal crosstalks in the regulation of key physiological events under normal and stressful conditions. For instance, the crosstalks of Aux-ABA, Aux-BRs, BRs-ABA, ET-ABA, BRs-ET, CKs-ABA, BRs-JAs, BRs-SA, and GAs-JAs have been shown to regulate a number of biological processes in plants. The phytohormonal crosstalk between two hormones can be antagonistic or synergistic or additive in action. Additionally, the signal transduction component(s) of one hormonal pathway may interplay with the signaling component(s) of other hormonal pathway(s).

The knowledge gained from the signal transduction studies of phytohormones has been practically valorized through genetic manipulation. Genetic engineering has enabled plant biologists to manipulate the signaling pathways of plant hormones for the development of crop varieties with improved yield and stress tolerance. Latest research findings have revolutionized the concept of phytohormonal studies in plants. The present book volume will describe the new facet of plant hormones; that is, not only phytohormones have been studied to understand their course of

actions in plants but also crosstalk implication of two or more hormones has become the target of plant scientists to manipulate the hormonal impact and to generate high-yielding varieties. In the preceding context, Chaps. 1–5 describe the metabolism, signaling, and genetic manipulation of classical hormones (Aux, ABA, CKs, ET, and GAs). Understanding the roles of emerging plant hormones, such as BRs, SA, JAs, and strigolactones, is of utmost significance to plant biologists. Chapters 6–9 of this book will apprise the readers about fundamentals and recent understandings of these emerging hormones. Implication of plant hormonal crosstalks under stressful conditions has just begun to be deciphered. Thus, to share the latest updates with the readers, the book will be concluding with chapters on phytohormonal crosstalks under abiotic and biotic stresses.

Overall, this volume will present our current understanding of phytohormonal signal transductions and crosstalk of phytohormones in plants as a regulation of key physiological processes. Every section will be concluded with application of biotechnological strategies based on modulation of the hormone contents or signal transduction pathway or crosstalk, enabling us to maintain agriculture in a sustainable manner.

We are grateful to the authors of various chapters of this book for writing their chapters meticulously and with great responsibility. We are extremely thankful to Dr. Kazuo Shinozaki, Director of RIKEN Center for Sustainable Resource Science, Japan; Prof. MPS Ishar, Vice-Chancellor, University of Jammu, India; and Prof. Pedro Berliner, Director and Dr. Shimon Rachmilevitch of Jacob Blaustein Institute for Desert Research, Ben-Gurion University, Israel, for providing overall support for our research and academic pursuits.

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We are quite hopeful that this book will be successful in updating the readers about the phytohormones and latest emerging trends.

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Auxin in Plant Growth and Stress Responses

Liu Liu*, Guangyan Guo*, Zhijuan Wang, Hongtao Ji, Fupeng Mu, and Xia Li

Abstract The phytohormone auxin has long been recognized for its essential role in plant growth and development. Recent advance indicated that auxin also plays critical roles in plant responses to environmental stresses. This has prompted investigation into molecular control of auxin homeostasis and plant growth in response to developmental and environmental stimuli. A simple two-step biosynthesis pathway from tryptophan to auxin has been defined. At its sites of action, three auxin receptor or co-receptor systems have been identified. Binding of auxin by ABP1 regulates ROP-GTPase-mediated gene expression and subcellular protein trafficking. Auxin perception by TIR1/AFB-Aux/IAA co-receptor and SKP2A activate auxin signaling and promote cell growth and cell division, respectively. Recent findings indicate that ABP1 functions upstream of TIR1/AFBs and negatively regulates the TIR1/AFB-Aux/IAA-mediated auxin signaling pathway, highlighting coordinate regulation of the signaling pathways mediated by different auxin receptor/co-receptors during plant growth and development. Recent advance reveals that environmental signals, such as high salinity and drought, induce modulations of auxin biosynthesis and the signaling pathway allowing for efficient cellular reprogramming of plant growth and

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development under stress. Research advance in auxin homeostatic control and response has led to success in manipulation of auxin biosynthesis and the signaling for improvement crops with desired agricultural traits.

Keywords Auxin • Biosynthesis • Auxin signaling • Abiotic stresses • Plastic development

Introduction

Growth is one of the most fundamental characteristics of living organisms. Plant growth is quite different from that of animals. Plant growth is caused by increases in both cell number and cell size, whereas growth of animals is a result of increased cell number. Another apparent difference between plant and animal growth is that plants maintain the capacity to grow throughout their life (the so-called indeterminate growth). In sharp contrast, animals have determinate growth and reach their final size before maturation. However, being multicellular organisms, plant and animal growth have a conspicuous feature in common: both plant and animal growth are regulated by hormones.

Auxin was the first plant growth hormones discovered, and their name was derived from the Greek word αυξειν (*auxein* means "to grow or to increase"). Their promoting role in plant growth was first noted by Charles Darwin and his son Francis in studying phototropism of coleoptile of canary grass (*Phalaris canariensis*) and was documented in the remarkable book entitled *The Power of Movement in Plants* published in 1888 (Darwin and Darwin 1888). The existence of auxin in the tip of oat (*Avena sativa*) that can move and regulate phototropism of coleoptile of oat was unequivocally demonstrated by Frits Went in 1926. IAA (indole-3-acetic acid), the principal of auxin in higher plants, was isolated by Kenneth V. Thimann in the 1930s (Thimann 1936). IAA and several other chemicals with similar structure and physiological activity in inducing cell elongation of stems were named as auxin in 1954 (Stowe and Thimann 1954).

In the past 80 years after auxin isolation, extensive studies have been conducted to investigate biological and physiological roles of auxin in plant growth and development. Up to date, no mutant lacking auxin has been identified. The findings have demonstrated that auxin is phytohormone that plays vital roles in plant growth and development, including leaf abscission and development of floral bud and fruit (Davies 2010). Notably, it has been proved that auxin is central regulator of root growth (Overvoorde et al. 2010). Therefore, endogenous and synthetic auxin with similar activity has been widely used in global agriculture and horticulture for more than 60 years. At the same time, numerous studies have been conducted to elucidate where auxin is synthesized, how it is transported to the sites of action, and how auxin becomes inactive after fulfilling their function (Ljung et al. 2005). Accordingly, a great deal of researches has focused on uncovering the molecular responses of plant cells to auxin.

In *Arabidopsis*, a two-step biosynthesis pathway from tryptophan to auxin has been well defined (Zhao 2012; Mashiguchi et al. 2011). A series of auxin transporters and carriers localized at the plasma membrane or the endoplasmic reticulum have been shown to be responsible for regulation of auxin homeostasis, including the location and amount of auxin, thereby the duration of auxin signaling and responses. At its sites of action, auxin is first perceived by three well-recognized receptor/co-receptor systems. Among them, TIR1/AFB-Aux/IAA (TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX-AUX/INDOLE-3-ACETIC ACID INDUCIBLE) co-receptor, the first identified and best characterized receptor system, regulates transcription of downstream auxin-responsive genes in nucleus (Villalobos et al. 2012) while newly identified auxin receptors SKP2A (S-phase Kinase-Associated Protein 2A) and ABP1 (Auxin Binding Protein 1) have been shown to mainly repress cell division during cell cycle and subcellular protein trafficking, respectively (Jurado et al. 2008a, 2010; Chen et al. 2001; Robert et al. 2010). The research advances have also highlighted the coordination of three auxin receptor systems in rapid and accurate activation of auxin signaling and responses (Chapman and Estelle 2009). Whether auxin biosynthesis, homeostasis control, and signaling pathway are conserved in various plants needs to be further characterized.

In addition to the pivotal roles of auxin during *Arabidopsis* growth and development, the functional analysis of auxin in plant response to environmental cues and plastic development have become an attractive new research area. There has been a sharp increase in deciphering the functions of auxin in plastic root development under nutrient deficiency (low nitrogen and phosphate) and abiotic stresses (e.g., salt stress) (Park et al. 2007; He et al. 2005; Gilbert et al. 2000), besides the well-known role of auxin in gravitropism and phototropism (Noh et al. 2003). These findings not only provided novel insights into the regulatory roles of auxin but also broadened the horizon of future auxin research.

Auxin Biosynthesis and Metabolism

Auxin Biosynthesis

IAA is the primary plant auxin and is predominantly synthesized in rapidly growing tissues, especially in shoot apical meristems, young leaves, and developing fruits and seeds (Ljung et al. 2001). Recently, it has been shown that root tips can also synthesize auxin that regulates root architecture together with the shoot-derived auxin (Aloni et al. 2006).

Because of the structure similarity between IAA and tryptophan (Trp), Trp has long been considered as the precursor of IAA. The compelling evidence has demonstrated that IAA is mainly converted from Trp in *Arabidopsis*, which is the so-called Trp-dependent pathway (Cohen et al. 2003). An enormous body of evidence has indicated existence of multiple pathways through which plants can convert Trp to IAA. During the past two decades, great progresses have been made in

understanding the biochemical mechanism of auxin biosynthesis, especially the mechanism of how Trp is converted to IAA. In this review, we will summarize the progress in the Trp-dependent auxin biosynthesis pathway.

A Simple Two-Step Biosynthesis Pathway for Auxin

Until very recently a complete two-step auxin biosynthesis pathway through which Trp is converted to IAA in plants was established (Mashiguchi et al. 2011; Won et al. 2011). In this pathway, the first step is that TAAl/SAV3 (TRYPTOPHAN AMINOTRANSFERASE OF *ARABIDOPSIS* 1/WEAK ETHYLENE INSENSITIVE 8/SHADE AVOIDANCE 3/CYTOKININ INDUCED ROOT CURLING1) converts Trp to indole-3-pyruvate (IPA), followed by converting IPA to IAA by the members of YUCCA (YUC) flavin monooxygenases family (Mashiguchi et al. 2011; Zhao 2012) (Fig. 1).

Conversion of Trp to IPA by TAA1

The indole-3-pyruvate (IPA) has long been considered as the most common intermediate in the Trp-dependent pathway for IAA biosynthesis (Cooney and Nonhebel 1991; Nonhebel et al. 1993). However, the role of IPA and the enzymes catalyzing the reaction from Trp to IPA in plant auxin biosynthesis are recently discovered by three independent genetic studies (Stepanova et al. 2008; Tao et al. 2008; Yamada et al. 2009). Interestingly, these studies were performed to identify the mutants with altered response of mutants to shade (*sav3*), ethylene (*wei8*), and NPA (an auxin transport inhibitor) (*tir2*) in *Arabidopsis*. However, despite of the original phenotypes in genetic screens, it turned out that these mutant phenotypes are due to mutations in a gene encoding *Arabidopsis* aminotransferase TAA1 that can convert Trp to IPA in vitro and is involved in auxin biosynthesis. The *taa1* mutants including *sav3*/*taa1*, *wei8*, and *tir2* show a decreased IAA synthesis and reduced expression of the auxin-responsive genes (Tao et al. 2008; Stepanova et al. 2008; Won et al. 2011; Yamada et al. 2009). Furthermore, it has been proved that the phenotypes of *taa1* can be partially rescued by a synthetic auxin picloram or IAA (Stepanova et al. 2008; Tao et al. 2008). Further experiments also demonstrate that simultaneous inactivation of *TAA1* and its close homologs *TAR1* and *TAR2* causes developmental defects similar to those of well-known auxin mutants (Stepanova et al. 2008). These findings provide strong evidence that the TAA1 and its close homologs play critical roles in auxin biosynthesis and plant development.

TAA1 and TARs are enzymes dependent on pyridoxal-50-phosphate (PLP) and are conserved in the plant kingdom. It is highly likely that TAA1 and its homologs act similarly to convert Trp to IPA in various plants to regulate plant growth and development. Functional analysis of TAA1 homolog genes in other species will provide novel insights into understanding the regulatory mechanisms controlling auxin biosynthesis in plants.

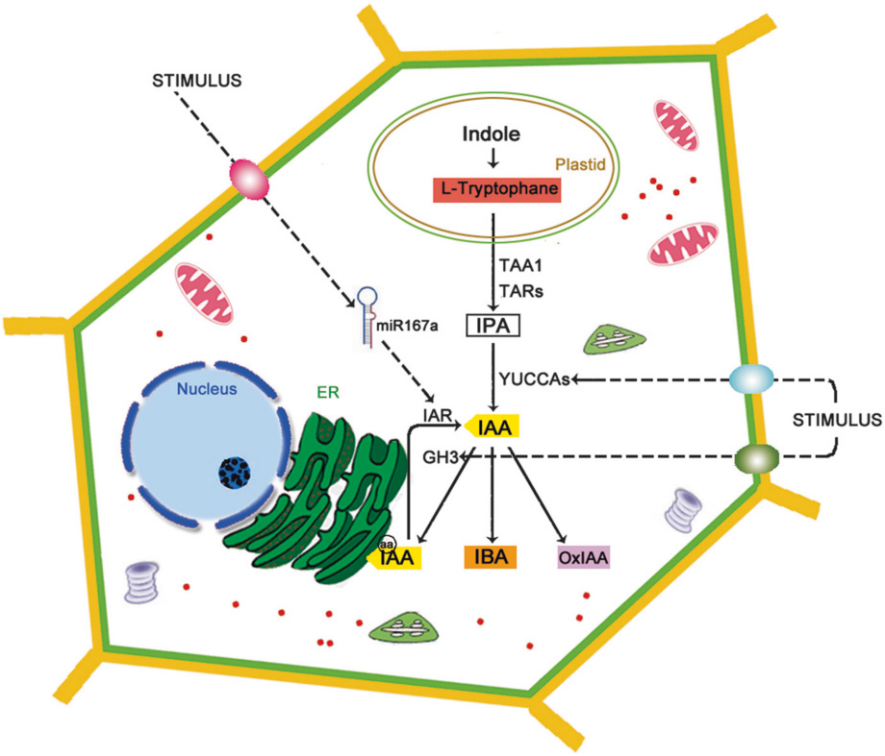

Fig. 1 Auxin synthesis and homeostasis. L-Tryptophan is the precursor of cell-synthesized indole-3-acetic acid (IAA). In the simple two-step Trp-dependent pathway, L-Trp is converted to indole-3-pyruvate (IPA) by TAA1, followed by YUCCAs converting IPA to IAA. In order to regulate IAA level, plant cells possess multiple ways to transform active IAA into inactive forms. IAA can be conjugated to other chemicals, such as sugar, amino acid, and glucan. As shown in the figure, IAA can be converted to IAA–amino acid conjugates by Gretchen Hagen 3 (GH3), which is localized to endoplasmic reticulum (ER). In addition, IAA can also be transformed into inactive indole-3-butyric acid (IBA), or be catabolized into 2-oxoindole-3-acetic acid (oxIAA). IAA level is balanced by GH3 and IAA-ALANINE RESISTANT (IAR), a gene targeted by miR167a encoding a hydrolase which can release IAA from inactive IAA–Ala form. Developmental and environmental stimuli modulate auxin homeostasis and subsequence plant growth by regulating IAA biosynthesis and catabolic pathways

The Rate-Limiting Step Catalyzed by YUC in Auxin Biosynthesis Pathway

Despite of important role of TAA1 and its homologs in the first step of auxin biosynthesis, the observation that the transgenic plants overexpressing TAA1 do not exhibit auxin overproduction phenotypes (Zhou et al. 2011) suggests that the TAA1-catalyzed step may not be a rate-limiting step in auxin biosynthesis. Indeed, the second step converting IPA to IAA catalyzed by YUC flavin

monooxygenases has been demonstrated as a rate-limiting step in a Trp-dependent auxin biosynthesis pathway (Zhao et al. 2001). Developmental defects in the *yuc* mutants can be rescued by in situ auxin production, and most importantly, overexpression of *YUC* genes encoding YUC flavin monooxygenases leads to auxin overproduction.

The role of YUC in auxin biosynthesis was first discovered in characterization of a dominant and fertile *yuc* mutant showing developmental phenotypes due to the elevated level of endogenous auxin (Zhao et al. 2001). *YUC* encodes a flavin monooxygenase (FMO)-like enzyme and is determined as a key auxin biosynthesis enzyme based on the genetic and physiological results, in particular the effect of overexpression of *YUC* in *Arabidopsis* on auxin overproduction (Zhou et al. 2011). Eleven *YUC* genes are identified in *Arabidopsis*, and genetic studies have shown that members of the *YUC* family function redundantly during plant growth and development (Cheng et al. 2006, 2007). For example, overexpression of single *YUC* gene in *Arabidopsis* and in other plant species leads to auxin overproduction and the corresponding phenotypes. Notably, loss-of-function mutation in a single *YUC* gene does not obviously influence plant development, whereas simultaneous inactivation of several *YUC* genes, such as *YUC1*, *YUC2*, *YUC4*, and *YUC6*, leads to apparent developmental defects in embryogenesis, seedling growth, floral development, etc. in *Arabidopsis* (Cheng et al. 2006, 2007), which is similar to that of well-known auxin mutants (Gälweiler et al. 1998; Dharmasiri et al. 2005a). Importantly, complementation of the developmental defects of the loss-of-function *yuc* mutants by overexpressing *iaaM*, a bacterial auxin biosynthesis gene, under the control of a *YUC* promoter demonstrates that *YUC* genes are essential for auxin biosynthesis and plant development (Cheng et al. 2006).

The very recent exciting breakthrough in auxin biosynthesis is the elucidation of biochemical mechanism of YUC in catalyzing the conversion from IPA to IAA (Dai et al. 2013). Using a recombinant *Arabidopsis* YUC6 containing FAD as a cofactor as an example, the authors provide evidence that YUC6 convert IPA to IAA through three sequential reactions using NADPH and oxygen. At the first step, the YUC6 catalyzes the reduction of the FAD cofactor to FADH(−) by NADPH. FADH(−) then forms a flavin-C4a-(hydro)peroxy intermediate by reacting with oxygen, followed by the reaction of the C4a-intermediate with IPA to produce IAA as the final chemical step. Thus, this work not only confirms the important role of YUC in auxin biosynthesis but also deciphers chemical mechanism that occurs during the flavin monooxygenase-catalyzed conversion from IPA to IAA in plants.

Genome-wide comparative analysis shows that *YUC* genes exist in all of the sequenced plant genomes. The important roles of *YUC* genes regulating auxin biosynthesis have also been experimentally validated in various plants, such as rice (Gallavotti et al. 2008). These results suggest that YUC flavin monooxygenases have a conserved role in coordinated regulation of the rate-determining step in auxin biosynthesis and subsequent plant growth and development.

A Second Pathway Converting Trp to IAA by Cytochrome P450s (IAOx Pathway)

Biochemical analyses have shown that multiple pathways from Trp to IAA exist for the auxin biosynthesis. In addition to the two-step auxin biosynthesis pathway, recent genetic studies have identified several genes, which regulate conversion from Trp to IAA through an important intermediate indole-3-acetaldoxime (IAOx). One key step in this pathway has been defined. During this step, Trp is converted to IAOx by *CYP79B2* and *CYP79B3*. The evidence for defining this reaction comes from identification and functional analysis of *CYP79B2* and *CYP79B3*, which encode two cytochrome P450s (Zhao et al. 2002). Overexpression of *CYP79B2* leads to elevated levels of free auxin and auxin overproduction phenotypes similar to the known IAA overproduction mutants such as *yuc* (Zhao et al. 2002). By contrast, the loss-of-function c*yp79b2 cyp79b3* double mutant contains reduced levels of IAA and displays the corresponding phenotypes, such as short hypocotyls and smaller stature, because of partial auxin deficiency (Zhao et al. 2002). The results show that the altered contents of auxin in the *CYP79B2* overexpression lines and c*yp79b2 cyp79b3* double mutant are due to the changes in IAOx.

Existence of the IAOx pathway is also supported by the biochemical and molecular analysis of loss-of-function mutants *sur1* and *sur2* showing similar typical auxin overproduction phenotypes (Delarue et al. 1998). SUR1 and SUR2 are involved in catalyzing the conversion from IAOx to indolic glucosinolates, a key intermediate to IAA (Delarue et al. 1998). Loss-of-function *sur2* mutant blocks the production of glucosinolates resulting in an increased IAOx flux and subsequent elevated level of IAA biosynthesis (Delarue et al. 1998). Further studies show that *SUR2* encoding the cytochrome P450 CYP83B1 has enzymatic activity of synthesizing 1-*aci*-nitro-2-indolyl-ethane from IAOx (Delarue et al. 1998; Barlier et al. 2000), thereby defining the first step in generating indolic glucosinolates from IAOx. *SUR1* encodes a C–S lyase that catalyzes the conversion of *S*-alkylthiohydroximate to thiohydroximic acid, a key reaction in indolic glucosinolate biosynthesis (Boerjan et al. 1995; Mikkelsen et al. 2004). Inactivation of *SUR1* disrupts glucosinolate production leading to the accumulation of upstream intermediates including IAOx and an increase in IAA (Boerjan et al. 1995). Taken together, these works established the catalytic role of these cytochrome P450s in converting Trp to IAOx and demonstrated existence of a parallel pathway (also termed IAOx pathway) in IAA biosynthesis (Mikkelsen et al. 2000).

Up to date, it is still not clear how IAOx is converted to IAA. Several studies have shown that IAOx is the precursor of indole-3-acetonitrile (IAN) and indole-3-acetaldehyde, which can then be used to generate IAA by nitrilases (Kobayashi et al. 1993) and aldehyde oxidases (Brumos et al. 2013), respectively. Recent biochemical analysis of the mutants suggests that indole-3-acetamide (IAM) is probably also an important intermediate in converting IAOx to IAA, but the genes and enzymes for producing IAM from IAOx are not known (Brumos et al. 2013). Although the IAOx pathway converting Trp to IAA plays a role during growth and development in *Arabidopsis*, the current results indicate that the IAOx pathway may

not be the mainly common IAA biosynthesis route in plants. The prediction comes from the observations including subtle phenotype and undetectable IAOx in *Arabidopsis cyp79b2 cyp79b3* double mutants, undetectable level of IAOx in monocots like rice and maize (Sugawara et al. 2009), and no apparent CYP79B2 and CYP79B3 orthologs found in monocots, such as rice and maize (Sugawara et al. 2009). Thus, many questions need to be answered, including how IAOx is converted to IAA, what are the key enzymes that catalyze the reactions, and whether the IAOx pathway is universal in the plant kingdom.

Auxin Conjugation and Degradation

Auxin is a hormone molecule whose activity levels are most important for its regulatory roles during plant cell, organ, and tissue development. Therefore, the precise regulation of auxin levels is an essential mechanism to fine-tune the activity of this powerful hormone during plant growth and development. After auxin is synthesized and completes it action, auxin must be attenuated to prevent overreaction. There are also two ways, conjugation with amino acids and sugars and degradation, to reduce active IAA (Normanly 2010; Barbez et al. 2012) (Fig. 1).

IAA Conjugation

Conjugation of hormone molecules with amino acids and sugars is a common mechanism to convert the active form to the inactive form. It has been shown that in many plant tissues, auxin is mainly in combination with a variety of sugars, sugar alcohols, amino acids, and proteins (Wood 1985). In this way, conjugated IAA can be stored locally or transported over long distances (Wood 1985). So far, there are basically two types of conjugated IAA found in *Arabidopsis*. One is ester-conjugated IAA, which is derived from conjugation of IAA with indole acetyl glucose, inositol, glycoproteins, glucan, or simple ester compounds, and the other is to combine IAA with amino acids, proteins, and peptides through amide connection.

Plenty of evidence shows that IAA–amino acid conjugates play an important role in auxin homeostasis. In 2005, Staswick group identified a family of *Arabidopsis GH3* (*Gretchen Hagen 3*) genes that encode an IAA-amido synthase and are responsible for production of IAA–amino acid conjugates (Hagen and Guilfoyle 1985; Wright et al. 1987; Li et al. 1991). Biochemical analysis has demonstrated that several recombinant GH3 enzymes are able to catalyze conjugation between IAA and amino acids, such as alanine (Ala), aspartic acid (Asp), phenylalanine (Phe), and tryptophan (Trp) (Staswick et al. 2005). Furthermore, loss-of-function mutants of the *GH3* genes *GH3.1*, *GH3.2*, *GH3.5*, and *GH3.17* show increased sensitivity to auxin (Staswick et al. 2005), while overexpression of a *GH3* gene reduces auxin levels in the plants resulting in a dwarfed phenotype. The results confirm that *GH3* genes are important regulators in maintaining auxin homeostasis by conjugating free IAA to amino acids (Staswick et al. 2005). IAA–amino acid conjugation is also

found in other plants. In rice, *GH3-8* gene encoding an IAA–amino acid synthetase promotes formation of IAA–Asp conjugates to reduce the auxin-induced cell wall loosening (Ding et al. 2008).

The conjugation process between IAA and sugar, glucan, and ester compounds is less understood. In *Arabidopsis*, the enzyme catalyze formation of methyl-esterified IAA (MeIAA) has been identified (Qin et al. 2005). The enzyme IAA carboxyl methyltransferase 1 (IAMT1) is a member of carboxyl methyltransferases family that can methylate the carboxyl side chain of IAA. The study has shown that overexpression of *IAMT1* gene leads to dramatic hyponastic leaf phenotypes (Qin et al. 2005). Most importantly, conjugation has been considered as an efficient pathway to rapidly regulate hormone contents because it is reversible. For example, during seed germination in maize, the IAA–inositol conjugates are transported from endosperm to the coleoptile by phloem and are then hydrolyzed to free IAA. It is noteworthy that most free IAA produced in the top of the maize coleoptile is hydrolyzed from IAA–inositol conjugates in seeds (Woodward and Bartel 2005; Ludwig-Müller 2011).

IAA Degradation

IAA levels can also be regulated by degradation, an irreversible mechanism through which the indole nuclear or chemical side chain is modified, causing auxin activity removed (Grambow and Langenbeck-Schwich 1983). The catalytic catabolism of IAA has been extensively studied. Physiological and biochemical results indicate that peroxidases are the enzymes that catalyze the catabolism of IAA into 3-methylene hydroxy indole (3-methyleneoxindole) (Meudt 1967). However, overexpression of peroxidase (POD) does not affect IAA content in *Arabidopsis* (Grambow and Langenbeck-Schwich 1983). Thus, it is possible that the peroxidase oxidation of IAA is not the main route for IAA catabolism in plants.

Recently, it has been shown that 2-oxoindole-3-acetic acid (oxIAA) and oxIAA-glucose (oxIAA-Glc) are the major degradation metabolites in rice, maize, and beans (Östin et al. 1998; Kai et al. 2007; Novák et al. 2012). OxIAA and oxIAA-Glc are induced by IAA treatment (Östin et al. 1998) or induction of IAA biosynthesis (Band et al. 2012), and the levels of oxIAA and oxIAA-Glc are markedly increased in the IAA overproduction plants (Stepanova et al. 2011; Novák et al. 2012). However, the genes involving in the IAA catabolism have not been identified, and the molecular mechanisms underlying IAA degradation still remain elusive (Fig. 1).

Auxin Homeostasis Control in Response to Environmental Stresses

Plants grow in a constantly changing environment over entire life cycle. As sessile organisms, plants regulate their growth and development according to both endogenous and environmental factors, such as high salinity, water status, and high or low temperature. During evolution, plants have evolved adaptive mechanisms to

optimize their development and survive the stress conditions. Plant hormones have been recognized as key regulators in plant adaptation. Among them, abscisic acid (ABA) is a well-recognized stress hormone that plays key roles in seed germination and plant growth in response to abiotic stresses, such as drought and salt stress (Lee and Luan 2012). During the past five decades, extensive studies have been conducted on ABA biosynthesis pathways and the regulation of ABA homeostasis and the signaling pathway under stress conditions (Verslues and Zhu 2005). Some studies have also demonstrated that ethylene is also involved in plant adaptation in response to abiotic stresses (Wang et al. 1990). Recently, accumulating evidence indicates that almost all the plant hormones, such as salicylic acid (SA), gibberellins (GAs), brassinosteroids (BR), and strigolactones, also somehow participate in regulation of plant development and adaptation to stresses (Hayat and Ahmad 2007; Davies 2010; Clouse et al. 1992; Gomez-Roldan et al. 2008). As an essential hormone molecule during plant growth and development, the roles of auxin in plant stress responses have drawn the scientists' attention focusing on the mechanisms of auxin homeostasis control and developmental plasticity under abiotic stresses, especially on salt stress, drought, and low temperature. Here we will briefly summarize the recent advances in adaptive adjustment of auxin biosynthesis and homeostasis and their roles in plant response to drought, salt stress, and low temperature.

Auxin and Plant Response to Salt Stress

Soil salinization is a global problem restricting agricultural production. High salinity causes multiple cellular stresses including osmotic stress, ion toxicity, nutritional deficiency, oxidative stress, and a series of secondary stresses, such as oxidative damage and metabolic toxicity (Hasegawa et al. 2000). As a result, salt stress causes reduced plant growth and photosynthesis, increased energy consumption, and accelerated aging and death of plants (Wang et al. 2003; Chaves et al. 2009; Zhu 2001). Most importantly, salinity has become an important environmental stress limiting crop yield in arid and semiarid areas (Pitman and Läuchli 2002). Therefore, the physiological and molecular mechanisms of plants to cope with salt stress have long been recognized as important scientific questions. However, majority of past researches focused on understanding the regulation of ion homeostasis control and osmotic stress response of plants, the regulatory roles of the individual hormones, and the interaction between growth hormones have just drawn attention.

There are still very little information on the effects of salt stress on auxin biosynthesis and the levels of auxin in the stressed plants, especially in the tissues or organs. The changes in auxin contents have been noted. However, whether auxin is increased or decreased under salt stress conditions remains controversial. A few studies reported that the increased level of IAA is correlated with the reduced plant growth (Ribaut and Pilet 1994), whereas some physiological researches show that salt stress causes great reduction in IAA in rice leaves (Prakash and Prathapasenan 1990; Nilsen and Orcutt 1996), tomato (Nilsen and Orcutt 1996), and wheat roots

(Shakirova et al. 2003). Recently, strong evidence shows that under mild salt stress, the auxin levels are maintained almost unchanged in both shoots and root tips in *Arabidopsis*. It is shown that maintenance of auxin homeostasis in these tissues of the stressed plants is regulated by the SOS (Salt Overly Sensitive) signaling pathway (Zhao et al. 2011). Research demonstrated that the auxin homeostasis in roots that is essential for lateral root formation and growth is regulated by the SOS signaling pathway. Loss-of-function mutant *sos3* shows substantially reduced auxin leading to abortion of lateral root formation and emergence and increased sensitivity to salt. Exogenous application of auxin in the growth medium containing NaCl can restore the lateral root development of *sos3* mutants under salt stress (Fig. 2). These findings confirm that maintenance of auxin homeostasis is an important adaptive mechanism for plant root growth to survive salt stress. However, whether the reduced level of auxin in *Arabidopsis* is caused by downregulation of biosynthesis pathway or stimulation of auxin catabolism remains elusive. Expression analysis of the *GH3* genes in *Sorghum bicolor* reveals that *SbGH3* is expressed at low level under normal conditions and is highly induced by salt stress (Wang et al. 2010). The result indicates that IAA conjugation may be involved in reduction of active IAA in the stressed plants. Further understanding of auxin homeostasis control in plants will provide novel insights into the molecular mechanisms of plant adaptation to saline soil.

Auxin and Plant Response to Drought Stress

Understanding the mechanism of plant response to drought and improvement of drought tolerance of crops is one of the fundamental questions in plant biology. The remarkable features of plants grown under drought conditions are stunted growth and shortened life cycle (Vinocur and Altman 2005). Therefore, it is quite apparent that auxin should participate in the adjustment of the development of plants. Genome-wide gene expression profiling shows that transcription level of auxin-responsive genes including the genes involved in auxin metabolism is changed in response to dehydration (Ghanashyam and Jain 2009). However, almost all the researches in plant drought tolerance focus on ABA. To date, only a few studies report the roles of auxin content and the auxin signaling pathway in plant responses to drought (Popko et al. 2010).

Understanding the roles of auxin comes from the results that disruption or overexpression of the genes encoding the key enzymes in auxin metabolism results in altered stress response of plants. For example, activation of *YUC7* gene elevates auxin levels and enhances drought tolerance of *Arabidopsis* (Im Kim et al. 2013; Lee and Luan 2012). Very recently, *YUC6* has also been shown to be involved in plant tolerance to drought in potato (Im Kim et al. 2013). Overexpression of *Arabidopsis YUC6* in potato causes auxin overproduction of phenotypes and enhanced drought tolerance. These results suggest that high levels of auxin are required for drought tolerance of plants, and the Trp-dependent auxin biosynthesis pathway plays critical role in the upregulation of auxin contents under water stress.

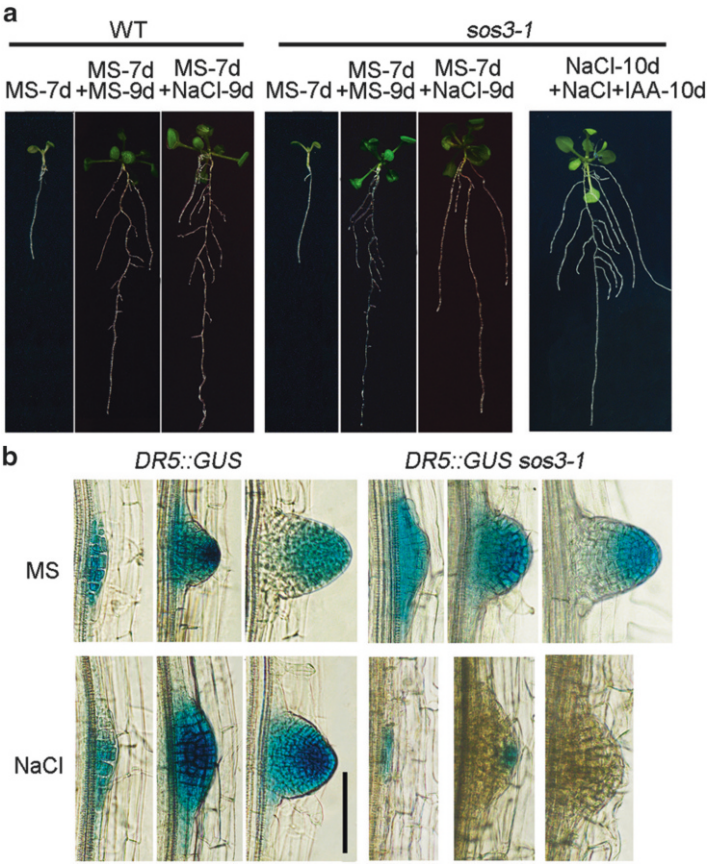

Fig. 2 Auxin is essential for lateral root development under salt stress. *sos3-1* mutant shows reduced auxin leading to abortion of lateral root formation and emergence and increased sensitivity to salt (Zhao et al. 2011). **a.** *sos3-1* mutant shows less lateral roots under 30 mM NaCl treatment. Both the wild-type and *sos3-1* seeds were sowed on the MS plates and grown for 7 days. Subsequently, the plants were transferred to the MS plates containing 30 mM NaCl, grown for an additional 9 days, and the lateral roots were compared. Exogenous application of auxin restores the lateral root development of *sos3-1* mutant under salt stress. The *sos3-1* seeds were sowed on the MS plates containing 30 mM NaCl and grown for 10 days. Subsequently, the plants were transferred to the MS plates containing 30 mM NaCl and 75 nM NAA and grown for an additional 10 days. **b.** Auxin accumulation is lower in the *sos3-1* in response to NaCl treatment. The *DR5*::*GUS* construct was analyzed in wild-type or *sos3-1* mutant for the free auxin accumulation. *DR5*::*GUS* construct shows GUS activity in sites where auxin accumulates

In addition to Trp-dependent pathway, free IAA derived from IAA conjugates also contributes to the increased levels of IAA and subsequent drought tolerance. It has been shown that overexpression of *OsGH3-2* catalyzing IAA conjugation with amino acids results in reduced free IAA level and increased sensitivity to drought (Du et al. 2012). Because alteration in *OsGH3-2* expression also changes

the level of ABA in the stressed plants, it is hypothesized that *OsGH3-2* regulates plant drought tolerance through modulating both free IAA and ABA homeostasis in rice (Du et al. 2012). It is apparent that IAA catabolism also plays an important role in maintaining IAA homeostasis when plants are subjected to water stress. Indeed, *GH3.8* and *GH3.13* also have functions in plant response to drought in rice (Ding et al. 2008; Zhang et al. 2009). Very recent report shows that *IAA-ALANINE RESISTANT 3* (*IAR3*), targeted by miR167a, encoding IAA-amido hydrolase that converts an inactive form of auxin, IAA–Ala conjugates, to free IAA is required for plant drought tolerance (Kinoshita et al. 2012). Notably, loss-of-function *iar3* mutants exhibit significantly higher sensitivity to drought than the wild type (Kinoshita et al. 2012). These results support the notion that IAA is required for plant drought tolerance. Recent works have indicated that cross talk between ABA and IAA signaling pathways modulates plant growth and survival under drought conditions (Du et al. 2013). In this aspect, the transcription factor R2R3-type MYB, MYB96, has been shown to be a molecular link that regulates the lateral root meristem activity through modulating cross talk between ABA and auxin under drought conditions (Du et al. 2013).

Plant drought tolerance is a complex trait and is unlikely controlled by single gene or single hormone. It is conceivable that there must be a complex network involving multiple hormones to fine-tune the plastic development and successive reproduction of plants under drought conditions. In the future, many questions about how the drought signal is perceived and transduced to the downstream effectors to modulate auxin contents and how ABA signaling integrates with IAA homeostasis control system still remain to be answered.

Auxin Perception, Transduction, and Attenuation

As a phytohormone molecule, auxin needs to be transported from the sites of auxin synthesis to the tissues and organs that generate appropriate responses. To do so, a perception system consisting of multiple receptor proteins has evolved to specifically recognize auxin, thereby activating a signal transduction cascade that leads to cell-type-specific responses. After providing rapid responses to developmental or environmental cues, the receptors are often rapidly attenuated in the signaling to avoid overreacting and abnormal growth.

Auxin Perception and Signaling Transduction

The word perception, derived from the Latin *perceptio*, means the organization, identification, and interpretation of sensory information. For plant hormones, perception starts with the specific binding of receptors with hormone molecules. To date, three proteins ABP1, TIR1/AFB, and SKP2A have been recognized as

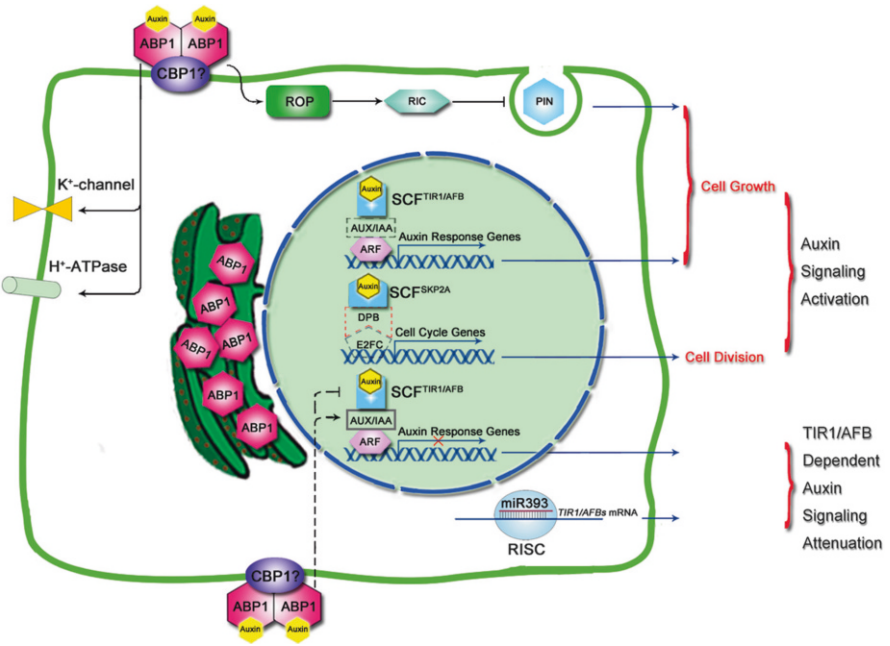

Fig. 3 A model of auxin perception, signal transduction, and attenuation. Auxin Binding Protein 1 (ABP1) is anchored by C-TERMINAL PEPTIDE-BINDING PROTEIN 1 (CBP1) with the plasma membrane. When binding to IAA, ABP1 influences the ion fluxes (such as H^+ and K^+) and inhibits clathrin-mediated PIN endocytosis through ROP-RIC (guanidine triphosphate hydrolases of plants-ROP interactive crib motif-containing proteins) pathway. Auxin can also bind to TIR1/AFB (TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX) co-receptors to regulate the expression of auxin responding genes and promote cell growth. In addition, auxin can regulate cell cycle through binding to the third receptor SKP2A (S-phase Kinase-Associated Protein 2A). The attenuation of the auxin signaling can occur at several levels. It is known that miR393 negatively regulate the expression levels of *TIR1* and *AFBs* through direct cleavage of their mRNAs and ABP1 negatively regulates the $SCF^{TIR1/AFB}$ (Skp1-Cullin-F-box) pathway through increasing the AUX/IAA stability

auxin receptors based on their strict structural and steric binding specificity with auxin (Peer 2013). Accumulating evidence has shown that each auxin receptor-mediated auxin signaling cascade plays a diverse regulatory role during plant growth and development (Fig. 3).

ABP1-Mediated Auxin Perception and Signaling Transduction

ABP1 was first identified as an auxin binding protein in maize (*Zea mays* L.) more than 40 years ago (Hertel et al. 1972). However, the ZmABP1 protein, a 22-kDa glycoprotein, was purified until 1985 (Löbler and Klämbt 1985), and the gene encoding ABP1 protein was eventually cloned 4 years later (Hesse et al. 1989;

Jones and Venis 1989). Biochemical analysis proves that ABP1 can specifically bind auxin (Jones and Venis 1989). ABP1 protein is originally detected on the endoplasmic reticulum (ER) of maize coleoptiles (Ray 1977). Indeed, a signal typical for luminal proteins of the ER consisting of the tetrapeptide ^{198}Lys-Asp-Glu-Leu (KDEL) is found at the C-terminus of the protein (Hesse et al. 1989; Inohara et al. 1989; Tillmann et al. 1989). However, the subcellular localization analysis shows that ABP1 is also localized at the plasma membrane/apoplast interface (Jones and Herman 1993; Diekmann et al. 1995). It is believed that the majority of ABP1 protein is in the ER, whereas a small fraction of ABP1 is at the plasma membrane/apoplast interface (Jones and Herman 1993). The crystal structure analysis of ABP1 protein suggests that the binding pocket of ABP1 is predominantly hydrophobic on the apoplastic side, suggesting that ABP1 binds auxin and perceives the auxin signaling outside of the plant cells (Woo et al. 2002). It is likely that the ER-localized ABP1 is transferred to the plasma membrane/apoplast interface and activates auxin signaling and response (Diekmann et al. 1995).

It has been shown that *ABP1* gene is induced by auxin in plants and activation is required for auxin-mediated responses (Hou et al. 2006). For example, overexpression of *Arabidopsis ABP1* in tobacco leaf strips results in an increase in auxin-mediated cell expansion, whereas induction of *ABP1* in intact plants leads to larger leaf cells although the leaves have normal morphology (Jones et al. 1998). *ABP1* expression is also required for auxin-mediated protoplast swelling (Steffens et al. 2001). A null mutation in *ABP1* also causes embryo lethality in *Arabidopsis* (Chen et al. 2001). However, it is noteworthy that *ABP1* plays a critical role in regulating the transition from the globular embryo to the bilaterally symmetrical structure during embryo development, because the early embryonic development is comparable to the wild-type control (Chen et al. 2001). These results support the role of ABP1 as an auxin receptor controlling plant growth and development (Jones et al. 1998).

Although ABP1 has been recognized as an auxin receptor, the further modeling studies of how ABP1 monomers bind auxin suggest ABP1 may require a co-receptor in order to effectively activate the signaling. So far, the co-receptor(s) has not been identified, but CBP1 (C-TERMINAL PEPTIDE-BINDING PROTEIN 1), which is a plasma membrane glycosylphosphatidylinositol (GPI)-anchored copper oxidase with homology to *Arabidopsis* SKEWED5 (SKU5) from maize, has been shown to participate in anchoring ABP1 to the plasma membrane (Shimomura 2006). Whether CBP1 functions as co-receptor or what ABP1 co-receptor(s) is still needs to be investigated.

Recently, the genetic and biochemical results show that ABP1 transmits the auxin signal through ROP-GTPase (guanidine triphosphate hydrolases of plants (Rho)-related GTPases of plants) and their associating RICs (ROP Interactive CRIB motif-containing proteins) (Xu et al. 2010). In the ROP-GTPase-mediated cascade, ABP1 regulates clathrin-mediated endocytosis of PIN (PIN-FORMED) auxin efflux carrier on the plasma membrane in pavement cells, guard cells, and root cells (Xu et al. 2010; Chen et al. 2012b; Lin et al. 2012). When exposed to auxin, ABP1 can rapidly activate ROPs (Murphy and Peer 2012) to inhibit ROP-RIC-mediated regulation of PIN endocytosis (Robert et al. 2010). To date, ROP2-RIC4 and

ROP6-RIC1 have been shown to function downstream of ABP1 in auxin signaling (Xu et al. 2010). In addition, ABP1 regulates clathrin-mediated endocytosis of PIN at the plasma membrane and the trans-Golgi network (Robert et al. 2010). Thus, it is widely acknowledged that ABP1 mediates non-transcriptional auxin signaling that quickly modulates cell-, tissue-, or organ-specific auxin response during growth and development. These rapid responses include auxin-mediated activation or deactivation of ion channels, transporters, and the proton pump ATPase across the plasma membrane, reflecting in response to auxin (Rück et al. 1993; Thiel et al. 1993; Zimmermann et al. 1994; Barbier-Brygoo et al. 1996). However, the molecular mechanisms underlying these rapid responses to auxin remain largely unknown. The possibility that ABP1 also mediates auxin signaling at the transcriptional level cannot be excluded, as a number of indirect evidences have already suggested the transcriptional regulatory role of ABP1 in auxin signaling and responses (Tromas et al. 2009, 2013).

TIR1-Mediated Auxin Perception and Signaling

The TIR1 is the first widely accepted auxin receptor, and the TIR1/AFB-auxin-Aux/IAA co-receptor system has been extensively characterized (Kepinski and Leyser 2005; Dharmasiri et al. 2005a; Tan et al. 2007). Interaction between the TIR1 and auxin results in degradation of Aux/IAA proteins that represses the auxin signaling, thereby activating ARF (AUXIN-RESPONSIVE FACTOR) transcription factors and the downstream signaling components (Tan et al. 2007; Mockaitis and Estelle 2008). The *TIR1* gene was first identified in a genetic screening with defects in auxin transport and/or auxin response (Ruegger et al. 1998). The *tir1* mutants show a variety of auxin-regulated growth defects including hypocotyl elongation and lateral root formation, indicating that TIR1 is required for normal response to auxin. The TIR1 protein contains 18 leucine-rich repeats (LRRs) (Tan et al. 2007) and an F-box motif with high sequence similarity to the yeast Grr1p (glucose repression-resistant 1 protein) and the human SKP2 protein which mediates the ubiquitination and subsequent proteasomal degradation of target proteins (Ruegger et al. 1998; Tan et al. 2007). The following studies demonstrate that *Arabidopsis* TIR1 forms a ubiquitin–ligase (E3) complex SCF^{TIR1} (Skp1-Cullin-F-box) with ASK (*Arabidopsis* Skp1-like protein) and AtCUL1 to degrade AUX/IAA proteins, such as AXR2/IAA7 and AXR3/IAA17 (Gray et al. 1999). In 2011, Gray et al. showed that auxin stimulates binding of SCFTIR1 to the AUX/IAA protein, resulting in the latter to be degraded. In the year of 2005, it was demonstrated separately by two papers that auxin can bind directly to SCF^{TIR1} (Dharmasiri et al. 2005a; Kepinski and Leyser 2005), thus confirming TIR1/AFB-auxin-Aux/IAA co-receptor system (Fig. 3).

There are six genes encoding TIR1 and AFB1-5 in *Arabidopsis*, which contain highly conserved sequences that bind to auxin (Lokerse and Weijers 2009; Calderon-Villalobos et al. 2010). However, they play varied roles in modulating the auxin signaling. For example, TIR1 and AFB2 are positive regulators of the auxin signaling (Dharmasiri et al. 2005b; Parry et al. 2009), while the AFB4 functions as a

negative regulator of the signaling (Greenham et al. 2011). Interestingly, a total of 29 AUX/IAA proteins are found in *Arabidopsis* (Liscum and Reed 2002). Therefore, TIR1/AFB proteins may have different binding activities to the AUX/IAA proteins at different levels of auxin, in different cells and tissues or in response to different developmental and environmental cues. The finding that the interactions between TIR1/AFB and AUX/IAA proteins and the interaction pairs are determined by the auxin concentrations (Villalobos et al. 2012) supports the above notion.

It has been well known for decades that auxin regulates expression of many genes (Abel and Theologis 1996). The compelling evidence shows that the TIR1/AFB-AUX/IAA co-receptor system is essential for activation of the auxin-responsive genes (Goda et al. 2008; Chapman and Estelle 2009). Now, it is quite clear that AUX/IAA proteins interact with ARFs to activate or repress the auxin-responsive gene expression (Weijers et al. 2005). There are 23 ARF proteins found in *Arabidopsis*, some of which are transcriptional activators (e.g., ARF5-ARF8 and ARF19), whereas others are transcriptional repressors, such as ARF2-ARF4 and ARF9 (Guilfoyle and Hagen 2007). AUX/IAA proteins interact with the ARFs at the promoters of the auxin-responsive genes to block ARF transcription activity and expression of the target genes in the absence of auxin. In the presence of auxin, binding of auxin to TIR1/ABFs promotes its interaction with AUX/IAA proteins resulting in the latter's degradation, thereby removing the repression of AUX/IAAs on the transcriptional activity of ARFs to activate the expression of the auxin-responsive genes (Ulmasov et al. 1997a, b; Kim et al. 1997).

Despite all these breakthroughs, many questions remain to be answered. The immediate questions include how three families of key proteins in the TIR1/AFB-AUX/IAA-ARFs pathway group to dynamically mediate auxin signaling and generate appropriate responses and what their specific downstream responsive genes are. Further studies using a combinatorial approach integrating application of new technology will help to decipher the molecular mechanism underlying the TIR1/AFB-AUX/IAA co-receptor system-mediated auxin signaling and plant responses.

SKP2A-Mediated Auxin Perception and Signaling

Because auxin modulates many biological processes, multiple auxin receptors are expected. Indeed, mutations of the known auxin receptors cause pleiotropic phenotypes which cannot be completely explained by these receptors and the corresponding cascade, such as cell cycle control (Gray et al. 1999; Chen et al. 2001). These observations encourage exploration of new auxin receptors. In mammals, the F-box protein SKP2 (S-phase kinase-associated protein 2) is a member of an SCF complex and plays a key role in cell cycle progression (Frescas and Pagano 2008). Thus, F-box protein SKP2A was identified in *Arabidopsis* based on sequence similarity to the human SKP2. The studies reveal that SKP2A is also a part of an SCF complex in *Arabidopsis* (del Pozo et al. 2002) and controls ubiquitin-dependent degradation of two cell division transcriptional factors, E2FC (E2 promoter

transcription factor C) and DPB (E2F dimerization partner B) (del Pozo et al. 2006). Further evidence reveals the role of SKP2A in mediating the auxin signaling. For example, the levels of nuclear protein SKP2A are reduced in the presence of auxin (Jurado et al. 2010), and accumulation of SKP2A protein is significantly reduced in the *axr2-1* and *axr3-1* mutants (Jurado et al. 2008a, b). Also, loss-of-function *skp2a* mutant exhibits auxin-tolerant phenotypes (Jurado et al. 2010). The critical evidence supporting SKP2A as an auxin receptor is its ability to directly bind auxin at the auxin binding site as predicted by comparative computational structure analysis using the TIR1 as a reference (Jurado et al. 2010; Mach 2010). Thus, SKP2A has been identified as the third auxin receptor.

SCF^{SKP2A} complex is a key regulator of the G1/S checkpoint in cell cycle progression, where some regulatory proteins need to be degraded to allow dividing cells enter the next phase. *Arabidopsis* SCF^{SKP2A} complex also positively regulates the cell cycle and functions almost in a same way to $SCF^{TIR1/AFB}$. In the absence of auxin or low auxin, transcription factors E2FC and DPB form a heterodimer that bind to the promoters of cell cycle genes and repress transcription of a subset of E2FC target genes. When auxin binds to SCF^{SKP2A}, the auxin SCF^{SKP2A} complex promotes ubiquitinylation and degradation of phosphorylated E2FC and DPB (del Pozo et al. 2006), activating transcription of cell cycle genes that function in cell cycle control. Since SKP2A is the newly discovered auxin receptor, much work is needed to be done to elucidate the entire mechanism of SKP2A in mediating the auxin signaling and cell cycle (Fig. 3).

Auxin Signaling Attenuation

After the auxin receptors transmit the signaling generating rapid responses to developmental and environmental stimuli, the signaling is often rapidly attenuated. Failure to switch the signaling off results in abnormal growth, and with attenuation, plant cells can also reset the system to prepare for the next response to a new stimulus (Peer 2013). Attenuation can occur at several levels, including removal of the stimuli, catabolism of auxin, and deactivation of receptors, and the signaling components at transcriptional or posttranscriptional levels. The mechanisms of the auxin signaling attenuation at different location within a cell/tissue/organ may vary. However, very little is known about how the auxin signaling is turned off in various auxin-mediated processes to date.

As mentioned above, auxin can be removed through the catabolism pathways, oxidation, and conjugation (Woodward and Bartel 2005; Normanly 2010). Recently, reactive oxygen species (ROS) has been shown to induce the oxidation of IAA to oxIAA (oxindole-3-acetic acid) (Peer et al. 2013). This result highlights the mechanism through which ROS regulates active auxin removal and the signaling attenuation, and the finding may be of particular importance for attenuation of auxin response under stress conditions. The control of attenuation also occurs at the level of the auxin receptors. It has been shown that microRNA (miRNA) miR393 plays an

important role in auxin signaling attenuation, which directly targets *TIR1* and *AFBs* (Navarro et al. 2006; Si-Ammour et al. 2011), therefore negatively regulating the expression levels of *TIR1* and *AFBs* through cleavage of their mRNAs. Importantly, miR393 modulates varied responses by cleaving different *TIR1* and *AFB* transcripts, although all the members of *TIR1* and *AFB* are the putative targets of miR393 (Si-Ammour et al. 2011). For example, in bacteria-infected (*Pseudomonas syringae*) *Arabidopsis* leaves, miR393 regulates flagellin22 (Flg22)-triggered enhanced innate immunity in response to bacterial infection through cleavage of *TIR1*, *AFB2*, and *AFB3* transcripts (Navarro et al. 2006), whereas in roots, miR393 specifically cleaves *AFB3* mRNAs to regulate root response to nitrate (Vidal et al. 2010). Thus, miR393 can attenuate the auxin signaling and response via reducing the negative control of TIR1 and AFB on AUX/IAA leading to transcriptional repression of the downstream auxin-responsive genes (Fig. 3).

A most recent study shows that ABP1 is a negative regulator of the $SCF^{TIR1/AFB}$ pathway (Tromas et al. 2013). The genetic analysis reveals that *ABP1* functions upstream of *TIR1/AFBs* in regulating root growth. Further molecular and biochemical evidence demonstrate that *ABP1* does not regulate *TIR1/AFB* expression but negatively affects the stability of AUX/IAA proteins. *ABP1* knockdown promotes degradation of AUX/IAA proteins without affecting its role on endocytosis. Negative regulation of SCF-mediated control of AUX/IAA by ABP1 provides an important regulatory mechanism to tightly control cross talk between the auxin signaling mediated by different receptors and fine-tune responses of cells/tissues/organs/plant to auxin during growth and development and under stress conditions (Fig. 3).

The auxin signaling is among the best characterized pathways. However, it is apparent that many questions remain to be answered. These include how the auxin signaling is precisely attenuated at various levels at the specific sites of action and whether there are negative feedback mechanisms in each receptor-mediated auxin signaling pathway and how these pathways coordinately regulate overall response to auxin.

Auxin Signaling Pathway Mediates Plant Responses to Abiotic Stresses

As mentioned earlier, salinity and drought affect auxin homeostasis, thereby causing plastic growth and development. Extensive genetic and molecular studies have demonstrated that the auxin transport is also involved in plant responses to environmental stimuli, such as low temperature, light, and gravity (Shibasaki et al. 2009; Buer and Muday 2004). In the past several years, there are some evidence pointing to the regulatory role of the auxin signaling in plant response to salinity and drought (Fang and Yang 2002; Iglesias et al. 2010; Chen et al. 2012a). Therefore, it is conceivable that the auxin signaling pathway also plays critical roles in plant responses to salinity and drought. In this section, we will briefly summarize the recent progress in the role of the auxin signaling in plant response to salt stress and drought conditions.

The evidence for regulatory role of the auxin signaling in plant response to salt stress and drought comes from the transcriptional profiling of plants treated with high salinity and drought, respectively. The results obtained from various plant species, such as *Arabidopsis* (Seki et al. 2002), rice (Jain and Khurana 2009; Song et al. 2009a), and sorghum (Wang et al. 2010), show many genes are upregulated or downregulated by salt stress and drought, among which many of the auxin-responsive genes display differential expression in response to the stress treatment. In particular, the members of the *Aux/IAA*, *SAUR*, and *ARF* gene families are differentially expressed under abiotic stresses, indicating the TIR-/AFB-mediated auxin signaling pathway is indeed involved in stress responses of plants to abiotic stress. Therefore, auxin signaling-mediated developmental plasticity may be a conserved adaptive mechanism for plants.

The direct evidence of involvement of the auxin signaling in plant stress tolerance is obtained from phenotypic analysis of the *tir1afb* auxin receptor mutants under abiotic stress (Iglesias et al. 2010). The *tir1afb2* mutant is more tolerant to salt stress. Interestingly, *tir1afb2* mutant contains less hydrogen peroxide and superoxide anion and increased antioxidant enzyme activities, exhibiting increased tolerance to oxidative stress. Thus, the auxin receptor mediates plant adaptive growth under salt stress. Further functional analysis of the miR393 provides strong evidence that *TIR1*- and *AFB2*-mediated plant responses to osmotic stress are also regulated by miR393 (Chen et al. 2012a). With certainty, these receptors are also important for biotic stress, because the *tir1afb2* mutant also showed altered sensitivity to SA (Iglesias et al. 2011). Taken together, these results suggest that TIR1/AFB receptors are required for not only plant growth and development but also plant adaptation to changing environment. It is possible that the TIR1/AFB receptors modulate plant responses to developmental and environmental stimuli through different downstream components in the auxin signaling pathway.

Further studies on the role of *IAA* genes support the notion. It is well known that high salinity delays seed germination and postgerminative development (Ayers 1952; Katerji et al. 2003). Most of the previous studies focus on the role of ABA. The recent results show that enhanced auxin signaling also plays a role during salt-induced delayed seed germination under salt stress. The results that *IAA30*, *IAA1*, and *IAA19* are induced by high salinity support the hypothesis (Park et al. 2011). Notably, it is found that a membrane-associated transcription factor NTM2 (NAC with transmembrane motif2) is translocated into nucleus after salt treatment where it can bind to the promoter of *IAA30* to activate expression of *IAA30*. Loss-of-function *ntm2* mutant abolishes the upregulation of *IAA30* and is more resistant to high salinity during seed germination. Overexpression of *IAA30* in the *ntm2* mutant restores the salt-resistant phenotype (Park et al. 2011). This finding suggests that the auxin signaling plays critical role in germination and postgerminative arrest induced by salt stress in *Arabidopsis* and provides evidence that seed germination and early development under abiotic stresses are modulated by cross talk of hormone signaling pathway. It is possible that different AUX/IAA family proteins function differently in plant responses to various environmental stimuli. However, the roles of individual AUX/IAA family protein in plant stress tolerance remain to be investigated.

To date, there is still very little information about the mechanism of auxin signaling-mediated stress tolerance. However, findings above highlight the importance of auxin signaling in stress responses of plants and point out new research directions to further understand the molecular mechanism of the complex trait of stress tolerance. Abiotic stresses including drought and high salinity affect almost all the developmental stages of plants during their entire life cycle. The responses of plants to various stresses or to the different levels of the same stress are quite different. For example, drought and salt shortens or prolongs plant life cycle, respectively. These observations suggest that plant growth and development are dynamically controlled by a complex regulatory network. Other questions include how these signaling pathways coordinate and integrate with stress signaling or ABA signaling to fine-tune plant growth and development for better survival under stress conditions. Future research will further our understanding regarding the molecular mechanisms of plant tolerance to salinity and drought and will help us to manipulate drought and salt tolerance of crops.

Biotechnological Manipulation of Auxin Biosynthesis and Signaling in Agriculture

With the increasing of global population, people have become concerned about whether agriculture can keep up with population growth. In the past several decades, there is great success in genetic manipulation of important traits of economically important crops, such as insect tolerance of cotton and herbicide resistance of cotton and soybean (Dunwell 2000; Owen and Zelaya 2005) as well as drought tolerance of maize (Mashiguchi et al. 2011; Castiglioni et al. 2008), making us believe that genetic engineering is a powerful approach to improve the agronomic traits of crops. Most strikingly, recent molecular genetics have demonstrated that mutations in a single gene in GA biosynthesis or the signaling pathway, such as reduced-height genes (*Rht*) in wheat (Peng et al. 1999; Hedden 2003) and a rice semidwarf gene (*sd1*) (Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002), result in the development of high-yielding varieties of cereal grains, referred to as an evolutionary breakthrough in agriculture. This significant achievement suggests that biotechnological manipulation of hormone biosynthesis and signaling pathways might be a potential approach for future improvement of crops with high yield and quality.

Auxin is the most important plant growth regulator. In addition to its roles in cellular elongation and expansion, it has long been noticed that auxin plays central roles in apical dominance (Thimann and Skoog 1934), formation of lateral roots and adventitious roots (Sabatini et al. 1999; Casimiro et al. 2001; Blilou et al. 2005; Teale et al. 2005; Haissig 1972; Gutierrez et al. 2012), onset of leaf abscission (Noh and Amasino 1999; Hong et al. 2000; Tucker et al. 2002; Ellis et al. 2005), fruit development (Veluthambi and Poovaiah 1984; Else et al. 2004; Goetz et al. 2006), and vascular differentiation (Sachs 1981; Aloni 1987; Mattsson et al. 2003) in various agricultural or horticultural important crops. According to these regulatory

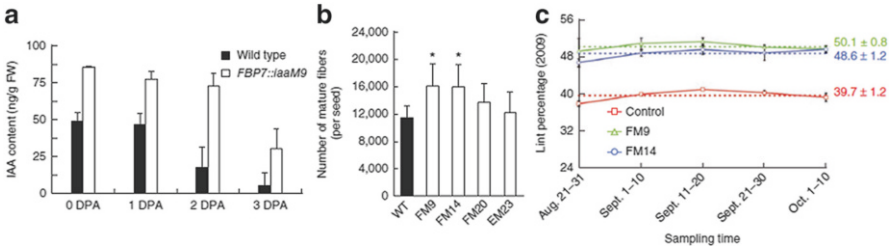

Fig. 4 Overexpression of auxin biosynthetic gene *iaaM* in cotton ovules increases the number of lint fibers (Zhang et al. 2011). **a.** Endogenous IAA level in ovules of transgenic lines is increased; DPA: days post anthesis. **b.** The transgenic lines show increased number of mature fibers; FW9 and FW14 are two transgenic lines derived from *FBP7*::*iaaM*. **c.** Variation of lint percentage over five sampling times in the trials of 2009

roles of auxin, artificial auxin, such as 1-napthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), has been broadly used in agriculture and horticulture for more than 60 years. These commercial applications include prevention of fruit drop, induction of parthenocarpic (seedless) fruits, and promotion of rooting of plant cuttings in propagation. These successful applications in agriculture and horticulture suggest that delicate manipulation of auxin levels and auxin responses must confer the desired agricultural or horticultural traits of crops. Although there has been some progress in manipulating auxin biosynthesis and signaling pathway, the successful cases of genetic improved crops with desired traits remain very limited.

Recent progress has demonstrated that ovule epidermis-specific expression of *iaaM* gene in the auxin biosynthesis pathway resulted in increased level of auxin and an eventual increase by greater than 15% in lint yield (Zhang et al. 2011). Because IAA is accumulated in cotton fiber initials (Beasley 1973), Zhang et al. attempted to investigate whether increase in IAA content through a genetic engineering approach can enhance the yield and quality of cotton. They expressed *iaaM* gene under control of the promoter of the petunia MADS box gene Floral Binding protein 7 (*FBP7*) (Zhang et al. 2011). A 4-year field trial of the transgenic cotton plants overexpressing *iaaM* shows that the transgenic plants contain increased levels of IAA in the epidermis of ovules at the fiber initiation stage and the markedly increased lint fibers (Fig. 4). Thus, manipulation of IAA concentration using a transgenic approach has led to solution of a long-standing problem in cotton. In tomato, the transgenic plants overexpressing the *Pseudomonas syringae iaaM* gene also have increased IAA and produce seedless fruits (Rotino et al. 1997). It is clear that *iaaM* gene can be used as a potential target for improving agricultural traits of crops.

In addition to *iaaM* gene, *YUC* and *TAA* families controlling the auxin biosynthesis pathway could also be the putative targets for genetic engineering of crops, because overexpression of *YUC1* results in apparent development phenotypes in *Arabidopsis* (Zhao et al. 2001) and crops, such as maize and rice, possessing homologue genes of *YUC*. It has been shown that knockout of *SPARSE INFLORESCENCE1* (*SPI1*) gene results in reduced number of tassels, ears, and spikelets because of failure to initiate the branch meristems and spikelet pair meristems (Gallavotti et al. 2008).

Loss of function in the *VANISHING TASSEL2* (*VT2*) gene, a co-ortholog of *IAA1* converting Trp to IPA, shows similar phenotypes in lateral organ formation (Phillips et al. 2011). In rice, constitutive overexpression of *Arabidopsis YUC1* causes leaf and root growth inhibition, whereas downregulation of *YUC1* results in dwarfisms of shoots and shortened root elongation (Yamamoto et al. 2007).

There are also several attempts to manipulate the level of GH3, an amino acid conjugase, which is responsible for conjugating IAA to the inactive form. It has been shown that overexpression of *OsMGH3* (*OsMADS1 regulated GH3* domain-encoding gene)/*OsGH3-8* in rice affects plant architecture (Ding et al. 2008; Yadav et al. 2011). The transgenic rice plants overexpressing *OsMGH3*/*OsGH3-8* exhibit dwarf and tufted shape with reduced internode length, apical dominance, and branching panicles. Overexpression of *OsGH3-2* in rice also leads to IAA deficiency phenotype, such as dwarfism, smaller leaf and panicles, and increased leaf angle (Du et al. 2012). Overexpression of *OsIAGLU* (rice IAA-glucose synthase gene) encoding an enzyme catalyzing IAA-glucose conjugation increases the tiller and panicle number but decreased the plant height and panicle length in rice (Choi et al. 2013). Their results showed that IAA levels dramatically affect the development of vegetative and reproductive organs, especially those important for grain yield of crop plants. However, it is still far away from the successful manipulation of important traits of crops.

Knowledge and understanding of auxin perception and signaling transduction have been almost completely obtained from *Arabidopsis*. The current advance shows that the auxin signaling is quite conserved in plants. Indeed, all the transgenic plants overexpressing rice *IAA1*, *IAA3*, and *OsIAA4* show developmental phenotypes in shoot development and root architecture (Nakamura et al. 2006; Song et al. 2009b; Song and Xu 2013). Overexpression of *SAUR39* also causes reduced growth of shoot and root, smaller vascular tissue, and lower yield (Kant et al. 2009).

Taken together, the auxin levels and its maxima in a tissue/organ as well as a plant are varied and dynamically regulated during organ formation and development. The auxin receptors and the downstream components also show varied subcellular localization and expression patterns during plant development. Although the auxin metabolism and signaling pathways are conserved, it is conceivable that there must be genes or regulatory mechanisms unique for different plant species. Therefore delicate design in genetic manipulation of auxin metabolism and the signaling is needed to achieve the desired performance in agriculture.

As mentioned earlier, auxin homeostasis and signaling are very important for plant responses to stress. Therefore, some efforts have been made to enhance the stress tolerance to abiotic stresses, such as drought and salinity in crops, through manipulating auxin levels. A little progress has been made in genetic manipulation of IAA contents and response sensitivity for enhanced stress tolerance. For example, overexpression of *Arabidopsis YUC6* in potato dramatically enhances plant tolerance to water deficit (Fig. 5) (Im Kim et al. 2013). The transgenic potato plants show high-auxin developmental phenotypes, such as greater height and erect stature, longer petioles, narrow and downward-curling leaves, and longevity. Under drought conditions, these transgenic plants are more tolerant to drought

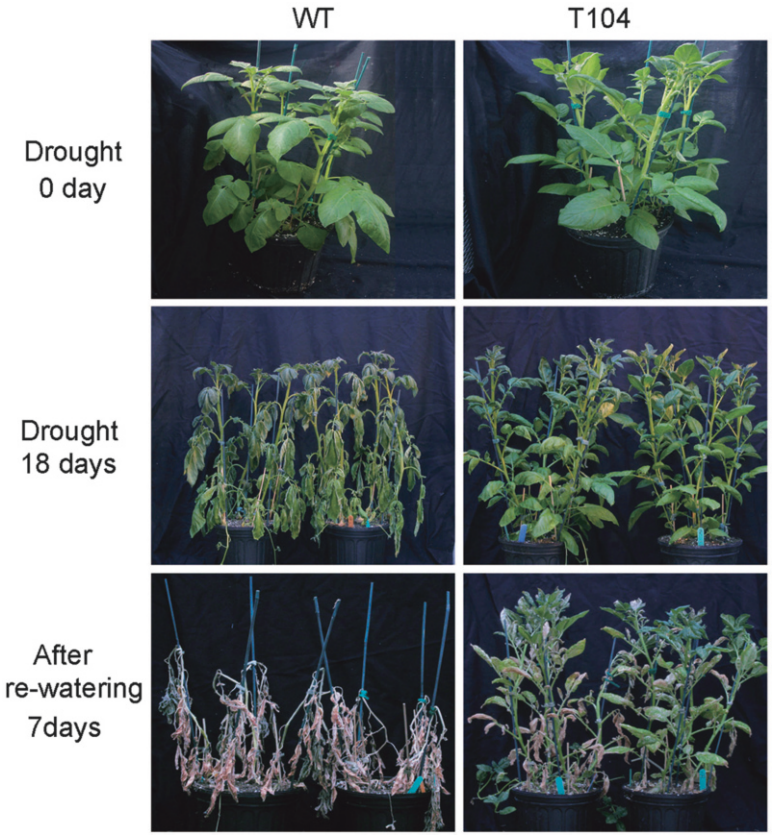

Fig. 5 Transgenic potato plants overexpressing the *Arabidopsis thaliana YUC6* (*AtYUC6*) are more tolerant to drought. Comparison of well-watered 4-month-old transgenic (T104) and untransformed plants (WT) before withdrawal of watering (drought 0 day), and at 18 days (drought 18 days) after withholding of water and at 7 days after rewatering (After rewatering for 7 days) (Im Kim et al. 2013)

(Im Kim et al. 2013). It is proposed that the drought-resistance phenotype of transgenic potato plants overexpressing *Arabidopsis YUC6* may be due to reduced ROS content (Im Kim et al. 2013). These results suggest that elevation of auxin level through genetic engineering may be a strategy to improve plant drought resistance. It has been reported that overexpression of *OsGH3.13* resulted in reduced auxin and increased levels of IAA–Ala and IAA–Asp conjugates that also conferred enhanced drought tolerance of the transgenic plants (Zhang et al. 2009). The result is controversial to the results obtained in *Arabidopsis* (Lee et al. 2012) and potato (Im Kim et al. 2013). This may give a hint of complexity of auxin in plant stress tolerance. With increasing understanding of auxin metabolism and its signaling, and its cross talk with other stress hormones as well as the corresponding signaling pathways, we believe that improvement of stress tolerance of crops through genetic manipulation is just around the corner.

Conclusions and Future Perspectives

Plants belong to one huge group of living organisms, which were traditionally divided into two groups, the other is animals. Plants are the main source of the world's molecular oxygen, the basis of the earth's ecologies, and mankind's basic foods. It is predicted that there are approximately 300–315 thousand plant species with varied sizes, stature, lifestyle, and growth conditions. Hormonal regulation plays central role in modulating plant growth and development and determining their stature and life cycle. It is clear that auxin is an essential growth regulator of plant growth and development. In the past three decades, we have made great breakthroughs in molecular mechanisms of auxin homeostasis control and plant responses to auxin in model plants such as *Arabidopsis* and rice. However, the regulation of homeostasis and response of plants to auxin is very complex in a species and becomes even more complex in different species.

To date, a complete two-step pathway of auxin biosynthesis has been determined. The immediate question is how this pathway is involved in cell-/tissue-/organ-specific growth regulation. Other questions include the following: what the other pathways are in various plant species and how these pathways are integrated in regulating local auxin levels and maintaining auxin maxima and gradients required for optimal growth. We also need to further characterize how active auxin is coordinately regulated by biosynthesis and catabolism pathways to maintain auxin homeostasis during plant growth and development.

While in the auxin signaling pathway, three auxin receptor ABP1 and co-receptor complexes $SCF^{TIR1/AFBs}$ and SCF^{SKP2A} have been identified, the downstream signaling components of each receptor and their mediated biological processes start to be understood. However, many questions also need to be answered. Are there any other auxin receptor/co-receptors? Does these signaling pathways-mediated different receptor/co-receptors regulate separate developmental processes, and if not, how are these pathways integrated to fine tune plant cell/tissue/organ and plant growth and development? It is also critical to understand the attenuation of auxin signaling at multiple levels. Interaction between auxin and other hormonal signaling pathways is also among the future researches.

The most important difference between plant and animal development is that plants develop postembryonically. Thus, auxin homeostasis control is particularly important for growth and reproductive success under constantly changing environments (Fig. 6). It is noteworthy that cross talk between auxin and stress signalings and integrative regulation of the hormone signaling pathways are particularly important for elucidation of the molecular mechanisms of developmental plasticity and plant adaptation to stress conditions. The knowledge gained in these research studies will definitely prove to be beneficial to future genetic improvement of crops with desirable architecture and high/stable yield.

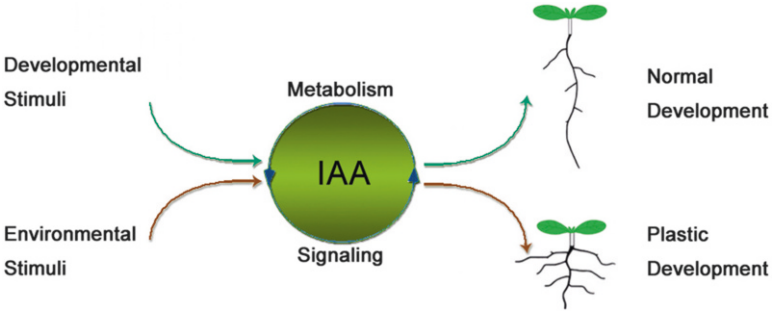

Fig. 6 A schematic model of auxin-mediated plant development and plastic development

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Abscisic Acid Implication in Plant Growth and Stress Responses

Hiroaki Fujii

Abstract Abscisic acid (ABA) is an important phytohormone regulating various physiological aspects in plants such as seed maturation, dormancy, seedling growth, and stomatal behaviour. In this chapter, a global picture with recent findings in ABA metabolism and responses is overviewed. Because of putting the priority on simplicity, to understand historical importance, you should refer to other reviews such as Cutler et al. (Annu Rev Plant Biol 61:651–79, 2010). In recent years, many enzymes responsible for the synthesis and catabolism of ABA have been identified, almost completing the main pathway of ABA production. In ABA-responding cells, there are sets of core components in the ABA reception system, which regulates multiple responses including induction of gene expression and alteration of ion transport. Many players modify the core components to produce sophisticated reactions. The possibility of modification of the pathways at the molecular level to improve crop productivity will be discussed in the final section.

Keywords Drought • PYR/PYL • PP2C • SnRK2 • Transcription factor • Ion channel

Abbreviations

AAO	ABSCISIC ALDEHYDE OXIDASE
ABA1-4	ABSCISIC ACID DEFICIENT 1–4
ABCG	ATP-BINDING CASSETTE G
ABF	ABSCISIC ACID-RESPONSIVE ELEMENT BINDING FACTOR
ABI	ABSCISIC ACID INSENSITIVE

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AFP	ABI FIVE BINDING PROTEIN
AKS	ABA-RESPONSIVE KINASE SUBSTRATE
AREB	ABSCISIC ACID-RESPONSIVE ELEMENT BINDING PROTEIN
AtrbohF	*A. thaliana* respiratory burst oxidase homologue F
CDPK	CALCIUM-DEPENDENT PROTEIN KINASE
CPK	CALCIUM-DEPENDENT PROTEIN KINASE
CYP	CYTOCHROME P450
DWA	DWD HYPERSENSITIVE TO ABA
GORK	GUARD-CELL OUTWARD-RECTIFYING K^+ CHANNEL
HAB	HOMOLOGY TO ABI
HAI	HIGHLY ABSCISIC ACID INDUCED
KAT	POTASSIUM CHANNEL IN *ARABIDOPSIS THALIANA*
KEG	KEEP ON GOING
KUP	K^+ UPTAKE TRANSPORTER
MAPK	MITOGEN-ACTIVATED PROTEIN KINASES
NCED	9-*cis* EPOXYCAROTENOID DIOXYGENASE
NRT	NITRATE TRANSPORTER
OST	OPEN STOMATA
PDR12	PLEIOTROPIC DRUG RESISTANCE12
PP	PROTEIN PHOSPHATASE
PYL	PYR1-LIKE
PYR	PYRABACTIN RESISTANCE
QUAC	QUICK ACTIVATING ANION CHANNEL
RCAR	REGULATORY COMPONENT OF ABA RECEPTOR
RHA2a	RING-H2 FINGER A2a
SCS	SnRK2-INTERACTING CALCIUM SENSOR
SDIR	SALT- AND DROUGHT-INDUCED RING FINGER
SDR	SHORT-CHAIN DEHYDROGENASE/REDUCTASE
SLAC	SLOW ANION CHANNEL ASSOCIATED
SLAH	SLAC1 HOMOLOGUE
SnRK	SNF1-RELATED PROTEIN KINASE
SUMO	SMALL UBIQUITIN-LIKE MODIFIER
ZEP	ZEAXANTHIN EPOXIDASE

ABA Metabolism and Transportation

Many genetic and biochemical studies have revealed the ABA biosynthesis pathway in *Arabidopsis* (Fig. 1). Abscisic acid is mainly synthesized from the carotenoid alcohol, zeaxanthin. ZEAXANTHIN EPOXIDASE (ZEP) catalyses zeaxanthin to all-*trans*-violaxanthin in plastids. Mutation of the ZEP gene (*aba1*) causes an ABA-deficient phenotype (Marin et al. 1996). All-*trans*-violaxanthin is converted to all-trans-neoxanthin or 9-*cis*-violaxanthin, although the exact enzymes in these steps are still unclear. ABSCISIC ACID DEFICIENT 4 (ABA4) is involved in neoxanthin synthesis. An *aba4* mutant lacks neoxanthin but still contains ABA, even though the

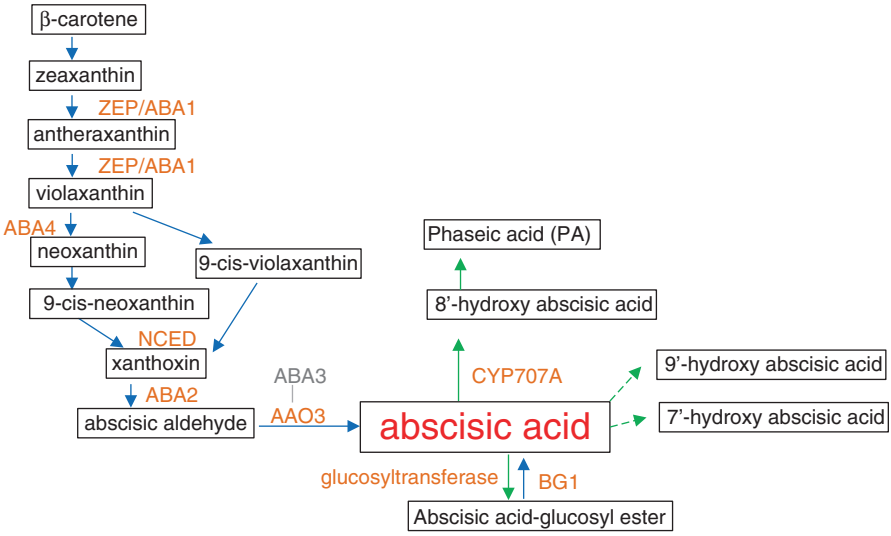

Fig. 1 Scheme of ABA metabolism. ABA is synthesized (*blue arrows*) and deactivated (*green arrows*) through several steps. Major enzymes responsible to each reaction are shown in *orange*

amount is reduced, indicating that converting to neoxanthin is not an essential step (North et al. 2007). 9-*cis* EPOXYCAROTENOID DIOXYGENASE (NCED) can catalyse both all-*trans*-neoxanthin and 9-*cis*-violaxanthin to xanthoxin. From this point, reactions occur in the cytosol. Xanthoxin is converted to abscisic aldehyde by SHORT-CHAIN DEHYDROGENASE/REDUCTASE (SDR), which is the causal gene for the *aba2* mutant (Rook et al. 2001). Finally, abscisic aldehyde oxidase (AAO) catalyses abscisic aldehyde to abscisic acid. AAO needs a molybdenum cofactor, which is impaired in the *aba3* mutant (Seo et al. 2000). Some other bypass/backup pathways may work in some tissues (Seo and Koshiba 2002).

The ABA amount is also regulated by the other side, catabolism (Nambara and Marion-Poll 2005). The 8′-hydroxylation is the predominant step, which is mediated by the cytochrome P450 monooxygenase CYP707A (Krochko et al. 1998), followed by converting to phaseic acid (PA, Milborrow et al. 1988). The 7′-hydroxylation and the 9′-hydroxylation also exist, although they are considered to be minor (Okamoto et al. 2011).

ABA is accumulated under osmotic stress as well as during seed maturation. To change the amount of ABA, some of the steps above should be regulated. Since osmotic stress induces a minor increase in the protein amount of ZEP, AAO3, and ABA3, even though their mRNA expression is induced (Liotenberg et al. 1999; Seo et al. 2000; Xiong et al. 2001), an increase of NCED3 may contribute the largest to ABA accumulation under osmotic stress (Iuchi et al. 2001). In seeds, in addition to maternal ABA, the expression of NCED5, NCED6, and NCED9 in later embryos is essential for ABA-mediated dormancy (Frey et al. 2012). In addition, there is a positive feedback loop. Exogenous ABA induces the expression of ABA1, AAO3, and ABA3 (Xiong et al. 2002). That may induce an efficient response, although it

adds complexity for researchers to unveil the ABA pathway. On the other hand, when seeds are imbibed, cyp707a2 expression is induced and plays crucial roles in breaking the dormancy (Liu et al. 2009).

Besides permanent catabolism, temporal deactivation is considered to change the amount of active ABA. ABA is conjugated with glucose, forming ABA-glucosyl ester (ABA-GE) by a glucosyltransferase (Xu et al. 2002). In reverse, β-glucosidase1 (BG1) hydrolyses ABA-GE to ABA (Lee et al. 2006). Since ABA-GE is inactive (Kepka et al. 2011), this system may provide temporal storage for a rapid response.

Another mechanism mediating ABA function is spatial regulation; ABA is sometimes transported to other cells. The expression of some ABA synthesis genes is unequal among cell types (Koiwai et al. 2004; Endo et al. 2008). For a long time, researchers believed that under drought conditions ABA was synthesized in roots, while ABA responses happened in leaves (Davies and Zhang 1991). In a recent report, guard-cell-specific expression of ABA3 in the *aba3* background rescued the wilted phenotype of leaves, indicating that ABA synthesis in guard cells was enough for the guard-cell responses to the dryness of leaves (Bauer et al. 2013). It, however, is still possible that different systems work during root-sensing dehydration. Mutants of the ABA transporter show their importance in ABA signalling. Two ATP-binding cassette (ABC) transporters are involved in ABA transportation. ATP-BINDING CASSETTE G40 (ABCG40 aka PDR12) is highly expressed in guard cells and can mediate the uptake of ABA (Kang et al. 2010), whereas ABCG25 is highly expressed in vascular cells and can mediate the efflux of ABA (Kuromori et al. 2010). Interestingly, the nitrate transporter NRT1.2 can also import ABA into yeast cells and the sensitivity to ABA of the *nrt1.2* mutant is lower than that of the wild type (Kanno et al. 2012). Thus, transportation can be another target to modify the ABA pathways.

Signal Perception and Execution of ABA-Induced Responses

Receptor and Signal Transduction: PYR/PYL-PP2C-SnRK2

Signal transduction in the cells responding to a molecule should be initiated by receptors, which bind to the molecule. The goals of the transduction are regulation of effectors such as a transcription factor and an ion transporter (Pandey et al. 2007).

A family of START proteins, PYRABACTIN RESISTANCE 1 (PYR1) and PYR1-LIKE (PYR/PYL aka RCAR), are cytosolic ABA receptors (Ma et al. 2009; Park et al. 2009). PYR/PYLs bind to ABA. ABA-bound PYR/PYLs inhibit clade A of PROTEIN PHOSPHATASE 2C (PP2C), including ABSCISIC ACID INSENSITIVE (ABI)1, ABI2, and HOMOLOGY TO ABI (HAB) 1. Without ABA, the PP2Cs dephosphorylate and inhibit SNF1-RELATED PROTEIN KINASES 2 (SnRK2s) (Umezawa et al. 2009; Vlad et al. 2009; Fujii et al. 2009). When ABA-bound PYR/PYLs inhibit PP2C, SnRK2s are released from the inhibition by PP2C, resulting in SnRK2-mediated phosphorylation of transcription factors ABA-RESPONSIBLE

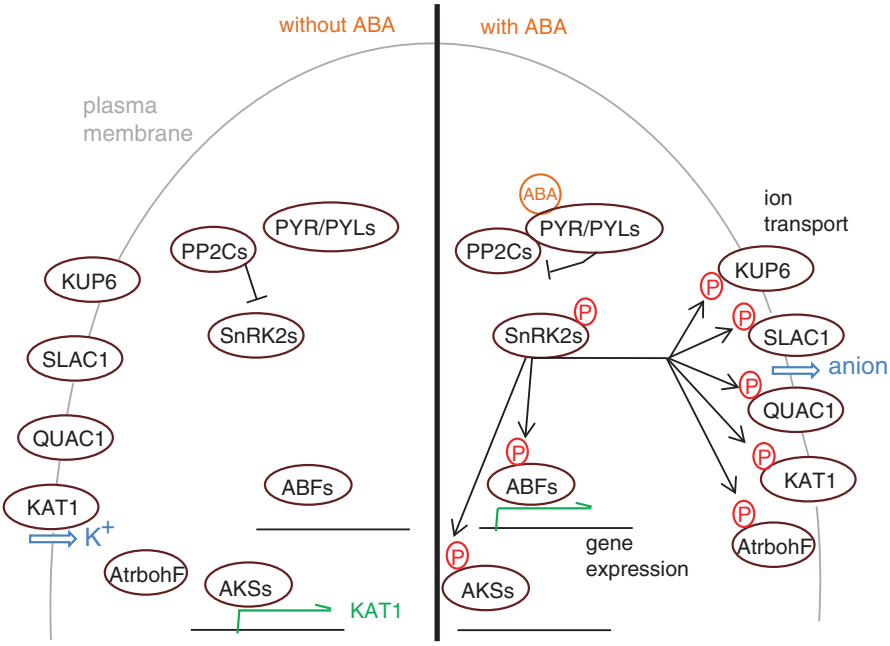

Fig. 2 Scheme of the PYR/PYL-PP2C-SnRK2 pathways. ABA-bound PYR/PYLs inhibit PP2C, resulting in activation of SnRK2s. SnRK2s phosphorylate and regulate various targets working on ion transport, gene expression and so on

ELEMENT BINDING FACTORS (ABFs aka AREB: Fig. 2). Because recombinant proteins of these four components can reconstitute the entire pathway from ABA to regulation of transcription factors in vitro, these are defined as core components of the pathway (Fujii et al. 2009). Genetic studies revealed that the core components were essential for ABA signalling. In an *snrk2.2/3/6* triple mutant, all examined ABA responses were eliminated (Fujii and Zhu 2009; Fujita et al. 2009; Nakashima et al. 2009). The *abi1-1* mutation on ABI1 that inhibits interaction to PYR/PYLs (G180D, Park et al. 2009) renders the dominant ABA-insensitive phenotype (Koornneef et al. 1984; Leung et al. 1997).

Even though the ABA pathway sounds simple in the explanation above, there are a lot of complexities even in the core components (Cutler et al. 2010). The PYR/PYL family consists of 14 members in *Arabidopsis*, although it is not yet confirmed whether PYL13 works as an ABA receptor (Fujii et al. 2009). Some of them work redundantly, since a *pyr1/pyl1/2/4/5/8* sextuple mutant is insensitive to ABA, while any single mutant is not (Gonzalez-Guzman et al. 2012). The more genes are disrupted, the less sensitivity is shown. This fact suggests that the protein amount of the total family is important. On the other hand, each member may have its special function. In terms of the oligomeric state of their apo forms, PYR/PYLs are divided into two classes. PYR1 and PYL1, 2, and 3 form dimers when they are in their apo forms, while PYL4–10 are always monomers (Dupeux et al. 2011). In addition,

PYL3 forms two states of dimers (Zhang et al. 2012). Dimerization alters the energy threshold for dissociation, which is required for their inhibitory function on PP2C. Monomeric PYLs sometimes inhibit some PP2Cs even in the absence of ABA (Hao et al. 2011).

Clade A PP2C consists of nine members in *Arabidopsis*. ABI1, ABI2, AHG1, AHG3, HAB1, and HAB2 work redundantly as negative regulators in the ABA pathway as mentioned above (Nishimura et al. 2007; Rubio et al. 2009). The other three HIGHLY ABSCISIC ACID INDUCED (HAI)1, HAI2, and HAI3 also work in the ABA pathway as negative regulators in post-germination growth, although the phenotype of mutants is weaker than the mutants of the other 6 (Bhaskara et al. 2012). In terms of radicle emergence, however, the triple mutant shows ABA insensitivity, meaning their opposite effect from the other PP2Cs (Bhaskara et al. 2012). The mechanisms regulating this phenomenon remain obscure.

Even though the SnRK2 family consists of ten members in *Arabidopsis*, SnRK2.1, SnRK2.4, SnRK2.5, SnRK2.9, and SnRK2.10 are not activated and SnRK2.7 and SnRK2.8 are just weakly activated by ABA (Boudsocq et al. 2004). Thus, three SnRK2s, 2.2, 2.3, and 2.6, are in the ABA pathways. Though germination is mainly mediated by SnRK2.2 and SnRK2.3 (Fujii et al. 2007) while guard-cell regulation is mainly mediated by SnRK2.6 (Mustilli et al. 2002; Yoshida et al. 2002), they also work redundantly in both regulations (Fujii and Zhu 2009).

The combination of members of the core components may be important for the function of the pathway. Though this functional importance is unclear, interactions between PYR/PYLs and PP2Cs have some preferences (Park et al. 2009; Ma et al. 2009; Bhaskara et al. 2012). The spatiotemporal expression pattern of core components varies among family members. Some of them, such as ABI1, ABI2 (Leung et al. 1997), and PYL4 (Park et al. 2009), are also regulated by environmental conditions such as ABA treatment. In addition, the core components are regulated post-translationally. Several proteins have been identified to modify the activity of the pathway. A Rho-like small GTPase ROP11 negatively modifies PYL9-mediated suppression of ABI1 (Li et al. 2012). The calcium-binding protein SCS (SnRK2-interacting calcium sensor) inhibits SnRK2 activity in a calcium-dependent manner (Bucholc et al. 2011). Thus, the signalling depends on the condition of the cell as well as on developmentally defined cell types. Finally, the signalling is branched at SnRK2s because SnRK2s phosphorylate several substrates, as discussed in the next section.

SnRK2 Substrates

The ABF/AREBs are beta-Zip-type transcription factors and bind to the ABA-responsive element (ABRE, PyACGTGG/TC). ABRE is located in the promoter regions of many ABA-induced genes and is important for ABA-induced gene expression (Hattori et al. 2002). AREB1, AREB2/ABF4, and ABF3 redundantly play pivotal roles in ABA signalling under water-deficient conditions (Yoshida et al. 2010). The *abi5* mutant was identified in screening for ABA insensitivity in

germination (Finkelstein and Lynch 2000) and seedling growth (Lopez-Molina and Chua 2000). ABF3 also works in germination redundantly with ABI5 (Finkelstein et al. 2005). Because overexpression of ABI5 itself cannot induce full activation (Uno et al. 2000), post-translational modification is needed. Phosphorylation is needed for the full activation of ABFs (Furihata et al. 2006), and SnRK2s phosphorylate ABFs in the ABA pathway (Johnson et al. 2002; Kobayashi et al. 2005; Furihata et al. 2006; Fujii and Zhu 2009; Fujii et al. 2009). In addition to SnRK2s, CALCIUM-DEPENDENT PROTEIN KINASES (CPKs/CDPKs) also can phosphorylate ABFs (Zhu et al. 2007).

The phosphorylation may be the final step for ABF-mediated transcription. Before that, several mechanisms regulate the amount of ABFs. First, expression is transcriptionally regulated. Since overexpression of ABI5 can rescue the ABA-insensitive phenotype of the *abi3* mutant, ABI3 may regulate the transcription of ABI5 (Lopez-Molina et al. 2002). The amount of ABI3 is regulated by alternative splicing (Sugliani et al. 2010; Carvalho et al. 2010) and by ubiquitin E3 ligase AIP2 (Zhang et al. 2005). Several WRKY transcription factors involved in ABA signalling are considered to be regulators of ABF transcription. WRKY63/ABO3 positively regulates ABF2 (Ren et al. 2010), while WRKY40 negatively regulates ABI5 (Shang et al. 2010). WRKY18 and WRKY60, which are induced by WRKY18 and WRKY40 (Chen et al. 2010), also repress ABI5 (Liu et al. 2012). Since the WRKY family may antagonize another WRKY in their heterodimer or through transcription (Chen et al. 2010), the regulation is complicated.

The protein amount of ABFs is also regulated post-transcriptionally. Ubiquitin-proteasome systems play critical roles in the controlled degradation of the ABI5 protein. An E3 ligase, KEEP ON GOING (KEG), is important for keeping ABI5 amounts low without stress and then *keg* mutants are hypersensitive to ABA (Stone et al. 2006). Under ABA, ubiquitination of KEG itself is facilitated in a phosphorylation-dependent manner, resulting in less ubiquitination of ABI5 (Liu and Stone 2010). In addition, the substrate receptors for CUL4-based E3 ligases, DWD HYPERSENSITIVE TO ABA (DWA)1, DWA2, and DWA3, work in reducing the amount of ABI5 (Lee et al. 2010, 2011). Another ubiquitin E3 ligase, SALT- AND DROUGHT-INDUCED RING FINGER (SDIR)1, which positively works in the ABA pathway, is also an upstream regulator of ABFs (Zhang et al. 2007). Protein degradation mediated by the N-end rule pathway, which regulates the half-life of proteins through the identity of the amino-terminal residue (Bachmair et al. 1986), may also function upstream of ABFs (Holman et al. 2009).

Sumoylation is another modification of ABI5. SIZ1-mediated sumoylation of ABI5 negatively regulates the ABA pathway (Miura et al. 2009). The *siz1* mutant shows hypersensitivity to ABA, although the amount of ABI5 is less in the *siz1* mutant. The authors suggest that sumoylation brings ABI5 into an inactivated status (Miura et al. 2009). Sumoylation is important in other aspects of the ABA pathways, since overexpression of SUMO1 or SUMO2 induces an insensitive phenotype to ABA in spite of the higher induction of some ABA-induced gene expression (Lois et al. 2003). Moreover, ABI FIVE BINDING PROTEINS (AFPs) with unknown molecular functions negatively regulate the amount of ABI5 (Lopez-Molina et al. 2003).

SnRK2 can phosphorylate another transcription-factor family, ABA-RESPONSIVE KINASE SUBSTRATE (AKS), which consists of AKS1, AKS2, and AKS3 (Takahashi et al. 2013). AKSs induce the expression of the inward-rectifying potassium channel KAT1 (potassium channel in *Arabidopsis thaliana*) in the absence of ABA. In the presence of ABA, AKSs are phosphorylated and deactivated in the guard cells, resulting in the reduction of KAT1 expression. This response does not affect ABA-induced stomatal closure in the short term but alters the reactivity of stomata in the long term (Takahashi et al. 2013). In maize, homologue of SnRK2.6 also phosphorylates the SNAC1-type transcription factor (Vilela et al. 2013).

Another important substrate of SnRK2s is an S-type anion channel, SLOW ANION CHANNEL ASSOCIATED (SLAC)1. SLAC1 is preferentially expressed in guard cells and is essential for ABA-induced stomatal closure (Vahisalu et al. 2008). Activated SLAC1 can export anions, including chloride and nitrate. The release of anions induces the release of potassium, resulting in lower turgor pressure. SnRK2.6 may phosphorylate S120 of SLAC1, which is important for channel activity although the S120D mutation, which introduces a negative charge to mimic phosphorylation, cannot make the channel constitutively active (Geiger et al. 2009; Lee et al. 2009). Another important residue of SLAC1 is S59, which CPK6 can phosphorylate (Brandt et al. 2012). The role of CPK will be summarized below. In guard cells, besides SLAC1, SLAC1 homologue (SLAH)3 also contributes to nitrate release. CPK21 can, but SnRK2.6 cannot, activate SLAH3 (Geiger et al. 2011). SnRK2.6 also activates another type (R-type) of anion channel, the QUICK ACTIVATING ANION CHANNEL (QUAC)1, which mediates the efflux of malate and sulphate (Imes et al. 2013).

The inward-rectifying potassium channel KAT1 is another target of SnRK2s. ABA-activated SnRK2.6 purified from T87 cells can phosphorylate the C-terminal region (T306 and T308) of KAT1. Point mutations at T306 to mimic phosphorylation reduce KAT1 activity (Sato et al. 2009). Thus, in the presence of ABA, activated SnRK2s phosphorylate KAT1, resulting in less potassium influx, which contributes to keep stomata open in the absence of ABA. As another potassium regulator, the K^+ uptake transporter KUP6 is also phosphorylated by SnRK2.6 (Osakabe et al. 2013). When KUP6, KUP8, and the outward-rectifying K^+ channel GORK are mutated, ABA-mediated stomatal closing is disrupted (Osakabe et al. 2013).

SnRK2.6 also phosphorylates NADPH oxidase. Recombinant SnRK2.6 purified from *E. coli* phosphorylates the N-terminal domain of *A. thaliana* respiratory burst oxidase homologue F (AtrbohF, Sirichandra et al. 2009). This phosphorylation may explain the results that SnRK2.6 acts upstream of reactive oxygen species in the ABA response of guard cells (Mustilli et al. 2002).

Components Other than the SnRK2 Pathway

CPK/CDPKs, such as CPK3, CPK6, CPK21, and CPK23, are another possible kinase family regulating stomatal opening by ABA (Mori et al. 2006) through SLAC1 (Geiger et al. 2010). Their roles in vivo, however, are complicated. Even

though CPK23 can activate SLAC1 when they are heterologously expressed in *Xenopus* oocytes (Geiger et al. 2010), a *cpk23* mutant is more sensitive to ABA (Ma and Wu 2007) or is indistinguishable from the wild type (Merilo et al. 2013) in terms of stomatal aperture. Besides, a *cpk4/11* double mutant is less sensitive to ABA (Zhu et al. 2007), whereas CPK12, which is located close to CPK4 and 11 on the phylogenetic tree (Hrabak et al. 2003), negatively regulates the ABA pathway (Zhao et al. 2011). Moreover, the ABA response is impaired in a *cpk10* mutant (Zou et al. 2010) and CPK32 overexpression enhances the ABA response (Choi et al. 2005). ABA-induced stomatal closure, however, was not dramatically impaired in *cpk10*, *cpk4cpk11*, and *cpk32cpk7cpk8* (Hubbard et al. 2012). The relationship between the phosphorylation by SnRK2s and the phosphorylation by CDPKs is unclear. If they work redundantly in the ABA pathway, cell status in terms of Ca^{2+} concentration changes the strength of the signals since CDPK needs Ca^{2+} for its activation. It is also possible that CDPKs mainly work independently of ABA. If ABA activates CDPK through an increase of Ca^{2+} concentration, the signalling between the ABA perception and an increase of Ca^{2+} will be clarified without CDPKs themselves.

Clade A of PP2C may work several points other than dephosphorylation of SnRK2s. ABI1 directly dephosphorylates SLAC1 (Brandt et al. 2012). This can interpret the fact that ABA-mediated regulation of SLAC1 can be reconstituted in *Xenopus* oocytes with CDPKs instead of SnRK2s (Geiger et al. 2010; Brandt et al. 2012). ABI1 also directly interacts with and dephosphorylates ABFs (Antoni et al. 2012; Lynch et al. 2012). Thus, without ABA, the PP2Cs stop the signals in multiple ways. To stop the signal on ABFs, PROTEIN PHOSPHATASE 6 (PP6) can also dephosphorylate ABI5 (Dai et al. 2013).

Besides the PYR/PYL-PP2C-SnRK2 pathway, huge numbers of proteins are identified to be involved in the ABA responses. Since it is impossible to cite everything, just some examples are given below. Some proteins are reported as ABA receptors. The H subunit of Mg-chelatase (CHLH) specifically binds ABA and mediates ABA signalling as a positive regulator in seed germination, post-germination growth, and stomatal movement in *Arabidopsis* (Shen et al. 2006; Wu et al. 2009). CHLH can alter the localization of WRKY transcription factors, which bind to the promoters of ABA-responsive genes (Shang et al. 2010). Other groups, however, reported controversial results (Müller and Hansson 2009; Tsuzuki et al. 2011). GPCR-type G proteins (GTG) 1 and 2 specifically bind ABA and function as a class of membrane-localized ABA receptors (Pandey et al. 2009). A structural study with and without ABA is needed to reveal the regulating mechanisms directly downstream of these receptors.

Moreover, many proteins, such as mitogen-activated protein kinases (MAPKs) (Jammes et al. 2009) and E3 ubiquitin ligase RING-H2 FINGER A2 (RHA2)a (Bu et al. 2009), have also been reported to work in the ABA responses. SIZ1-mediated sumoylation of MYB30 is also involved in the ABA pathway (Zheng et al. 2012). The relationships of these proteins to the PYR/PYL-PP2C-SnRK2 pathway should be clarified in the future.

Biotechnological Manipulation of ABA Homeostasis and Signalling in Agriculture

The ABA synthesis pathway and the respective genes are highly conserved in angiosperms (Xiong and Zhu 2003). The mechanism of ABA perception might be broadly conserved, because the core components of the ABA-responsive pathway have been found in several crop plants such as tomato (Sun et al. 2011), strawberry (Chai et al. 2011), rice (Kim et al. 2012), and grape (Boneh et al. 2012).

The first target of manipulation of ABA signalling is dehydration tolerance. As well as stomatal regulation, growth rate is also modified by ABA under dehydration such as root elongation (Spollen et al. 2000). In addition to survival under drought, water usage under well-watered conditions can be reduced by enhancement of ABA signalling (Duan et al. 2007; Kim and van Iersel 2011). Several studies show that overexpression of ABA biosynthetic enzymes enhances drought tolerance. In model plants, overexpression of NCED3 increases stress tolerance under short-term stress (Iuchi et al. 2001). These may be mainly caused by lower transpiration rate. The yield of crop plants is also improved by the modification of ABA synthesis. A transgenic rice expressing ABA3/LOS5 is tolerant to drought, resulting in better yields (Xiao et al. 2009).

In addition to modification of the ABA synthesis pathway, the ABA-responsive pathways can also be improved. Moreover, modification of the responsive pathways more easily restricts the effects within a specific feature than does modification of the synthesis pathway. Overexpression of PYR/PYLs improves drought tolerance in *Arabidopsis* (Ma et al. 2009; Santiago et al. 2009a; Saavedra et al. 2010). Overexpression of SnRK2a also improves drought tolerance in *Arabidopsis* (Umezawa et al. 2004) and salt tolerance in rice (Diédhiou et al. 2008).

Alterations in regulators of the core components may also improve the tolerance. Transgenic canola expressing antisense RNA to *era1*, which is a negative modulator of the pathway (Allen et al. 2002), displayed an enhanced yield under a mild drought stress (Wang et al. 2005).

Thus, mediation of the ABA pathway is a good scheme for the improvement of crops. ABA responses, however, are not simple. ABA induces numerous responses in several aspects and the concentration of ABA is important under some conditions. For example, a higher concentration of ABA inhibits seedling growth, while a lower concentration (less than 1 μM) of ABA enhances it (Parcy et al. 1994).

Just overexpression of stress-related genes frequently has several effects other than the expected tolerance to stress. For example, high tolerance to stress frequently accompanies growth retardation. Since growth retardation itself may be a mechanism to confer tolerance to plants, it cannot be separated directly. In that case, stress-induced promoters are useful to restrict the expression under stress conditions (Kasuga et al. 2004). Since hypersensitivity to ABA confers not only tolerance to dehydration but also inhibition of seed germination and seedling growth, expression only under stress may be useful. A transgenic rice expressing ABA3/LOS5 under the dehydration-inducible HVA22 promoter is tolerant to drought, resulting in better yields (Xiao et al. 2009).

As another modification to restrict the effects to expected aspects, it is possible to use the specificity of receptors. The PYR/PYL family consists of 14 members. Some of their roles are overlapped redundantly, while some are specific (Park et al. 2009; Antoni et al. 2013). Since some ligands, such as pyrabactin (Park et al. 2009), do not activate all types of receptors, the ligands can activate some parts of the pathways that ABA activates. Such specificity has been used to distinguish the features of specific pathways in basic biology (Okamoto et al. 2013) and is expected to contribute to improving agriculture in the near future. In addition, recent biotechnology can invent totally new things that never exist in nature. A structural study revealed the molecular basis of interaction between ligands and residues of the receptors (Melcher et al. 2009; Miyazono et al. 2009; Nishimura et al. 2009; Santiago et al. 2009b; Yin et al. 2009). New ligands can be designed and synthesized based on the information at the molecular level. As well as ligands, receptors can be modified. Randomly induced mutations in receptors are generated by PCR with low-fidelity polymerases and are screened. The modified receptor driven by a tissue-specific promoter will enable the responses only in the expected tissue.

Besides responses to dehydration, modification of the ABA pathways on other aspects may also be beneficial. ABA is important for seed dormancy. While easy and quick germination is good for cultivating, unexpected germination, such as pre-harvest sprouting, causes substantial losses in seed yield and quality of cereal crops (Morris et al. 1989; Bewley 1997; Liu et al. 2013). Wheat mutants increasing ABA sensitivity show higher seed dormancy (Schramm et al. 2013). Temporally programmed manipulation or conditional induction of ABA sensitivity may provide controlled germination, making significant profits.

Even though ethylene is a well-known hormone at the stage of fruit ripening, ABA is also involved in fruit ripening such as in tomato (Mizrahi et al. 1975; Zhang et al. 2009), strawberry (Chai et al. 2011), and banana (Jiang et al. 2000). ABA may be upstream of ethylene or may be independent of ethylene (Giovannoni 2001). Since the timing of ripening is one of the critical factors for commercial success, this is another target of mediating ABA signalling. Not only fruit ripening but also leaf senescence is controlled by ABA. Generally, ABA facilitates senescence (Zeevaart and Creelman 1988). Interestingly, pyrabactin antagonizes the effect of ABA in senescence (Arrom and Munné-Bosch 2012), suggesting the complexity of ABA pathways and the possibility to separate one from another.

The roles of ABA in biotic stress are complicated (Ton et al. 2009). The closing stomata, which can be triggered by ABA, is a defence response at the first phase. At a later phase, however, ABA enhances susceptibility to several pathogens (Bari and Jones 2009). For that phase, reducing the ABA response may help plants to resist the pathogens. Interaction with other hormones is important in this aspect (Ton et al. 2009).

ABA also influences the circadian clock (Hanano et al. 2006). As well as carbon metabolism (Cardi et al. 2011), ABA is involved in the synthesis pathway of secondary metabolites, some of which are important for the quality of products, such as proanthocyanidins in persimmon (Akagi et al. 2012). Thus, there are massive possibilities that modification of the ABA pathways connects to benefits.

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Cytokinin Regulation of Plant Growth and Stress Responses

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Abstract Plant hormones cytokinins stimulate cell division; regulate shoot and root development; promote leaf growth and flower, fruit, and seed formation; stabilize photosynthetic machinery; suppress senescence; and enhance sink strength and nitrogen acquisition. Cytokinin signaling is mediated by multistep phosphorelay. Binding of the cytokinin molecule to CHASE domain of the histidine kinase receptors triggers an autophosphorylation of the histidine domain and subsequent intramolecular transfer to receiver domain. Phosphoryl group is then transmitted to histidine phosphotransfer proteins and subsequently to type B response regulators (transcription factors) in the nucleus. Phosphotransfer proteins interact also with transcription factors CRFs (cytokinin response factors) that represent signaling side branch. The signal strength is regulated by cytokinin metabolism, which controls levels of active cytokinins, through feedback inhibition of signal transduction via type A response regulators (primary response genes), S-nitrosylation of phosphotransfer proteins, and/or proteasome degradation of type B and type A response regulators. Practical applications of cytokinins include their use in in vitro micropropagation, stimulation of flower branching, crop tillering or berry formation, and prolongation of fruit or tuber shelf life. Targeted elevation of cytokinin levels was found to increase the tolerance of plants to abiotic stresses, at least partially by diminishing the negative stress effects on photosynthesis. Recently, function of cytokinins in biotic stress responses has been also recognized. Full utilization of cytokinin potential to improve plant productivity by regulation of plant development has been until now limited by the necessity of targeting modulation of their levels or signal transduction in a time- and tissue-specific manner.

Keywords Cytokinin • Receptor • Response regulator • Application • Stress response

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In plants, the cytokinins (CKs) were defined as substances stimulating cell division (cytokinesis) in tissue cultures (Miller et al. 1955). Apart of this effect, CKs exhibit a wide range of physiological functions, including regulation of shoot and root apical meristems, stimulation of branching, vascular development, chloroplast differentiation, stabilization of the structure and function of the photosynthetic machinery, delay of senescence, stomata opening, and elevation of the sink strength and nutritional signaling (Mok and Mok 2001; Spichal 2012; Chernyadev 2009; Kiba et al. 2011; Ruffel et al. 2011). Naturally occurring CKs are N^6-substituted adenine derivatives with either isoprenoid or aromatic side chain. Due to their predominance in plant tissues, attention has been focused mainly on isoprenoid CK research. The first identified natural CK, *trans*-zeatin (tZ), was named according to the species from which it was isolated (*Zea mays*, Letham 1963). Later on, other physiologically active CKs were identified: isopentenyladenine (iP), *cis*-zeatin (cZ), and dihydrozeatin (DHZ) as well as their ribosides (Sakakibara 2006). According to the activity in bioassays as well as the affinity to CK receptors, the most active CK is tZ, followed by iP (Spichal et al. 2004). Considerably less active cZ also widely occurs, being highly abundant in some species, especially in the monocots (Gajdosova et al. 2011). Apart from the species specificity, cZ seems to play a role in the stress responses (Dobra et al. 2010). DHZ was found predominantly in dormant seeds and apical buds, where it may serve as a source of active CKs before acceleration of de novo biosynthesis after germination (Frebort et al. 2011). CKs were detected not only in land plants but also in algae (Ordog et al. 2004), mosses (von Schwartzenberg et al. 2007), cyanobacteria, or symbiotic bacteria (Droog et al. 1997). Production of CKs by biotroph pathogens, e.g., *Agrobacterium tumefaciens* (Akiyoshi et al. 1984) or *Rhodococcus fascians* (Crespi et al. 1994), is part of their plant invading strategy. Detailed phylogenetic analysis of the occurrence of CKs and components of their signaling pathway was described by Spichal (2012).

Biosynthesis and Metabolism of CKs

The rate-limiting step of CK biosynthesis is transfer of isopentenyl moiety from dimethylallyl diphosphate (DMAPP) or its hydroxylated derivative (HMBDP) to adenosine 5′-phosphate (ATP and ADP in plants or AMP in bacteria). This reaction is catalyzed by isopentenyltransferase (IPT). In fact, CK biosynthetic genes were first characterized in *A. tumefaciens*. Plant *IPTs* were identified in *Arabidopsis thaliana* based on the homology with bacterial genes (Kakimoto 2001; Takei et al. 2001a). Plant IPTs strongly prefer ATP and ADP to AMP as well as DMAPP. The CK side chain may originate either from the plastid methylerythritol phosphate (MEP) pathway or from the cytoplasmic mevalonate (MEV) pathway. The prevalence of plastid pathway, in case of tZ and iP, was reported by Kasahara et al. (2004). The scheme of CK metabolism is shown in Fig. 1.

Some IPTs (AtIPT2 and AtIPT9) are tRNA-IPTs that prenylate adenine moieties adjacent to the 3′-end of the anticodon of specific tRNAs. This modification

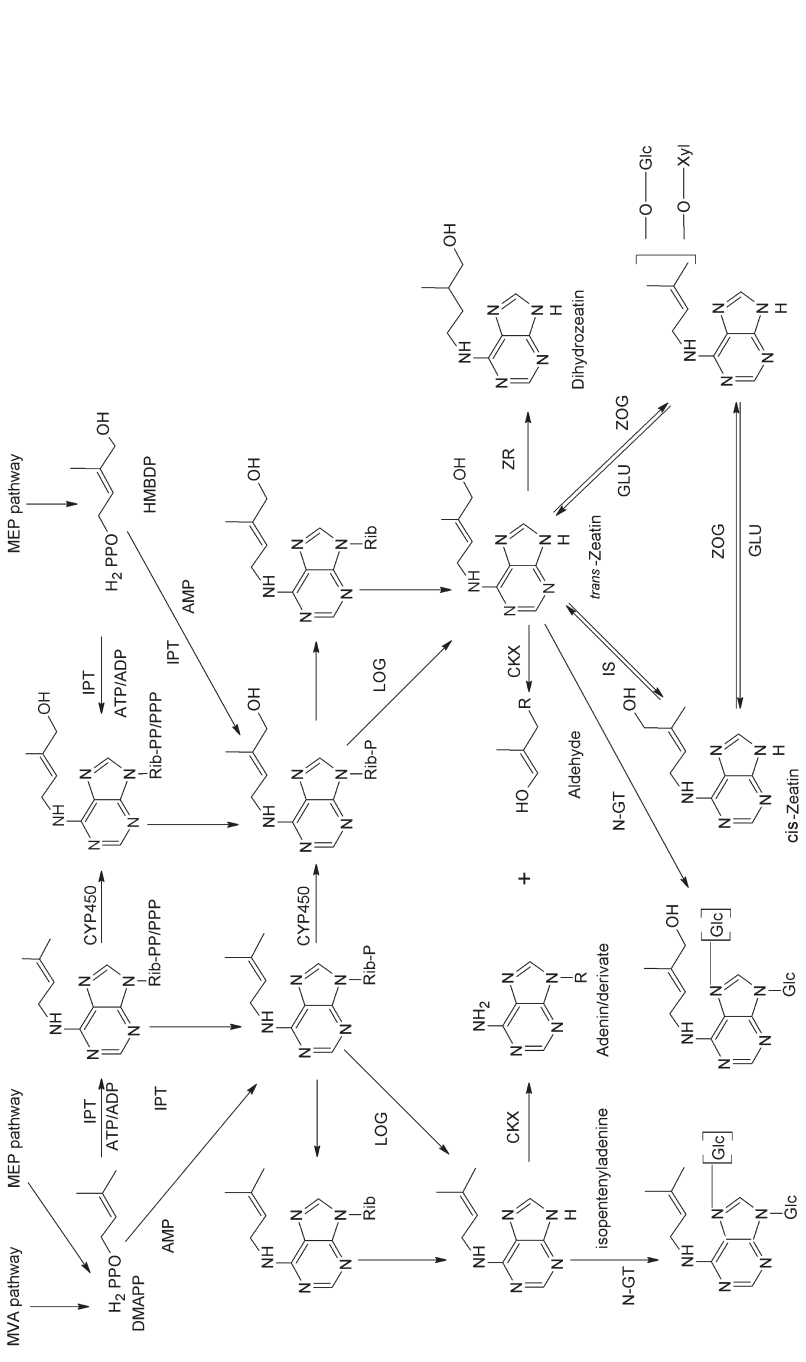

Fig. 1 The scheme of cytokinin metabolism. Donor of isoprenoid side chain can originate either from plastid methylerythritol phosphate (MEP) or from the cytoplasmic mevalonate (MEV) pathway. *IPT* isopentenyltransferase, *CYP450* cytochrome P450 monooxygenase, *LOG* cytokinin phosphoribohydrolase (LONELY GUY), *CKX* cytokinin oxidase/dehydrogenase, *ZOG* zeatin *O*-glucosyltransferase, *N-GT* cytokinin *N*-glucosyltransferase, *GLU* β-glucosidase, *IS* zeatin *cis-trans* isomerase, *ZR* zeatin reductase (adapted from Frebort et al. 2011, J Exp Bot 62: 2431–2452)

supports codon–anticodon interaction, as indicated by the disturbed interactions in bacterial tRNA mutants lacking CK moiety at A_{37} (Einset et al. 1976). Highly prevailing CK moiety in tRNAs is cZ. The tRNA degradation has been considered as an important source of cZ (Kamada-Nobusada and Sakakibara 2009).

In plants, the IPT-catalyzed reaction results in iP nucleotides that may be hydroxylated with cytochrome P450 monooxygenases (CYP735A1 and CYP735A2, Takei et al. 2004). As CK nucleotides are not active, gradual cleavage to nucleosides (i.e., ribosides) and bases by nucleotidase and nucleosidase was anticipated. Later on, CK-activating enzyme phosphoribohydrolase LOG (LONELY GUY), which converts all CK mononucleotides directly to the CK bases, was found (Kurakawa et al. 2007).

Due to the high physiological activity of CKs, the levels of the active forms need to be strictly regulated with respect to the plant developmental stage as well as environmental conditions. The key enzymes downregulating CK levels are cytokinin oxidases/dehydrogenases (CKX), which cleave the N^6 side chain. Oxidative cleavage of CKs was first described by Paces et al. (1971). Later on, the enzyme was identified as cytokinin oxidase (Whitty and Hall 1974). The structure of CKX was first reported by Houba-Hérin et al. (1999). Galuszka et al. (2001) reclassified the enzyme as a dehydrogenase possessing slight oxidase activity. The substrates for CKXs are CKs with unsaturated side chain (iP, tZ, and cZ), while DHZ, containing a saturated side chain, and CKs with aromatic ring at N^6 position are resistant to these enzymes.

Another important mechanism of suppression of CK activity is their glycosylation. Irreversible glucosylation occurs at the adenine ring in N^7 or N^9 position. CK *N*-glucosyltransferase was first isolated from radish (Entsch and Letham 1979). O-glucosylation (or much less frequent O-xylosylation) of the side chains containing a hydroxyl group or quite rarely observed N^3-glucosylation is reversible (Mok and Mok 2001). The enzymes *trans*-zeatin *O*-glucosyltransferase, *trans*-zeatin *O*-xylosyltransferase, and *cis*-zeatin *O*-glucosyltransferase were identified by Martin et al. (1999a, b, 2001, respectively). CK O- and N^3-glucosides can be hydrolyzed back by glucosidases (Brzobohaty et al. 1993). Very rare CK modification is the binding of L-alanine to N^9 position of purine ring reported in lupine (Entsch et al. 1983) or the formation of methylthioderivatives in *R. fascians* (Pertry et al. 2009). Recently, in vitro interaction between CKs and NO (or more precisely with the product of NO reaction with superoxide–peroxynitrite) was reported (Liu et al. 2013). The authors identified several nitro- and nitroso-*trans*-zeatin and iP derivatives and suggested that reaction of CKs with NO can control levels of reactive nitrogen species in vivo.

Signal Perception and Execution of CK-Induced Responses

The CK signaling pathway is mediated by the multistep phosphorelay, similar to a two-component system found in bacteria for perception of extracellular stimuli (Argueso et al. 2009).

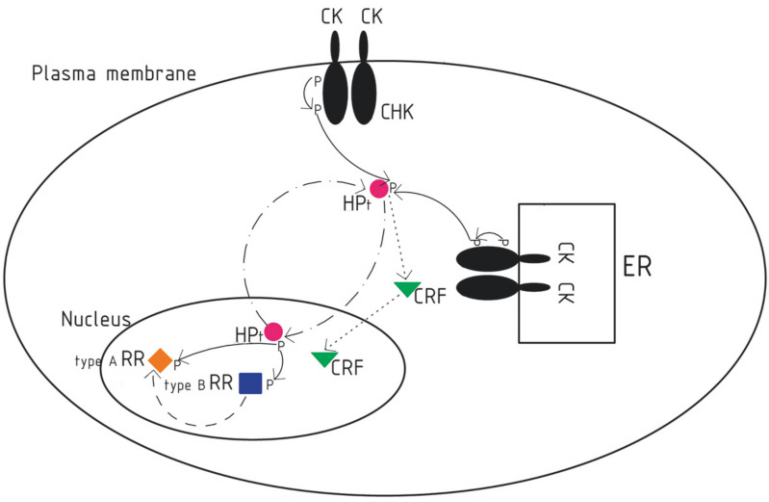

Fig. 2 The scheme of cytokinin signaling pathway mediated by phosphorelay. The binding of cytokinin molecule by a CHASE-domain histidine kinase receptor (CHK) at the endoplasmic reticulum or plasma membrane results in autophosphorylation of the histidine kinase domain and transfer of the phosphoryl group to the receiver domain. Phosphoryl group is then transferred to histidine phosphotransfer proteins (HPts) in the cytoplasm, and the phosphorylated HPts cycle between the cytoplasm and nucleus and pass the phosphoryl group to type B response regulators (type B RRs). The activated type B RRs stimulate transcription of cytokinin primary response genes, including those encoding type A response regulators (type A RRs), which negatively affect signal transduction, at least partially by competing with type B RRs for the phosphate transferred by HPts. The signal from HPts may also be transferred to other transcription factors, the cytokinin response factors (CRFs)

The scheme of cytokinin signaling pathway mediated by phosphorelay is shown in Fig. 2.

Cytokinin Receptors

CK molecule is bound by CHASE-domain-containing histidine kinase receptors (CHKs, Heyl et al. 2013). The receptors contain sensor CHASE domain, which serves for interaction with CKs (Heyl et al. 2007), histidine kinase (HK) domain, and receiver domain (West and Stock 2001; Ueguchi et al. 2001). The binding of CKs to CHASE domain triggers an autophosphorylation of the receptor and subsequent intramolecular transfer of phosphoryl group (Gruhn and Heyl 2013). The phosphoryl residue is transferred to histidine phosphotransfer proteins (HPts) and subsequently to type B response regulators (RRs) (Shi and Rashotte 2012). The first CK receptor was described in *Arabidopsis* as a two-component hybrid molecule that regulates vascular morphogenesis WOL (WOODEN LEG, Mahonen et al. 2000). This CK receptor kinase was named also as CRE1 (cytokinin response 1, Inoue et al. 2001) or AHK4 (*Arabidopsis* histidine kinase 4, Suzuki et al. 2001;

Yamada et al. 2001). Ueguchi et al. (2001) reported AHK4 together with two other CK receptors, the AHK2 and AHK3. The CK receptors exhibit partially overlapping activity, especially in roots, but play specific roles, given by their expression pattern and ligand specificity (Stolz et al. 2011). Recently, receptors were reported to be localized to the endoplasmic reticulum membrane (Caesar et al. 2011; Lomin et al. 2011; Wulfetange et al. 2011). However, their localization to plasma membrane can still be anticipated, too.

The ligand specificity of CK receptors is given by their CHASE domain, which was originally described as "Cyclase/Histidine kinase-Associated Sensing Extracellular" domain (Anantharaman and Aravind 2001; Mougel and Zhulin 2001). This full name was recently suggested to be changed to "Cyclase/Histidine kinase-Associated SEnsing" (Steklov et al. 2013). The crystal structure of the AHK4 CHASE domain was determined by Hothorn et al. (2011), who described also the AHK4 interactions with iP, tZ, benzyladenine, kinetin, and thidiazuron. The N-terminus of the AHK4 molecule folds into a long stalk α-helix, followed by the CHASE domain, which consists of two PAS-like (Per–Arnt–Sim-like) domains connected by a helical linker. The last β-strand of the membrane-proximal PAS domain (proximal to the C-terminus) is covalently linked to the N-terminus of the stalk helix by a disulfide bridge, which brings the flanking membrane helices into close proximity. The membrane-distal PAS domain forms binding cavity for CK molecule. In the lower part of the ligand-binding pocket, the central β-sheet of the PAS subdomain is lined by small hydrophobic residues, such as Ala and Gly. The hydrophobic upper part of the binding site is formed by two β-strands. The purine ring of CK molecule is oriented in the binding cavity by hydrogen bonds with Asp_{262} and Leu_{284}. Approximately 20 amino acid residues are in contact with tZ. Three water molecules in the cavity mediate the additional interactions, including the hydrogen bond between Thr_{294} and side-chain hydroxyl group of tZ, which is the reason for much higher affinity of AHK4 to tZ than cZ (Spichal et al. 2004).

Phylogenetic analysis of ca. 100 receptors (Steklov et al. 2013) indicated that CHASE domain (ca. 220 amino acids) together with adjacent domains (totally about 280 amino acid residues) is enclosed at both sides with transmembrane helices. These hydrophobic regions seem to play a role in the correct subcellular localization as well as in intramolecular signaling. One transmembrane region occurs between CHASE and downstream kinase domains. This means that CHASE domain and catalytic part of the protein are always located at different sides of the membrane. Specific mutations in this downstream transmembrane helix render receptors constitutively active regardless of the CK presence. The number of upstream transmembrane helices may vary among the receptor orthologous groups from 1 to 4. The CRE1/AHK4 orthologs possess only one upstream transmembrane region, whereas AHK2 orthologs have three or four transmembrane helices. In AHK3 orthologs, the number of upstream transmembrane regions may vary. Steklov et al. (2013) suggested that upstream transmembrane helices are predominantly responsible for receptor subcellular localization, while downstream helices are involved in the signal transduction. The conserved N-terminal helix α1, upstream of the CHASE domain, may fix the appropriate conformation of the distal PAS domain and may

regulate its movement upon CK binding. The substrate high-affinity binding results in specific conformational rearrangements of the PAS region in the sensory module. The signaling mechanisms of PAS domains were reviewed by Moglich et al. (2009). Signals originated within the conserved core generate structural and dynamic changes, which are propagated via amphipathic α-helical and coiled-coil linkers at the N- or C-termini of the core to the covalently attached effector domain. Many CK receptors were found to have a short coiled-coil motif that connects transmembrane helix with histidine kinase domain. Steklov et al. (2013) suggested that CK binding can affect the mode of interaction between ligand-binding PAS subdomains in the receptor dimer(s). Such change in the interaction mode of PAS subdomains might induce a mutual rotation of sensory modules relative to each other. The twist of sensory modules can in turn change the mutual position of transmembrane helices and cytoplasmic parts of receptors in dimer. As the histidine phosphorylation obviously occurs in *trans* by the parallel receptor, the change in relative position of receptors in dimer can switch on or off their kinase activity. Thus, formation of CK receptor complex results in HK activation and autophosphorylation of the conserved histidine in the catalytic module (West and Stock 2001). The phosphoryl group is then transferred intramolecularly to the conserved aspartate in the receiver domain. The HK domains of all *Arabidopsis* receptors have conserved histidine residue and five consensus motifs (H, N, G1, F, and G2). The receiver domain has conserved aspartate residue and three regions containing the conserved D, D, and K amino acid residues (Ueguchi et al. 2001). The subsequent transfer of CK signal is based on His–Asp phosphorelay (Grefen and Harter 2004; Muller and Sheen 2007; To and Kieber 2008; Schaller et al. 2011; Gupta and Rashotte 2012).

When ligand specificity of CK receptors was tested, AHK4 showed very high preference for tZ, followed by iP, and very low to cZ (Spichal et al. 2004). AHK3 showed only slight preference for tZ in comparison with tZR, iP, cZ, and DHZ. AHK2 has similar ligand specificity as AHK4 (Stolz et al. 2011). CK receptors differ also in their localization. *AHK4* is expressed predominantly in roots, especially in vascular cylinder and pericycle of primary roots. *AHK3* is expressed in rosette leaves, roots, stems, and flowers (Ueguchi et al. 2001; Higuchi et al. 2004). *AHK2* is expressed in leaves, roots, and flowers (Ueguchi et al. 2001). In accordance with their expression pattern, CK receptors were reported to be involved in the regulation of root vascular morphogenesis (Mahonen et al. 2000) and shoot vascular development (Hejatko et al. 2009), control of root meristem (Dello Ioio et al. 2007) and shoot apical meristem size and activity (Higuchi et al. 2004; Skylar et al. 2010), as well as retardation of leaf senescence (Kim et al. 2006) and abiotic stress responses (Tran et al. 2007; Jeon et al. 2010).

Since its identification, AHK4 role in regulation of root vascular development has been recognized. AHK4 is the main CK receptor involved in the control of root vascular tissues (Mahonen et al. 2000, 2006b). AHK2 and AHK3, together with CKI1 (CYTOKININ-INDEPENDENT1) HK, are important regulators of shoot vascular tissue development. Their mutation results in defects in procambium proliferation and absence of secondary growth (Hejatko et al. 2009). The size of the root meristem was found to be negatively affected by AHK3 signal transduction

(Dello Ioio et al. 2007). AHK3, but not the other CK receptors, plays a major role in CK-mediated chlorophyll retention and leaf longevity (Riefler et al. 2006; Kim et al. 2006). Homologues of AHK4 were reported to be indispensable for root nodulation in *Medicago truncatula* (Gonzalez-Rizzo et al. 2006) and *Lotus japonicus* (Tirichine et al. 2007). Cold-induced expression of a subset of type A *Arabidopsis* RR (*ARR*) genes, including *ARR5*, *ARR6*, *ARR7*, and *ARR15*, was shown to be mediated by the receptors AHK2 and AHK3 (Jeon et al. 2010).

It is interesting that AHK4 may function in the absence of CKs as a phosphatase, which dephosphorylates HPts and further suppresses CK signaling (Mahonen et al. 2006b).

Cytokinin Phosphotransfer Proteins

The components of CK signaling cascade downstream of receptors are the HPts that function as intermediate proteins to transfer the phosphoryl group from hybrid kinase receptors to downstream RRs (West and Stock 2001). In *Arabidopsis*, there are five authentic HPts (AHP1–5), which carry the conserved phospho-accepting His residue (Heyl and Schmulling 2003; Hutchison et al. 2006), and a pseudo-HPt (AHP6), which does not contain the conserved His residue necessary for phosphotransfer activity (Suzuki et al. 2000; Mahonen et al. 2006a). The AHPs have approximately 150 amino acids (Suzuki et al. 2000), except AHP4, which may occur in longer (145 aa) and shorter (127 aa) versions, and AHP5, which exhibits alternative splicing (Hradilova and Brzobohaty 2007). The authentic AHPs are positive regulators of CK signal transduction, which function to transfer phosphoryl group, obtained from AHKs, from the cytoplasm into the nucleus. Their continuous shuttling between the cytoplasm and nucleus was reported to be independent of their phosphorylation status (Punwani et al. 2010). The function of AHP4 is not clear; AHP4 had a slight positive effect in hypocotyl elongation assay, while in lateral root (LR) formation assay it acted as a negative regulator of CK response (Hutchison et al. 2006). However, it needs to be taken into account that *AHP4* transcription levels are very low in most tissues. AHP4 may play a role in specific developmental processes (e.g., anther endothecium formation, Jung et al. 2008). The pseudo-HPt AHP6 is a negative regulator of CK signaling. Its transcription is downregulated by CKs (Mahonen et al. 2006a). Recently, S-nitrosylation of AHP1 by NO at Cys_{115} was reported, which suppressed AHP1 phosphorylation and subsequent transfer of phosphoryl group to ARR1 (Feng et al. 2013). This finding indicates an important mechanism for regulation of CK-induced phosphorelay activity in plants.

The crystal structure of one HPt protein from maize, the ZmHP2, was determined several years ago (Sugawara et al. 2005). ZmHP2 contains four C-terminal helices that form an antiparallel bundle connected to two N-terminal helices by a β-turn. The phospho-accepting residue is His_{80}. The conserved residues surrounding His_{80} possibly act as a docking interface for receiver domains, while the

non-conserved residues seem to be responsible for specific activities of different HPt proteins. More recently, the crystal structure of MtHPt1, an HPt from *Medicago truncatula* (MtHPt1), was reported. The MtHPt1, with His_{79} as its phosphorylation site, consists of six α-helices, four of which form a C-terminal helix bundle. The coiled-coil structure of the bundle is stabilized by a network of S-aromatic interactions involving highly conserved sulfur-containing residues (Ruszkowski et al. 2013).

The *Arabidopsis AHP1* is expressed mainly in the roots; *AHP2*, *AHP3*, and *AHP5* transcripts are widely spread in plants (in roots, stems, leaves, flowers, and siliques). The highest *AHP2* expression is in roots and flowers, while *AHP3* is predominantly expressed in roots and leaves (Suzuki et al. 1998; Hradilova and Brzobohaty 2007). The *Arabidopsis* AHPs, especially AHP2, AHP3, and AHP5, were found to be negative regulators of the drought response (Nishiyama et al. 2013). The loss-of-function mutants of these three *AHP* genes exhibited strong drought tolerance, improved cell membrane integrity under stress conditions, and increased sensitivity to abscisic acid.

AHP6 is expressed in developing protoxylem and pericycle cells, shoot apices, and young leaves (Mahonen et al. 2006a). It promotes protoxylem formation by counteracting CK signaling (Mahonen et al. 2006a). AHP6 also functions as a CK repressor during early stages of lateral root (LR) development. AHP6 is expressed at different developmental stages during LR formation. It is required for the correct orientation of cell divisions at the onset of LR development. Recently, AHP6 was found to influence localization of the auxin efflux carrier PIN1 that is necessary for patterning the LR primordia (Moreira et al. 2013).

Cytokinin Response Regulators

As mentioned above, the HPts transport the phosphate signal, received from receptor AHKs, from the cytoplasm to the nucleus, and transfer the phosphoryl groups to response regulators (RRs) (Gupta and Rashotte 2012). In *Arabidopsis*, there are two main classes of RRs, type A and type B ARRs. Type B ARRs are transcription factors (Sakai et al. 2000) which upon phosphorylation of a conserved Asp residue activate transcription of CK response genes (including type A ARRs). The type B ARRs possess an N-terminal phospho-accepting receiver domain and a C-terminal output domain containing a GARP (GOLGI-ASSOCIATED RETROGRADE PROTEIN) family Myb-like DNA-binding and transactivating region. In addition, there is a conserved nuclear targeting sequence located in the Myb-like/B motif of the type B RRs (Imamura et al. 2001; Hosoda et al. 2002). Three subfamilies of type B ARRs may be distinguished. Subfamily I includes ARR1, ARR2, ARR10, ARR11, ARR12, ARR14, and ARR18. This subfamily is the most important in mediation of CK responses (Hwang and Sheen 2001; Sakai et al. 2001; Argyros et al. 2008). Subfamily II consists of ARR13 and ARR21, and subfamily III of ARR19 and ARR20. *ARR1*, *ARR2*, *ARR10*, and *ARR12* are expressed in young leaves. Their expression is restricted to the vascular tissues and hydathodes during

the leaf maturation. *ARR1*, *ARR2*, *ARR10*, *ARR11*, and *ARR12* are expressed in the roots, especially in root apical meristem and elongation zone (Birnbaum et al. 2003; Imamura et al. 2003; Mason et al. 2004; Tajima et al. 2004). The *ARR1* is expressed at similar level throughout the stele, endodermis, cortex, and epidermis, but *ARR10* is expressed at higher level in epidermis than in the other tissues (Mason et al. 2004; Birnbaum et al. 2003; Argyros et al. 2008).

The role of type B ARRs as positive regulators of CK signaling was demonstrated using *ARR2*-overexpressing plants which proved to be able to stimulate cell proliferation and shoot formation in the absence of exogenous CKs (Hwang and Sheen 2001). The fact that *ARR1*, *ARR2*, *ARR10*, and *ARR12* (but not *ARR11*, *ARR14*, *ARR18*, *ARR13*, *ARR19*, and *ARR20*) were able to complement *arr1arr12* mutant indicates functional diversities among the type B ARRs (Hill et al. 2013). The role of ARR1 and ARR12 in the control of cell division in shoot apical meristem seems to be mediated by transcriptional control of SHY2 (SHORT HYPOCOTYL 2), a suppressor of the auxin response (Dello Ioio et al. 2008). ARR2 was found to be activated downstream of AHK3 in the delay of leaf senescence (Kim et al. 2006). ARR1 and ARR12 were reported to suppress the expression of *AtHKT1;1* (*Arabidopsis thaliana* high-affinity K^+ transporter 1;1) that functions to remove sodium ions from the root xylem (Mason et al. 2010). ARR1 and ARR12 were thus suggested to delay the response to salinity stress.

The effects of the individual type B RRs on meristem size are generally consistent with their absolute transcript abundance, as well as with temporal changes in the expression (Hill et al. 2013). However, the ability of the type B RRs to stimulate transcription of CK response genes may be affected not only by the affinity or specificity to the target but also by potential interactions with HPts or transcriptional coregulators (Dortay et al. 2006; Kim et al. 2006). Promoter deletion analysis of the primary CK response gene *ARR6* showed that a combination of two extended motifs within the promoter is required to mediate the full transcriptional activation by ARR1 and other type B ARRs. The identification of a novel enhancer, which is not bound by the DNA-binding domain of ARR1, indicates that apart from type B RRs additional proteins might be involved in mediating the transcriptional CK response (Ramireddy et al. 2013).

The function of the type B RRs may be also affected by the protein stability (Kim et al. 2012). Recently, specific degradation of type B ARRs upon binding to a family of F-box proteins KMD (KISS ME DEADLY) was reported (Kim et al. 2013). KMD proteins form an S-PHASE KINASE-ASSOCIATED PROTEIN1 (SKP1)/cullin/F-box protein (SCF) E3 ubiquitin ligase complex and directly interact with type B ARR proteins. The *KMD* family members are broadly expressed, predominantly in shoot apical meristem (especially *KMD1* and *KMD2*) and in root tip (especially *KMD2* and *KMD3*) (Kim et al. 2006). They are localized both in the nucleus and in the cytoplasm. KMD proteins interact with ARR1, ARR12, and ARR20, less with ARR2 and ARR10. ARR1 and ARR12 were found unstable, readily to be degraded by proteasome, independently of CK presence. In contrast, degradation of ARR2 by proteasome requires CK-induced phosphorylation (Kim et al. 2012). KMD proteins seem to be key players of an important mechanism that is

responsible for reducing the levels of activated type B RRs, thereby preventing continued transcriptional activation by CKs (Kim et al. 2013). The representative members of type A ARRs, ARR4 and ARR7, were found not to be the substrates of KMD proteins.

Plant hormones regulate most physiological processes in an intensive cross talk. ARR2 seems to represent a link between CK and ethylene signaling pathways (Hass et al. 2004). ARR2 also makes a complex with TGA3 (TGACG-motif-binding transcription factor 3), a salicylic acid response factor. Salicylic acid signaling via NPR1 (NON-EXPRESSOR OF PATHOGENESIS-RELATED GENE1) enhanced binding of ARR2/TGA3 to the PR1 (PATHOGENESIS-RELATED PROTEIN1) promoter. CKs were thus found to promote resistance against *Pseudomonas syringae* in *Arabidopsis* (Choi et al. 2010).

Type B ARRs stimulate the expression of the type A *ARR*s (Hwang and Sheen 2001; Sakai et al. 2001), which are negative regulators of CK signaling and represent a negative feedback loop (CK signal switch-off). In *Arabidopsis*, ten type A ARRs were identified: ARR3, ARR4, ARR5, ARR6, ARR7, ARR8, ARR9, ARR15, ARR16, and ARR17 (To et al. 2004). These ARRs contain a phospho-accepting receiver domain, but no DNA-binding domain as do the type B ARRs. Phosphorylation of type A ARRs, for example, ARR5 and ARR7, at an aspartate of the phosphate receiver domain is a necessary prerequisite of their action as negative regulators (Lee et al. 2007; To et al. 2007). The mode of action of type A RRs seems to include competitive binding of the phosphoryl group from HPts at the expense of type B RRs. ARR5, ARR6, ARR7, and ARR15 were detected only in the nucleus; ARR4 and ARR16 were found both in the cytoplasm and in the nucleus (Hwang and Sheen 2001; Imamura et al. 2001).

Expression of several type A *ARR*s is rapidly induced by CKs, even after the inhibition of de novo protein synthesis (Brandstatter and Kieber 1998; Sakakibara et al. 1999; D'Agostino et al. 2000), suggesting that type A *ARRs* are CK primary response genes. To et al. (2007) specified a subset of type A ARRs stabilized by CKs, in part via phosphorylation (ARR5, ARR6, and ARR7), while ARR4 and ARR9 were not stabilized. The function of CKs as well as of proteasome in regulation of type A RR stability was studied by Ren et al. (2009). They found regulatory effect of CKs in case of ARR5, ARR6, ARR7, ARR8, ARR15, ARR16, and ARR17. Proteasome affected stability of ARR3, ARR5, ARR7, ARR8, ARR15, ARR16, and ARR17 (Ren et al. 2009).

Comparative analysis of *Arabidopsis* plants over-expressing individual members of type A *ARR*s showed their differential roles (Ren et al. 2009). The inhibitory effect of CKs on the primary root elongation was suppressed predominantly by *ARR3* and *ARR5* over-expression, followed by that of *ARR4*, *ARR16*, and *ARR17*. Inhibition of lateral root initiation was affected by most type A RRs, with the exception of ARR4, ARR5, and ARR7. Most type A RRs speeded up flowering, while only ARR16 was active in regulation of dark-induced leaf senescence. The strongest inhibition of CK-induced shoot formation was exhibited by ARR3, ARR5, ARR6, ARR16, and ARR17 (Ren et al. 2009). The expression of *ARR5*, *ARR6*, *ARR7*, and *ARR15* is repressed by transcription factor WUS (WUSCHEL,

"wuscheligen habitus"), in order to maintain optimal CK levels in shoot apical meristem (Leibfried et al. 2005). In root apical meristem, *ARR7* and *ARR15* transcription is positively regulated by auxin to maintain a balance between auxin and CK levels (Muller and Sheen 2008). A subset of type A *ARR*s, especially *ARR5*, *ARR6*, *ARR7*, and *ARR15*, are induced by cold (Jeon et al. 2010). Upregulation of type A *RR*s at the early phase of cold stress response is in accordance with transient downregulation of the active CK levels observed in winter wheat after exposure to cold (Kosova et al. 2012). ARR4 was found to represent a link between CKs and light signaling, interacting with phytochrome B (Fankhauser 2002; Sweere et al. 2001). ARR3 and ARR4 are involved in regulation of circadian rhythms (Salome et al. 2006).

Similar to type A RRs are the type C RRs, which are sometimes included into the type A RR group. Type C RRs have also only receiver domain. This domain is more related to the receiver domain of the hybrid histidine kinase receptors (Kiba et al. 2004; To and Kieber 2008). Additionally, type C RRs are not induced by CKs. This ARR group includes ARR22 and ARR24 (Gattolin et al. 2006). *ARR22* is expressed in flowers and developing pods, where it undergoes alternative splicing. Expression of *ARR24* was found restricted to pollen grains (Gattolin et al. 2006). Transcription of *ARR22* is induced by wounding, which may indicate a possible role of type C ARRs in response to biotic stresses (Gattolin et al. 2006). ARR22 interacts with AHP2, AHP3, and AHP5, acting as phosphohistidine phosphatase (Horak et al. 2008).

CRFs represent a side branch of CK signaling pathway. These proteins can interact directly with HPt proteins. The *Arabidopsis CRF*s are induced by CKs and belong to AP2-/ERF-like (APETALA2/ethylene-responsive factor) transcription factor family, distinct from type B RRs (GARP-/Myb-related family). CRFs share some targets with type B RRs but also activate some other genes (Rashotte et al. 2003, 2006). CRFs occur broadly in land plants (Rashotte and Goertzen 2010) and are involved in the normal development of embryos, cotyledons, and leaves (Rashotte et al. 2006).

Improvement of Plant Productivity with Biotechnological Manipulation of Cytokinin Biosynthesis and Signaling

The multiple physiological functions of CKs, which include regulation of germination, shoot and root development, leaf growth, flower and fruit formation, suppression of leaf senescence, enhancement of sink strength as well as uptake of nitrogen (Mok and Mok 2001), make this hormone class very perspective for practical applications. Unfortunately, effective regulation of particular physiological processes requires very precise time- and site-specific targeting of modulation of CK levels or signaling.

Until now, exogenous applications of CKs have been predominantly used in practice, to enhance shoot formation, branching, and tillering, to improve nitrogen acquisition, or to delay senescence (e.g., see Mala et al. 2013; Malabug et al. 2010; Gapper et al. 2005). Aromatic CKs are preferentially used, as these types of CKs are

not subjected to fast degradation by CKXs. N^6-(3-hydroxybenzyl)adenine (*meta*-topolin) became a very promising alternative for widely used N^6-benzyladenine (Strnad 1997). *meta*-Topoline has a high biological activity, which is in accordance with its relatively high affinity to AHK4 (Mok et al. 2005). As a good substrate of *trans*-zeatin *O*-glucosyltransferase (Mok et al. 2005), *meta*-topoline is metabolized to a storage *O*-glucoside, which can be gradually converted back to the active compound, in contrast to N^6-benzyladenine that is quickly *N*-glucosylated, resulting in a stable metabolite accumulated in basal parts of plants (Werbrouck et al. 1996). Recently, an alternative approach—suppression of degradation of endogenous CKs by inhibition of CKXs—has been tested (Zatloukal et al. 2008; Motte et al. 2013).

Since their discovery in 1955, CKs are routinely used in in vitro cultures for stimulation of shoot differentiation and propagation. Micropropagation techniques are used, for example, for cultivation of ornamental plants (orchids, chrysanthemums or carnations, e.g., see Ferreira et al. 2006), for multiplication of elite clones of forest trees (pine, elm, poplar, eucalyptus, and teak, e.g., see Mala et al. 2013), or for propagation of potato (Baroja-Fernandez et al. 2002). Exogenous CKs are used in classical horticulture to increase branching and thus the amount stem cuttings and flowers (Kaminek et al. 1987). Exogenous CKs have been also used for prolongation of the flower vase life, e.g., of gerberas (Danaee et al. 2011). Benzyladenine together with gibberellin was shown to promote plant growth and yield in three strawberry cultivars (Momenpour et al. 2011). When synthetic CK CPPU [*N*-(2-chloro-4-pyridyl)-*N*-phenylurea] and gibberellic acid were applied to various grapevine varieties at the fruit setting stage, these hormones increased berry size in Perlette, Superior, and Thompson Seedless cultivars. Gibberellin was found to enhance cell expansion, while CKs to increase cell number and density (Ben-Arie et al. 1997). CPPU was also found to increase berry mass and firmness, as well as cluster mass and compactness in *Vitis labrusca* and *V. labrusca* × *V. vinifera* in field trials (Zabadal and Bukovac 2006). Souza et al. (2010) reported positive effect of benzyladenine application on the quality of clusters of cv. Superior Seedless grapes. CKs were tested to increase a wheat grain yield by promotion of tillering. Their effect was significantly positive when the original plant density was low. In case of high plant density, the amount of seeds was also increased, but their size was reduced; thus, the yield was not enhanced.

CK application may also allow reduction of nitrogen fertilization, as CKs promote nitrogen acquisition (Takei et al. 2001b; Sykorova et al. 2008; Kiba et al. 2011; Pavlikova et al. 2012). This may solve the problems associated with high nitrogen levels in the field soils and underground waters which often result from heavy fertilization used to maintain high grain yields.

Surprisingly, CKs have been commercially used also in "non-plant" areas. Their antiaging effects were proved also for human skin, as evidenced by the fact that several types of cosmetics contain CKs (e.g., Pyratine-6 antiaging cream). Some CK analogues were found to block cell cycle progression not only in plant cells (Vesely et al. 1994) but also in humans (Vermeulen et al. 2002). These CK analogues were successfully tested as anticancer agents (Casati et al. 2011; Molinsky et al. 2013).

When genetic approach to elevation of CK levels is applied, it is necessary to prevent too strong *IPT* over-expression. High CK levels cause morphological abnormalities and very high levels may induce cell apoptosis (Mlejnek and Prochazka 2002). This problem was solved by Gan and Amasino (1995), who expressed *IPT* gene under the control of senescence-inducible promoter (*SAG12*) in tobacco. Stimulation of senescence program in plants resulted in an enhanced activity of *SAG12* promoter, leading to an increase of CK biosynthesis. Increased CK levels in turn suppressed the promoter activity, which prevented their overproduction. Repetition of these cycles allowed prolongation of the plant life-span. The *SAG:IPT* construct was successfully used in *Lactuca sativa* to delay developmental and post-harvest leaf senescence in mature lettuce heads (McCabe et al. 2001). No significant effect of transformation on the head diameter or fresh weight of leaves or roots was observed. Postponed plant senescence, accompanied by the delay in the loss of photosynthetic activity, was observed in maize expressing *ipt* under the control of a senescence-enhanced maize promoter (Robson et al. 2004). The elevation of CK content by expression of *SAG:ipt* in cassava plants delayed substantially the post-harvest senescence of cassava tuberous roots (Zhang et al. 2010). Prolongation of the shelf life may be very important in some developing regions, where cassava represents substantial part of the diet. When promotion of photosynthetic activity and delay of senescence are desirable, stimulation of CK biosynthesis is advantageous (Gregersen et al. 2013). In some cases, however, delay of leaf senescence may interfere with the developmental program. The over-expression of *SAG:ipt* in wheat prolonged substantially the vegetative period, increasing the sink strength of leaves, which interfered with grain filling (Sykorova et al. 2008). Due to the smaller seed size, no yield improvement was observed, in spite of their increased number. Apart from the changed sink/source relations, increase in seed number may result in plant exhaustion that limits the seed growth. A similar situation was observed after stimulation of flower branching in chrysanthemum plants by over-expression of *ipt* under *LEACO1* promoter, which resulted in substantial increase in flower number, but their diameter was smaller than in wild type (Khodakovskaya et al. 2009).

Recent climate changes have strengthened the demands for crops with improved stress tolerance, as unfavorable environmental conditions, including various abiotic and biotic stresses, may cause more than 50 % loss of the crop yield, especially in developing countries. As the response to the stress conditions requires vast re-programming of the metabolism to reallocate the energy supplies from the developmental programs to fast and effective stimulation of defense pathways, down-regulation of CK levels, associated with low growth rate, was tested. CK deficiency achieved by over-expression of *CKX* or downregulation of CK biosynthesis was found to increase substantially drought tolerance (Werner et al. 2001; Mytinova et al. 2010; Nishiyama et al. 2011). The same phenomenon has been described with knock-out mutants of CK receptor (Tran et al. 2010) and of AHP-encoding genes (Nishiyama et al. 2013). Apart from drought, also salinity or heat stress tolerance was improved (Nishiyama et al. 2011; Mackova et al. 2013). Constitutive *CKX* expression promotes growth of the root system, a trait that positively correlates with tolerance to water deficit (Tuberosa et al. 2002). However, it has strong negative

effect on the shoot growth. This drawback can be avoided by utilization of root-specific promoters. Targeting of *CKX* expression only to roots (Werner et al. 2010) resulted in plants which maintained enhanced root system, but their shoot phenotype was similar to wild type. Their stress tolerance was lower in comparison with *35S:CKX* plants but still significantly higher than that of wild type (Mackova et al. 2013).

Interestingly, opposite strategy—elevation of CK levels by expression of CK biosynthetic gene (*IPT*)—also resulted in strong elevation of abiotic stress tolerance. In contrast to constitutive elevation of CK levels, which was associated with high sensitivity to drought (*pssu:ipt*, Synkova et al. 1999), *IPT* over-expression under the senescence- or stress-inducible promoters (*SAG12*, *SARK*, or *rd29A*) enhanced tolerance to drought (Rivero et al. 2007, 2009, 2010; Merewitz et al. 2010, 2012; Peleg et al. 2011; Qin et al. 2011; Qiu et al. 2012; Kuppu et al. 2013), heat (Xing et al. 2009), salinity (Ghanem et al. 2011), cold (Hu et al. 2005; Belintani et al. 2012), or flooding (Huynh et al. 2005). The underlying mechanism seems to be diminishing of the stress-induced suppression of photosynthetic activity and stabilization of photosynthetic machinery (Rivero et al. 2009), which improves the energy supply. Moreover, transcription of many stress-inducible genes could be stimulated by CKs (Hare et al. 1997). Recent reports indicate intensive cross talk of CKs with salicylic acid and jasmonic acid and potential positive effect of CKs in biotic stress responses (Choi et al. 2010; Synkova et al. 2006). CK functions in stress responses were recently reviewed by Argueso et al. (2009) or Ha et al. (2012).

The CK functions in regulation of plant development offer unique opportunities to target different processes using suitable promoters. The over-expression of *ipt* under cysteine protease promoter resulted in transgenic rice plants with early flowering and higher number of emerged panicles (Liu et al. 2010). Expression of *ipt* under the control of seed-specific lectin promoter in tobacco promoted cell division in the embryo, resulting in an increase in the number of plerome cell layers and cell number in cotyledons (Ma et al. 2008). Dry weight of seeds was higher and transgenic seedlings grew faster.

The alternative approach to over-expression of biosynthetic gene is silencing of the expression of deactivating gene (*CKX*). Bartrina et al. (2011) reported that *Arabidopsis ckx3ckx5* double mutant formed larger inflorescence and floral meristems. Cellular differentiation was also retarded in this mutant, leading to higher cell number and larger flowers. Silencing of *HvCKX1* expression in barley and wheat resulted in higher grain yield (Zalewski et al. 2010). As *HvCKX1* exhibits high activity in the regulatory aleurone layer of the seeds, the positive effect of downregulation of CKX activity seems to be based on the increase of CK concentration in this layer with positive effect on sink strength and starch accumulation during grain filling (Zalabak et al. 2013).

The perspectives of genetic engineering of CK metabolism for the improvement of agricultural traits of crop plants were discussed by Zalabak et al. (2013), who also provided a comprehensive list of transgenic plants with altered expression of CK-related genes and their traits. The abovementioned data indicate that modulation of CK metabolism and/or signaling may represent a promising strategy for

improvement of plant productivity, especially in combination with suitable tissue- and time-specific promoters that allow direct control of grain filling or stimulation of inflorescence meristems.

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Roles of Ethylene in Plant Growth and Responses to Stresses

Biao Ma, Hui Chen, Shou-Yi Chen, and Jin-Song Zhang

Abstract Ethylene regulates many aspects of plant growth and development and responses to multiple biotic and abiotic stresses. The regulatory mechanisms of ethylene have been extensively studied during the past two decades. Ethylene is synthesized via a simple linear pathway, in which ACC synthase and ACC oxidase function as key enzymes. Ethylene biosynthesis is tightly controlled in response to various internal and external signals. A linear signaling pathway has been established on the basis of characterization of triple response mutants in *Arabidopsis*. Ethylene signal is perceived by a family of membrane-bound receptors and is transmitted by CTR1 and EIN2 and is then amplified through EIN3 and ERF transcription cascades. Ethylene interacts with other phytohormones in most developmental process. Biotechnological manipulation of ethylene actions at the level of biosynthesis, perception, and signal transduction has been successfully achieved in a number of plant species, especially crops. This chapter summarizes the recent advances in ethylene biosynthesis and its regulation, ethylene signal transduction, regulatory roles of ethylene in plant development and abiotic stress responses, cross talk with other hormones, and biotechnological applications in agriculture.

Keywords Ethylene signaling • Abiotic stress • Plant growth • Development • Crop

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Ethylene Biosynthesis

Ethylene Biosynthetic Pathway

Ethylene is synthesized via a simple biochemical pathway which involves three enzymatic reactions (Fig. 1): (1) activation of methionine (Met) to *S*-adenosyl-L-methionine (SAM) by SAM synthetase, (2) conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), and (3) oxygenation of ACC to ethylene by ACC oxidase (ACO) (Bleecker and Kende 2000; Lin et al. 2009a). The first step is common among all organisms. About 80 % of the cellular Met is activated to SAM (Hesse et al. 2004). Apart from being a precursor of ethylene, SAM is involved in synthetic reactions of many metabolites such as polyamines, nicotianamine, biotin, and glycinebetaine. In addition, SAM also serves as the major methyl donor for methylation reactions that modify nucleic acids,

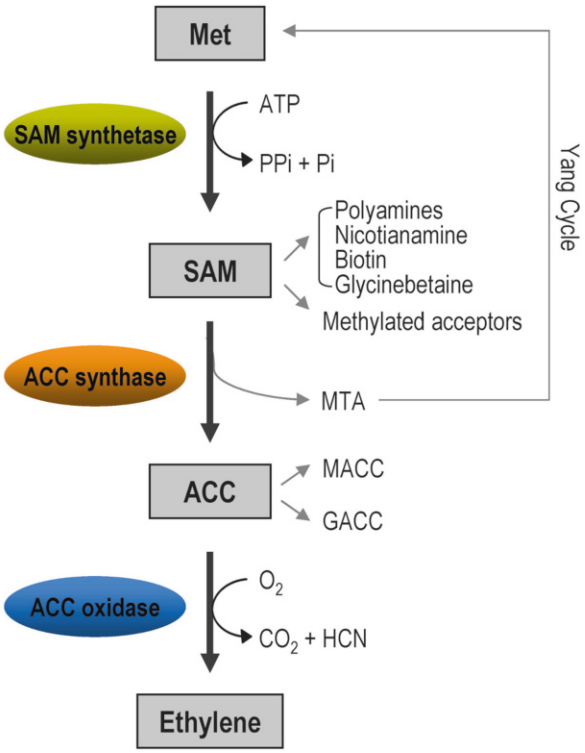

Fig. 1 Ethylene metabolic pathway. *Met* methionine, *SAM* *S*-adenosyl-L-methionine, *ACC* 1-amino-cyclopropane-1-carboxylic acid, *MTA* 5′-methylthioadenosine, *MACC* 1-(malonylamino) cyclopropane-1-carboxylic acid, *GACC* 1-(γ-L-glutamylamino)cyclopropane-1-carboxylic acid

proteins, and lipids. The second step, formation of ACC, is the major rate-limiting step of ethylene biosynthesis. ACS appears to be the prime targets for regulation of ethylene biosynthesis by a variety of signals. The 5′-methylthioadenosine (MTA), a by-product of this step, is recycled back to Met via the Yang cycle for another round of ethylene biosynthesis. In addition to be the precursor of ethylene, recent studies suggest that ACC might act directly as a signaling molecule to regulate plant development (Xu et al. 2008; Tsang et al. 2011; Tsuchisaka et al. 2009). In the final step, conversion of ACC to ethylene by ACO, a member of the oxygenase/oxidase superfamily, is oxygen dependent. When high levels of ethylene are produced at some situations such as fruit ripening, senescence, and wounding, ACO activity is also important for regulation of ethylene production (Alexander and Grierson 2002).

Ethylene Biosynthetic Genes

The key enzyme ACS is encoded by a multigene family in plants. There are nine authentic *ACS* genes in *Arabidopsis*. Each member displays distinct spatial and temporal expression pattern and is highly regulated by various internal and external signals (Peng et al. 2005; Tsuchisaka and Theologis 2004a). Biochemical analysis reveals that all the ACS isoforms are biochemically distinct (i.e., differences in their substrate affinities and k_{cat} values) (Yamagami et al. 2003). All the lines of evidence suggest divergent roles of the ACSs in plant growth and development. Lots of research has focused on the specific roles of individual *ACS* genes in response to developmental and environmental cues in various plant species. In tomato (*Lycopersicum esculentum*), for instance, *LeACS2* and *LeACS4* are found to be involved in fruit ripening (Barry et al. 1996, 2000). In rice (*Oryza sativa*), *OsACS1* and *OsACS5* are suggested to be responsible for the rapid elongation growth of submerged internode (Van Der Straeten et al. 2001; Zarembinski and Theologis 1997). The diversity of *ACS* gene family is further enhanced by heterodimerization among various ACS subunits. The nine ACS polypeptides of *Arabidopsis* can potentially form 45 homo- and heterodimers of which 25 are functional (Tsuchisaka and Theologis 2004b). A combinatorial interplay among different ACS subunits determines the relative ratio of active and inactive dimeric isozymes, which could contribute to the pleiotropic effects of ethylene by being able to operate in a broad gradient of SAM concentration in various tissues and cell types during plant growth and development (Tsuchisaka et al. 2009; Yamagami et al. 2003; Tsuchisaka and Theologis 2004b).

ACO is encoded by a small multigene family. The *Arabidopsis* genome encodes five *ACO* genes. In common with *ACS* genes, *ACO* display differential expression patterns during plant growth and development and in response to a wide range of developmental and environmental stimuli (reviewed in Dorling and McManus 2012). ACO proteins act as monomers which require ascorbate as a cofactor, but little is known about its biochemical diversity.

Regulation of Ethylene Biosynthesis

The levels of ethylene in different cell types and tissues are tightly regulated in response to developmental, hormonal, and environmental signals. Ethylene production during vegetative growth is maintained at basal level via feedback inhibition mechanisms, whereas plants produce high levels of ethylene via positive feedback regulation during specific developmental processes such as ripening and senescence or under stress conditions. Regulation of ethylene biosynthesis is mainly achieved through controlling the abundance of ACS and ACO enzymes at either transcriptional or protein levels.

Transcriptional Regulation

Transcriptional regulation of *ACS* and *ACO* genes by developmental, hormonal, and environmental signals has been extensively studied in various plant species. In tomato, identification of the ethylene biosynthetic genes involved in the transition from System I (auto-inhibition) to System II (autocatalytic) ethylene synthesis during fruit ripening gives a typical example for developmental regulation. It is found that *LeACS1A* and *LeACS6* are responsible for the production of basal ethylene (System I) in the pre-climacteric period, as the two genes are regulated by a negative feedback system (Barry et al. 2000). In contrast, the expression of *LeACS2*, *4* and *LeACO1*, *4* exhibits ripening-related increase and is upregulated through positive feedback by ethylene, which suggests that these genes are responsible for the production of climacteric (System II) ethylene (Barry et al. 2000; Alba et al. 2005). However, the nature of the developmental factors involved in this process is still largely unknown (Yokotani et al. 2009). In addition to developmental regulation, the expression of *ACS* and *ACO* genes is also regulated by various phytohormones such as auxin, gibberellic acid (GA), abscisic acid (ABA), brassinosteroid (BR), jasmonate, salicylate, and ethylene itself. For example, *AtACS2*, *4*, *5*, *6*, *7*, *8*, and *11* transcripts are induced by IAA in *Arabidopsis* roots (Tsuchisaka and Theologis 2004a; Wang et al. 2005). In etiolated rice seedlings, our results show that *OsACS2*, *6* and *OsACO3*, *5* are upregulated in the ABA-deficient mutants *mhz4* and *mhz5* (our unpublished data). As a stress hormone, ethylene is induced by various abiotic and biotic stresses through activating the transcription of a set of *ACS* and *ACO* genes (Wang et al. 2005). The stress conditions that have been extensively studied include drought, flooding, salt, chilling, ozone, wounding, hypoxia, and pathogen infection (Argueso et al. 2007).

For unraveling the regulatory mechanisms for *ACS* and *ACO* gene expression, a number of studies have been conducted to identify the *cis*-elements as well as the transcription factors. In the auxin-induced *Arabidopsis AtACS4* and melon (*Cucumis melo* L.) *CMe-ACS2* promoters, multiple auxin-responsive *cis*-elements have been identified based on motif alignment (Abel et al. 1995; Ishiki et al. 2000). For *ACO*

genes, lots of *cis*-elements responsive to a wide range of stimuli have been identified using *promoter*:*GUS* fusion strategy coupled with deletion analysis. The following are some examples: multiple ripening- and senescence-associated regions in tomato *LeACO1* promoter (Blume and Grierson 1997), two separate regions in melon *CmACO1* promoter in response to ethylene and wounding (Bouquin et al. 1997), ethylene-responsive elements in peach (*Prunus persica*) *PpACO1* promoter (Rasori et al. 2003), auxin and wounding responsive regions in loblolly pine (*Pinus taeda* L.) *PtACO1*, *2* promoters (Yuan and Dean 2010), and ethylene-related motifs in white clover (*Trifolium repens* L.) *TrACO2*, *3* promoters (Scott et al. 2010).

So far, four types of transcription factors responsible for transcriptional regulation of *ACS* and *ACO* genes have been identified. Tomato MADS-box transcription factor RIPENING INHIBITOR (RIN) activates the expression of *LeACS2*, *4* and *LeACO6* during fruit ripening via binding specifically to the CArG motif (Ito et al. 2008; Fujisawa et al. 2011, 2013). Tomato HD-zip homeobox protein LeHB-1 binds to the HD protein binding sequences in *LeACO1* promoter and activates the gene expression during floral organogenesis and ripening (Lin et al. 2008). Tomato ETHYLENE RESPONSE FACTOR2 (LeERF2) can specifically bind to the DRE/CRT element in *LeACO3* promoter and functions as a positive regulator in the feedback loop of ethylene induction (Zhang et al. 2009a). Similarly, banana (*Musa acuminata* AAA group, cv. Cavendish) MaERF11 can bind to the promoters of *MaACS1* and *MaACO1* to suppress their expression, whereas MaERF9 binds to *MaACO1* promoter and acts as a transcriptional activator (Xiao et al. 2013). Tomato Cys protease LeCP can directly bind to *LeACS2* promoter and activates the gene expression in response to fungi infection (Matarasso et al. 2005).

Posttranslational Regulation

Compared with transcriptional regulation, posttranslational regulation is a faster manner in response to rapid environmental changes (McClellan and Chang 2008). Many studies have demonstrated that the ACS proteins are subjected to posttranslational regulation via phosphorylation and proteasomal degradation. Based on the C-terminal sequences, ACS proteins can be divided into three groups: Type 1 ACS proteins (AtACS1, 2, and 6) contain three mitogen-activated protein kinase (MAPK) phosphorylation sites and one calcium-dependent protein kinase (CDPK) phosphorylation site. Type 2 ACS proteins (AtACS4, 5, 8, 9, and 11) contain a CDPK phosphorylation site and a Target Of ETO1 (TOE) domain that is required for interaction with ETO1 (ETHYLENE OVERPRODUCER 1) and ETO-like (EOL). Type 3 ACS (AtACS7) proteins have a truncated C-terminal that lacks the known motifs (Lyzenga and Stone 2012). Type 1 ACS proteins, such as AtACS2 and AtACS6, can be phosphorylated by MAPK6, which stabilize the proteins by blocking their proteasomal degradation (Liu and Zhang 2004). Dephosphorylation of type 1 ACSs by protein phosphatase 2A (PP2A) causes the protein to be unstable (Skottke et al. 2011). Type 2 ACS proteins, such as AtACS4, 5, and 9, are ubiquitinated by

CRL E3 ligases ETO1 and EOL1/2 and subjected to proteasomal degradation (Wang et al. 2004; Christians et al. 2009). Cytokinin or BR can stabilize the type 2 ACS proteins, yet the molecular mechanism remains to be elucidated (Hansen et al. 2009). Type 3 ACS protein AtACS7 is ubiquitinated and targeted for degradation by the RING-type E3 ligase XBAT32 (Lyzenga et al. 2012). Additionally, the phospho-specific binding protein 14-3-3 can interact with all three categories of ACS proteins and increases their stability (Yoon and Kieber 2013). For ACO, its protein turnover has not been reported so far.

Finally, in addition to the regulation of the key enzyme ACS and ACO, ethylene production can further be controlled by conjugation of ACC into biologically inactive forms. Malonylation of ACC to 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) by ACC-*N*-malonyltransferase decreases the level of active ACC in plants and thus reduces ethylene synthesis, and this process is reversible (Kionka and Amrhein 1984; Jiao et al. 1986). Another type of ACC conjugate is 1-(γ-L-glutamylamino)cyclopropane-1-carboxylic acid (GACC) catalyzed by γ-glutamyl transferase (Martin et al. 1995).

In summary, ethylene is synthesized via a well-characterized biochemical pathway, in which the two key enzymes, ACS and ACO, are both encoded by multigene families. Ethylene production is exquisitely controlled in response to various endogenous and environmental signals via regulation of the abundance of ACS and ACO enzymes at either transcriptional or posttranslational levels. Several corresponding transcription factors (LeRIN, LeHB-1, LeERF2, and LeCP), as well as MAPK6, PP2A, and E3 ligase (ETO1, EOL1/2, and XBAT32) involved in the regulation of ACS protein stability, have been identified. However, this is only the beginning for understanding of how plants regulate ethylene production; extensive studies are still needed to unravel the regulatory network in ethylene biosynthesis.

Signal Perception and Execution of Ethylene-Induced Responses

Ethylene Signal Transduction Pathway

A linear signaling pathway has been established on the basis of genetic analysis of *Arabidopsis* ethylene-responsive mutants (Fig. 2). Ethylene binds to a family of membrane-bound receptors that act as negative regulators in the signaling pathway. Binding of ethylene inactivates the receptors, resulting in the deactivation of downstream CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) that is a negative regulator of the pathway. ETHYLENE INSENSITIVE 2 (EIN2) acts downstream of CTR1 and positively regulates ethylene responses. The master transcription factor EIN3/EIL (EIN3-LIKE) acts downstream of EIN2 and directly activates expression of ERF transcription factors which in turn modulate the expression of various ethylene-responsive genes (Bleecker and Kende 2000).

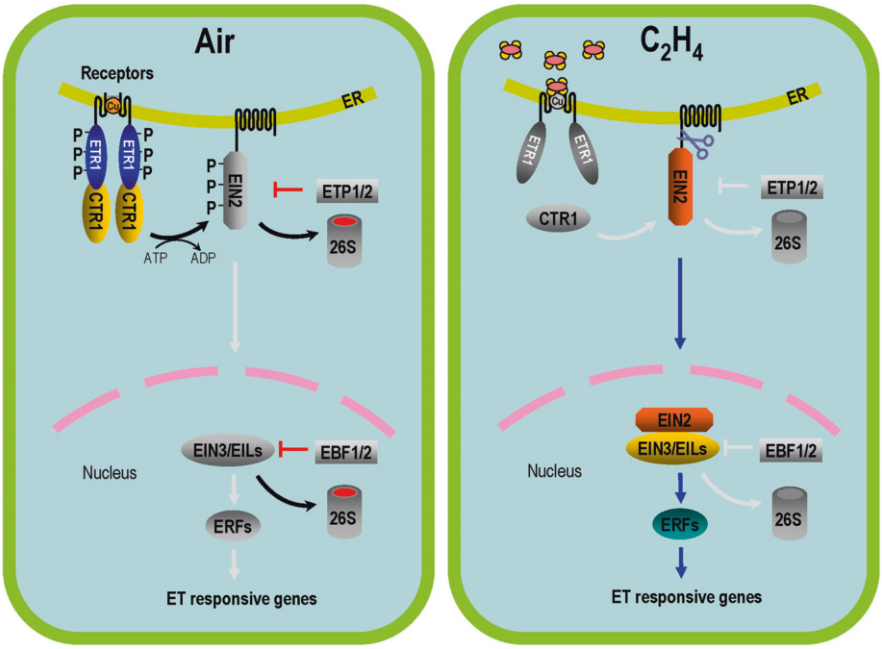

Fig. 2 A model for ethylene signaling. The ethylene receptors, CTR1 and EIN2 are all predominantly localized in the ER membranes. In the absence of ethylene (air), the highly phosphorylated ethylene receptors activate CTR1 kinase activity, which in turn phosphorylates EIN2, likely causing the degradation of EIN2 by F-box proteins EPT1 and EPT2. Meanwhile, EIN3/EILs are also subjected to proteasomal degradation mediated by F-box proteins EBF1 and EBF2. In the presence of ethylene (C_2H_4), ethylene binding inactivates the receptors by suppressing its phosphorylation, which consequently leads to deactivation of CTR1. The un-phosphorylated EIN2 is thus cleaved and its C-terminal domain is translocated into the nucleus, resulting in activation of EIN3/EILs and downstream transcriptional cascades. *ET* ethylene

Ethylene Receptor

Ethylene receptors are encoded by a small gene family. In *Arabidopsis* there are five members including ETHYLENE RESPONSE 1 (ETR1), ETHYLENE RESPONSE SENSOR 1(ERS1), ETR2, ERS2, and EIN4 (Chang et al. 1993; Hua et al. 1995, 1998; Sakai et al. 1998). The receptor proteins have similarity to bacterial two-component histidine (His) kinase receptor. Based on sequence similarity and protein structure, ethylene receptors are classified into two subfamilies (Bleecker et al. 1998). Subfamily I receptors (ETR1 and ERS1) have three transmembrane domains in the N-terminus containing the ethylene binding site and a GAF (cGMP phosphodiesterases/adenylyl cyclases/FhlA) domain in the middle portion that may mediate protein–protein interactions, followed by a His kinase domain with (ETR-type) or without (ERS-type) an attached receiver domain. Compared with subfamily I receptors, the subfamily II members (ETR2, ERS2, and EIN4) have an extra N-terminal

transmembrane domain that is predicted to be signal peptide and a diverged His kinase domain lacking some essential residues required for His kinase activity. The basic functional unit of ethylene receptors is disulfide-linked homodimer; meanwhile they can also form heterodimers and even higher-order clusters in planta (Schaller et al. 1995; Gao et al. 2008). High affinity of ethylene binding requires copper ion as a cofactor (Rodriguez et al. 1999), which is delivered by the copper transporter RESPONSIVE-TO-ANTAGONIST1 (RAN1) (Hirayama et al. 1999; Binder et al. 2010).

Ethylene receptors are integral membrane proteins predominantly localized in the endoplasmic reticulum (ER) (Chen et al. 2002; Grefen et al. 2007; Ma et al. 2006). The receptor proteins span the ER membrane three times with its N-terminus facing the luminal space and the large C-terminal portion lying on the cytosolic side as demonstrated using the melon CmERS1 receptor (Ma et al. 2006). Localization of ER resident proteins usually involves two mechanisms: static retention (keeping the proteins at a particular location within the ER) and dynamic retrieval (returning the proteins that have left the ER to the Golgi apparatus back to the ER). So far little is known about the ER localization mechanism for ethylene receptors. Noteworthy, the ETR1 receptor was observed at both the ER and Golgi apparatus in *Arabidopsis* root hair cells (Dong et al. 2008). This is indicative of a dynamic retrieval mechanism for the ER localization of ethylene receptors. Moreover, the copper transporter RAN1 has been found at the Golgi apparatus (Dunkley et al. 2006). Thus it is possible that the nascent receptor proteins might be sorted from the ER to the Golgi apparatus at where they accept the copper ions delivered by RNA1, and then are retrieved to the ER for ethylene perception (Ju and Chang 2012). Anyway, further characterization of the ER localization mechanism will provide more insights into biogenesis of ethylene receptors.

The subfamily I receptors have conserved His kinase domain. In vitro phosphorylation analyses have demonstrated that ETR1 and ERS1 receptors do possess canonical His autokinase activity (Gamble et al. 1998; Moussatche and Klee 2004). However, genetic studies revealed that the His kinase activity of ethylene receptors is not absolutely required for the receptor function, but can modulate signal output from the receptors possibly by affecting interactions with other signaling elements and/or phosphorylating other proteins to regulate their activity (Hall et al. 2012). As for the subfamily II receptors with diverged His kinase domain, we demonstrated that the tobacco (*Nicotiana tabacum*) NTHK1 and rice OsETR2 have serine/threonine (Ser/Thr) kinase activity (Xie et al. 2003; Wuriyanghan et al. 2009). The *Arabidopsis* subfamily II receptors ETR2, ERS2, and EIN4 are subsequently found to have Ser/Thr kinase activity (Moussatche and Klee 2004). The Ser/Thr kinase activity of NTHK1 plays a role in ethylene signaling and salt stress response when expressed in *Arabidopsis* (Chen et al. 2009). As in vivo evidence for ethylene receptor phosphorylation, phos-tag PAGE analysis using the native proteins has shown that tomato LeETR4 (subfamily II) and NR (subfamily I) receptors are multiple phosphorylated in planta and ethylene treatment can decrease the phosphorylation level, suggesting that the phosphorylation state of receptors is implicated in ethylene signal output in tomato fruits (Kamiyoshihara et al. 2012). At present, several lines

of evidence suggest that both the His and Ser/Thr kinase activity of ethylene receptors may play a role in modulating signal output from the receptors. However, more research is still required for confirming the exact function of ethylene receptor kinase activity.

On the membrane, ethylene receptors can physically interact with CTR1, EIN2, and REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1). The receptor–CTR1 interaction involves the kinase and receiver domains of receptors and the N-terminal regulatory domain of CTR1, through which ethylene receptors recruit the soluble protein CTR1 to the ER and regulate its activity (Clark et al. 1998; Zhong et al. 2008; Gao et al. 2003). On the other hand, CTR1 can also regulate the ETR1 N-terminal signaling by docking to the receptor (Xie et al. 2012). EIN2 interacts tightly with the kinase domain of ETR1, and the interaction is enhanced when the receptor kinase activity is blocked or upon ethylene binding (Bisson et al. 2009; Bisson and Groth 2010). The biological significance of receptor–EIN2 interaction is unclear. RTE1 is a novel membrane protein that specifically associates with ETR1 receptor (Resnick et al. 2006; Dong et al. 2010a). RTE1 activates ETR1 signaling possibly by promoting either ETR1 folding or stabilization of the ETR1 active conformation (Resnick et al. 2008). Ethylene receptors also interact with other proteins such as TRP1 and ECIP1 (Lin et al. 2009b; Lei et al. 2011). Interestingly, the MA3 domain-containing protein ECIP1 can interact with both ethylene receptors (ETR2 and EIN4) and EIN2 to affect ethylene response as well as salt stress response (Lei et al. 2011). The exact function of these interacting proteins is unclear. Collectively, increasing evidence establishes that ethylene receptors transmit ethylene signal to the downstream components via protein complexes. The molecular mechanisms involved in these processes remained to be elucidated. After ethylene perception, the ligand-bound receptors are subjected to proteasomal degradation, as demonstrated in *Arabidopsis* and tomato (Chen et al. 2007; Kevany et al. 2007).

CTR1

CTR1 is encoded by a single gene in *Arabidopsis* genome, whereas there are multiple *CTR*-like genes in other plant species such as tomato and rice (Adams-Phillips et al. 2004; Rzewuski and Sauter 2008). CTR1 is a key negative regulator of ethylene signaling. Without ethylene, CTR1 is activated by the receptors to inhibit downstream signaling components. Ethylene binding presumably causes a conformational change in the receptor–CTR1 complex, resulting in deactivation of CTR1 activity and thus releasing downstream components to initiate ethylene response. CTR1 protein consists of two distinct domains: the N-terminus is a putative regulatory domain that is responsible for association with ethylene receptors, while the C-terminus is a Raf-like Ser/Thr protein kinase domain. In vitro analysis and genetic study demonstrated that CTR1 has intrinsic Ser/Thr protein kinase activity with enzymatic properties similar to Raf-1, and the kinase activity is required for CTR1 function (Huang et al. 2003). Moreover, the physical association of CTR1 N-terminus with ethylene receptors is found to be crucial for CTR1 kinase activity

(Huang et al. 2003). Crystallographic analysis of CTR1 kinase domain shows that the active kinase domains form dimers, while inactive variants are monomers (Mayerhofer et al. 2012). These results, together, imply that the receptor–CTR1 association might facilitate CTR1 to form a dimer, an active isoform in the absence of ethylene. Recent study identified the authentic substrate of CTR1 kinase. CTR1 can interact with and directly phosphorylate the C-terminal domain of EIN2 in the absence of ethylene. Disruption of EIN2 phosphorylation sites results in constitutive activation of ethylene responses by a mechanism involving translocation of the EIN2 C-terminus to the nucleus (Ju et al. 2012).

Although CTR1 serves as a key regulator of ethylene response, a CTR1-independent pathway may exist in ethylene signaling, due to fact that *ctr1* null mutants still remain residual ethylene response and that the strong loss-of-function ethylene receptor mutants (e.g., *etr1-9ers1-3* double mutant) and the strong *ran1* mutant (*ran1-3*) display constitutive ethylene response phenotypes stronger than that of *ctr1* mutants (Huang et al. 2003; Qu et al. 2007; Woeste and Kieber 2000). Identification of the potential bypass pathway will help us to establish a complete ethylene signaling pathway.

EIN2

EIN2 is a central component of the ethylene signaling pathway. Loss-of-function mutations of *EIN2* lead to complete ethylene insensitivity in *Arabidopsis* (Alonso et al. 1999). EIN2-like proteins have been identified in other plant species. In rice, we recently identified *Osein2*/*mhz7* mutants by a genetic screen for ethylene insensitivity of etiolated rice seedlings (Ma et al. 2013). The rice ethylene response phenotype is different from that of triple response in *Arabidopsis*, namely ethylene inhibits rice root (both seminal and adventitious roots) growth but promotes the coleoptile elongation (Ma et al. 2010, 2013). The *Osein2*/*mhz7* mutants exhibit complete ethylene insensitivity in both root and coleoptile, and overexpression of *OsEIN2*/*MHZ7* confers constitutive and enhanced ethylene responses in the absence or presence of ethylene, respectively, suggesting that OsEIN2/MHZ7 is also an essential regulator of ethylene response in monocot plants. In addition, OsEIN2/MHZ7 also regulates yield-related traits and leaf senescence in rice.

EIN2 is encoded by a single gene in *Arabidopsis*. AtEIN2 is an integral membrane protein consisting of 12 predicted transmembrane domains at the N-terminus that has similarity to the mammalian Nramp metal transporters. However, no metal transport activity of EIN2 was observed so far. The C-terminus of EIN2 has no distinct motifs but is conserved in all the known EIN2 homologs from both dicot and monocot plants. EIN2 protein is localized at the ER membrane (Bisson et al. 2009). Without ethylene, EIN2 is subjected to proteasomal degradation by two F-box proteins EIN2-INTERACTING PROTEIN1 and 2 (ETP1/2); ethylene treatment results in the accumulation of EIN2 proteins via downregulation of ETP1/2 protein level (Qiao et al. 2009). Recent studies have identified a molecular mechanism of how EIN2 transduces the ethylene signal to downstream EIN3/EILs (Ju et al. 2012;

Qiao et al. 2012; Wen et al. 2012). In the absence of ethylene, EIN2 proteins reside in the ER membrane and are phosphorylated by CTR1; this phosphorylation may serve as a signal to target EIN2 for degradation. Ethylene perception inactivates CTR1 and triggers dephosphorylation as well as proteolytic cleavage of EIN2, resulting in the translocation of EIN2 C-terminal fragment to the nucleus. In the nucleus, EIN2 C-terminus may stabilize the transcription factors EIN3/EILs that in turn activate the transcriptional cascade resulting in the expression of ethylene-responsive genes.

EIN3/EILs and ERFs

Ethylene signals are amplified in the nucleus by a transcriptional cascade mediated by EIN3/EILs and ERFs. EIN3/EILs function as master transcription factors in ethylene signaling pathway (Chao et al. 1997). In the absence of ethylene, EIN3/EILs are constantly ubiquitinated and degraded by two F-box proteins EIN3-BINDING F-BOX PROTEIN1 and 2 (EBF1/2) (Guo and Ecker 2003; Potuschak et al. 2003). Ethylene stabilizes EIN3/EIL1 by promoting EBF1/2 proteasomal degradation, during which EIN2 is required (An et al. 2010). Interestingly, the *EBF2* gene expression is directly activated by EIN3, indicating that EBF2 serves as a control point in negative feedback regulation of ethylene signaling (Konishi and Yanagisawa 2008). The *EBF1/2* mRNAs are also subjected to posttranscriptional regulation by the 5′ to 3′ exoribonuclease EIN5/XRN4 (Olmedo et al. 2006). ERF1 is the first ERF identified in *Arabidopsis* as an immediate target of EIN3 (Solano et al. 1998). ERF proteins specially bind to the GCC-box in the promoters of target genes. The ERFs are a large gene family of transcription factors in plants. For example, there are 122 members in *Arabidopsis* and 139 members in rice (Nakano et al. 2006). This indicates that ERFs play important roles in many physiological aspects in plants. So far, only a few ERFs have been functionally characterized. Determination of the specific biological function of each of these ERFs should help us better understanding of the regulatory mechanisms of ethylene in plant growth and development. Moreover, due to their specificity of individual members, ERFs represent ideal targets for genetic manipulation to improve specific traits of plants.

Overall, in the past two decades, extensive studies have established the ethylene signal transduction pathway that is one of the best characterized signaling pathways of phytohormones. However, to fully understand the signaling mechanism, many questions remain to be addressed, such as the biochemical nature of ethylene receptor signaling, the molecular mechanism of CTR1 kinase activity regulation, the mechanism of EIN2 C-terminus stabilizing EIN3/EILs, and the CTR1-independent pathway. In addition, the studies on ethylene signaling have mainly focused on dicot plants at present; little is known about the signaling mechanism in monocot plants although all the signaling components are conserved. Considering that rice, a model plant of monocot, exhibits different ethylene responses and different botanical structures and shows only limited synteny at genome level compared to *Arabidopsis* (Ma et al. 2010), it is likely that monocot plants at least rice may possess both conserved and diverged mechanisms for ethylene signaling.

Regulation of Plant Growth and Development by Ethylene

Ethylene controls or influences numerous aspects of plant growth and development. Here we just focus on the developmental processes that are particularly important to agriculture.

Seed Germination

Ethylene is one of the phytohormones that play an essential role in seed germination. Application of ethylene stimulates seed germination in numerous plant species (Linkies and Leubner-Metzger 2012). Ethylene production begins with seed imbibition and increases during germination. Likewise, treatments that break seed dormancy often stimulate ethylene biosynthesis. In most species, ACO but not ACS is associated with the increase in ethylene biosynthesis during seed germination. For example, in *Lepidium sativum* and *Arabidopsis*, the *ACO2* transcripts and enzyme activity are upregulated during endosperm cap weakening and rupture, suggesting that ACO2 acts as a key enzyme in regulating ethylene production during the seed germination (Linkies et al. 2009). In addition to ethylene biosynthesis, ethylene signaling is also required in seed germination of many species. In *Arabidopsis*, for instance, ethylene-insensitive mutants *etr1-1* and *ein2* show delayed germination, whereas constitutive ethylene response mutant *ctr1* displays early germination (Subbiah and Reddy 2010). The *ERF* genes involved in seed germination have been identified in several species such as beech tree (*Fagus sylvatica*), sunflower (*Helianthus annuus*), and tomato (Jimenez et al. 2005; Oracz et al. 2008; Pirrello et al. 2006). Ethylene promotes seed germination by counteracting ABA effects via repressing its biosynthesis and signaling (see section "Ethylene Cross Talk with Other Hormones").

Vegetative Growth

Ethylene affects many aspects of vegetative growth of plants including root growth, hypocotyl elongation, leaf expansion, and stem growth, which determine the plant architecture (Vandenbussche et al. 2012). The regulation of root growth by ethylene has been most extensively studied. *Arabidopsis* mutants with enhanced ethylene production or constitutive ethylene response (i.e., *eto* and *ctr1*) display a short root phenotype in both dark-grown and light-grown seedlings, whereas the ethylene-insensitive mutants (e.g., *etr1-1* and *ein2*) show no root inhibition upon ethylene treatment, indicating a inhibitory role for ethylene in root growth. Ethylene inhibits root growth primarily by affecting cell elongation in the root elongation zone (Ruzicka et al. 2007). In the presence of ethylene, the trichoblast cell elongation ceases earlier due to differentiation into hair cells, resulting in a shorter elongation zone (Ruzicka et al. 2007). Ethylene inhibition of root growth largely depends on

auxin actions (Ruzicka et al. 2007). Ethylene promotes auxin biosynthesis in root apex by the activation of several auxin biosynthetic genes such as *WEI2/ASA1* (*WEAKLY ETHYLENE INSENSITIVE 2/ANTHRANILATE SYNTHASE α1*), *WEI7/ASB1* (*ANTHRANILATE SYNTHASE β1*), and *WEI8/TAA1* (*TRYPTOPHAN AMINOTRANSFERASE 1*) (Stepanova et al. 2005, 2008). Auxin produced in root apex is then transported to the elongation zone (basipetal transport) by the auxin influx carrier AUX1 and efflux carrier PIN2/EIR1 (PIN-FORMED2/ETHYLENE INSENSITIVE ROOT1) that are upregulated by ethylene in the root tips (Ruzicka et al. 2007; Stepanova et al. 2005). In the elongation zone, supraoptimal levels of auxin lead to inhibition of cell elongation (Strader et al. 2010). However, the molecular mechanism for auxin signaling involved in this root-inhibition process in the elongation zone is poorly understood so far (for details, see section "Ethylene Cross Talk with Other Hormones"). Apart from repressing primary root growth, ethylene also inhibits lethal root formation through reducing auxin levels in the mature root zone (Lewis et al. 2011). Additionally, ethylene promotes root hair development through interaction with auxin and jasmonates (Zhu et al. 2006a).

Ethylene has dual functions in the regulation of hypocotyl growth (Vandenbussche et al. 2012). In darkness, ethylene inhibits hypocotyl elongation of *Arabidopsis* seedlings. By contrast, ethylene can stimulate *Arabidopsis* hypocotyl elongation in the light (Smalle et al. 1997). Both inhibitory and stimulatory effects on hypocotyl elongation are resulted from affecting cell expansion rather than cell division (Smalle et al. 1997; Le et al. 2005). However the precise molecular mechanism of how ethylene switches between the two opposite functions remains to be elucidated. In comparison with *Arabidopsis*, ethylene promotes coleoptile elongation of rice seedlings even in darkness as described above (Ma et al. 2013). This suggests that the regulatory effects of ethylene on seedling growth are species dependent.

In adult plants (post-seedling growth), ethylene also plays a dual role in regulating stem and leaf growth, and the effects depend on species, environmental conditions, and developmental stages (reviewed in Dugardeyn and Van Der Straeten 2008). For example, ethylene represses leaf expansion and stem growth in most cases; however, it can promote internode elongation of deepwater rice. Similarly, under shade conditions, plants often produce more ethylene to extend their stems and petioles for optimal shade avoidance.

Flower Development

The vegetative-to-reproductive transition is a key step in plant life cycle. Flowering time is controlled by various factors including photoperiod, temperature, plant age, and GA (Song et al. 2013). Ethylene plays a role in the regulation of flowering timing, while the effects appear complicated. In *Arabidopsis*, the bolting time is earlier in *eto1* mutant but late in *ein2*, *eni3*, and *etr1-1* mutants, suggesting that ethylene promotes floral transition (Ogawara et al. 2003). However, *ctr1* mutant as well as the ACC-treated wild type shows delay in flowering, indicating an inhibitory role for ethylene in *Arabidopsis* flowering (Achard et al. 2007). Similarly, the opposite

effect of ethylene is observed in rice. Overexpression of *OsETR2* decreases ethylene sensitivity and delays floral transition, while suppression of *OsETR2* by RNAi enhances ethylene sensitivity and accelerates rice flowering, indicating that ethylene promotes rice flowering (Wuriyanghan et al. 2009). In contrast, the *osctr2* loss-of-function mutant and the transgenic lines overexpressing *OsCTR2* N-terminus exhibit constitutive ethylene response and delayed flowering phenotype, suggesting that ethylene represses floral transition in rice (Wang et al. 2013a). More surprisingly, both knockout and overexpression of *OsEIN2*/*MHZ7* result in delayed flowering in rice (our unpublished data). These contradictory observations may be due to different growth conditions, different genetic background, or different mechanisms employed by these signaling components. The repressive effect of ethylene on *Arabidopsis* floral transition is caused by a reduction of bioactive GA levels and inhibition of GA signaling, which in turn delays flowering via repression of the floral meristem identity genes *LEAFY* (*LFY*) and floral integrator gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Achard et al. 2007). Characterization of the stimulatory effect of ethylene on flowering will provide a more comprehensive understanding of ethylene-mediated floral transition.

Besides regulating floral transition, ethylene plays a key role in floral sex determination of some species. Cucumber (*Cucumis sativus*) is a model system for sex determination studies. Exogenous application of ethylene promotes femaleness (Iwahori et al. 1970). Cucumber generally produces male and female flowers separately on the same plant (monoecious type). Whereas the andromonoecious type produces bisexual and male flowers on the same plant, the hermaphroditic type bears only bisexual flowers, the gynoecious type bears only female flowers, and the androecious type has only male flowers. Initially all floral buds have both staminate and pistillate primordia. Selective arrest of the male or female organs results in these sex expression (Yamasaki et al. 2001). Extensive studies have revealed that sex determination of cucumber is largely controlled by *CsACS2* gene (Yamasaki et al. 2001; Kamachi et al. 1997; Boualem et al. 2009). Both the timing and the levels of *CsACS2* expression are correlated with the development of female flowers (Kamachi et al. 1997). Active CsACS2 inhibits the development of male organs and thus leads to female flower development, whereas mutations leading reduced to no enzyme activity of CsACS2 cause andromonoecy (Boualem et al. 2009). Similar function was identified for *CmACS-7*, a melon ortholog of *CsACS2*, indicating a conversed mechanism for sex determination in the cucurbitaceous plants (Boualem et al. 2008, 2009).

Fruit Ripening

Fruit ripening is a highly coordinated developmental process that leads to chlorophyll degradation, cell wall losing, texture change, aroma development, and accumulation of pigments, sugars, and acids. Ethylene plays a key role in promoting ripening of climacteric fruits, such as tomato, apple (*Malus* x *domestica*), peach, and banana. Climacteric fruits are characterized by a burst of respiration at the onset of ripening

along with a dramatic increase in ethylene production. Ethylene biosynthesis is essential for normal ripening of climacteric fruits. Two distinct systems are involved in the ethylene biosynthesis during fruit development and ripening. System I functions in the pre-climacteric period and is responsible for producing the basal ethylene through auto-inhibitory regulation; System II is responsible for autocatalytic ethylene production during ripening stage (McMurchie et al. 1972). Tomato is a model system for fruit ripening studies. As described in section "Regulation of Ethylene Biosynthesis," *LeACS1A* and *LeACS6* are responsible for the basal ethylene production of System I, while *LeACS2*, *4* and *LeACO1*, *4* are required for the climacteric ethylene synthesis of System II. The expression of these *ACS* and *ACO* genes is tightly controlled by some developmentally regulated transcription factors such as RIN, LeHB-1, LeERF2, and LeCP. Ethylene production is rapidly shut down at post-climacteric ripening stage as a consequence of reduced ACO activity (but not ACS activity) (Van de Poel et al. 2012). The termination of autocatalytic ethylene production of System II likely prevents the fruits from premature senescence and thus ensures seed maturation (Van de Poel et al. 2012).

Apart from ethylene biosynthesis, ethylene perception and signal transduction cascades are also important control points of fruit ripening. Tomato *Never-ripe* (*Nr*) mutant harbors a gain-of-function mutation in the ethylene receptor NR and thus confers ethylene insensitivity and nonripening phenotype (Wilkinson et al. 1995). On the other hand, fruit-specific suppression of *LeETR4* by RNAi causes early fruit ripening (Kevany et al. 2008). These results demonstrate that ethylene receptors negatively control the onset of fruit ripening. Tomato Green-Ripe (GR) is an ortholog of *Arabidopsis* RTE1 that is an ETR1-depedent negative regulator of ethylene response (Resnick et al. 2006; Barry and Giovannoni 2006). The nonripening phenotype of the dominant *Gr* mutant is a result of reduced ethylene sensitivity in fruit tissues (Barry and Giovannoni 2006). Tomato possesses three *CTR*-like genes, among which *LeCTR1* transcript increased during the onset of ripening (Adams-Phillips et al. 2004). However, the exact function of these *LeCTRs* in fruit ripening is so far unclear. LeEIN2 positively regulates fruit ripening, as suppression of *LeEIN2* by virus-induced gene silencing (VIGS) fruit system leads to delayed fruit development and ripening (Zhu et al. 2006b). Overexpression of *LeEIL1* in the *Nr* mutant can partially rescue the nonripening phenotype, indicating a role for *LeEIL1* in fruit ripening (Chen et al. 2004). Consistently, VIGS-mediated silencing of tomato *EBF1* and *EBF2* leads to earlier fruit ripening associated with constitutive ethylene responses (Yang et al. 2010). The *LeERF1* is directly involved in fruit ripening (Li et al. 2007). Overall, the regulatory mechanism of ethylene in fruit ripening identified in tomato is highly conserved in other climacteric fruits (reviewed in Bapat et al. 2010).

Leaf Senescence

Leaf senescence is the final stage of leaf development and involves recycling of nutrients from old leaves to developing organs such as fruits and seeds. This process is characterized by chlorophyll breakdown, loss of photosynthetic activity, and nutrient

remobilization. Leaf senescence highly depends on developmental age and is regulated by various internal signals and environmental cues (reviewed in Zhang and Zhou 2013). Ethylene promotes leaf senescence through upregulating the expression of senescence-associated genes and downregulating the transcription of photosynthesis-associated genes (Grbic and Bleecker 1995). Ethylene can only induce senescence in leaves that have reached a defined age and the effect increases with increasing leaf age (Jing et al. 2005). Ethylene signaling rather than its biosynthesis greatly influences the onset of leaf senescence. In *Arabidopsis*, dominant mutations in the ethylene receptors confer ethylene insensitivity and delay in leaf senescence (Sakai et al. 1998; Hua et al. 1998; Grbic and Bleecker 1995). Consistently, knockout of *ETR1* and *ERS1* (i.e., *etr1-9ers1-3* double mutant) causes premature leaf senescence (Qu et al. 2007). In common with the ethylene receptors, ethylene insensitivity conferred by loss of *EIN2* or *EIN3* delays leaf senescence (Chao et al. 1997; Oh et al. 1997). A recent study using overexpression and knockout strategy revealed that the AtERF4 and AtERF8 play an important role in ethylene-mediated leaf senescence (Koyama et al. 2013). In rice, *OsETR2* overexpressing transgenic plants are greener than WT at maturation stage (Wuriyanghan et al. 2009). Loss of *OsEIN2*/*MHZ7* delays dark-induced leaf senescence, whereas the overexpression lines exhibit premature senescence phenotypes (Ma et al. 2013). Loss of *OsCTR2* or overexpression of its N-terminus promotes senescence of detached rice leaves (Wang et al. 2013a). Overexpression of *OsRTH1* prevents ethylene-induced leaf senescence (Zhang et al. 2012). Taken together, these findings indicate that altering ethylene signaling can profoundly influence leaf senescence.

In summary, many aspects of plant growth and development are controlled by ethylene at levels of biosynthesis, signal perception, and/or signaling cascades. In most cases, however, the downstream responsive elements that ultimately regulate the individual biological processes remain unclear. Furthermore, ethylene usually acts through interacting with other phytohormones and/or developmental factors. A detailed understanding of the interplay of ethylene with different factors in fine control of plant growth and development is a challenge for the future research.

Regulatory Roles of Ethylene in Stress Responses

Ethylene plays various roles in plant growth and development. It is also involved in abiotic stress responses. Ethylene has long been regarded as a stress hormone. Its roles during flooding and submergence, in pathogen/defense response, and many other stresses have been well documented (Fukao and Bailey-Serres 2008; Van der Ent and Pieterse 2012). However, how ethylene and its signaling affect salt stress responses is largely unclear. Here, we focus on this issue mainly according to our own studies involving tobacco ethylene receptor genes and also the ethylene signaling or regulated genes from *Arabidopsis*.

Tobacco ethylene receptor genes *NTHK1* and *NTHK2* are subfamily II genes. Both are induced by wounding and osmotic stress. In situ mRNA hybridization and

immunohistochemistry analysis disclose that *NTHK1* mRNA and its protein are first produced in the palisade cell layer upon cutting/wounding and then gradually spread to other sponge cells of a leaf (Zhang et al. 2001; Xie et al. 2002). However, only *NTHK1* is induced by salt stress (Zhang et al. 2001), suggesting its roles in salt stress response.

NTHK1 overexpression increases salt sensitivity in both transgenic tobacco and transgenic *Arabidopsis* plants, in addition to the reduction of ethylene sensitivity and promotion of rosette/seedling growth (Cao et al. 2006, 2007). When the ethylene precursor ACC is included in the salt medium, the salt-stressed phenotype of *NTHK1*-overexpressing *Arabidopsis* is inhibited, indicating a positive role of ethylene in salt stress tolerance (Cao et al. 2007). It is interesting to note that the *NTHK1* transcripts are also induced by salt stress and cycloheximide (CHX) in transgenic plants. Further *NTHK1* promoter-GUS analysis reveals that the promoter activity can be induced by wounding but not by salt stress, suggesting that the salt-induction element may be present in the coding region but not in the promoter region (Zhou et al. 2006). We further made various truncations of the *NTHK1* genes and generated the overexpressing transgenic plants. Examination of these transgene expressions in response to salt or CHX demonstrates that the salt and CHX-responsive element were in the region coding for the transmembrane domains (Zhou et al. 2006). We propose that the transmembrane-coding region may contain an instable element, which can be targeted for degradation under normal condition by an unknown mechanism. Under salt/CHX treatment, proteins in this process are inhibited and *NTHK1* transcripts accumulate. Further study may shed light on the regulation of *NTHK1* transcript accumulation.

NTHK1 has various domains. Through truncation and transgenic analysis, we find that the presence of the kinase domain of NTHK1 is associated with salt sensitivity and large rosette phenotype (Zhou et al. 2006). Since NTHK1 has Ser/Thr kinase activity, we tested whether the kinase activity is required for salt response. N-box mutation in NTHK1 abolished the kinase activity and also disrupted its roles in salt stress response and ethylene response (Chen et al. 2009). However, this mutation only has partial effects on rosette growth and expressions of downstream genes including *AtNAC2*, *AtERF1*, and *AtCor6.6*. H-box mutation doesn't affect kinase activity or the salt/rosette phenotype. However, it may alter a few gene expressions. Compared with the subfamily I ethylene receptor NtETR1 from tobacco, which has His kinase activity, subfamily II receptor NTHK1 with Ser/Thr kinase activity has much stronger roles in the regulation of salt response, rosette growth, and ethylene response, indicating functional preference of ethylene receptors (Chen et al. 2009).

Arabidopsis ethylene receptor gain-of-function mutants *etr1-1* and *ein4-1* are also sensitive to salt stress, probably due to the active receptor signaling state (Cao et al. 2007). *EIN2* is the central component of ethylene signaling in *Arabidopsis*, and its mutants *ein2-1* and *ein2-5* are extremely sensitive to salt stress (Lei et al. 2011). EIN2 C-terminal end, which can be cleaved upon ethylene perception and translocated to the nucleus (Ju et al. 2012; Qiao et al. 2012; Wen et al. 2012), can rescue the salt phenotype of the mutant, indicating that active ethylene signaling is required for salt tolerance (Lei et al. 2011). An MA3 domain-containing protein

ECIP1 has been identified to interact with both ethylene receptors and the C-terminal end of EIN2 to negatively regulate salt response and ethylene response (Lei et al. 2011). Roles of EIN3 were also examined and the *ein3-1* single mutant appears to have no significant change in salt stress (Cao et al. 2007). However, the double mutant *ein3 eil1* is very sensitive to salt stress, similar to the response of *ein2* mutant, further demonstrating that ethylene signaling participates in salt tolerance (Lei et al. 2011).

Through microarray analysis, we have identified the *NTHK1*-regulated genes. Among these, *AtNAC2* can be induced by salt stress and ethylene but suppressed in *NTHK1*-overexpressing *Arabidopsis* plants. The ethylene induction but receptor suppression coincides with the negative regulation between ethylene and its receptors (Hua and Meyerowitz 1998). The salt induction of this gene required ethylene signaling pathway (He et al. 2005). Overexpression of the gene promotes lateral root formation. Later this gene was further found to play essential roles in leaf senescent process and salt-promoted senescence (Kim et al. 2009; Balazadeh et al. 2010). NIMA-related kinase NEK6 gene is another gene regulated by *NTHK1* overexpression. Both *NEK6* transcripts and proteins are induced by ACC and salt stress (Zhang et al. 2011). The other *NEK1* to *NEK4* and *NEK7* genes are also responsive to the two treatments, suggesting important roles of this small family gene in salt and ethylene responses. *NEK6* overexpression and mutant analysis discloses that NEK6 increases rosette growth, seed yield, and lateral root formation. The gene also promotes plant tolerance to salt and osmotic stresses (Zhang et al. 2011). NEK6 may function through suppression of ethylene biosynthesis and activation of cyclin genes. These analyses support that ethylene and receptor-regulated genes affect plant growth and salt response.

We also identified the NTHK1-interating proteins using yeast two-hybrid assay and these proteins can be induced by ethylene and/or salt stress (our unpublished data). Overexpression of the genes promotes plant growth but exerts different effects on stress response. We propose that ethylene induced these proteins and the proteins would associate with ethylene receptors to desensitize ethylene response for plant growth recovery after ethylene treatment. This may represent a feedback control mechanism for ethylene-regulated processes. Further studies should dissect the fine-tuning of the mechanism for ethylene regulation.

ERF-type transcription factor ERF1 acts downstream of EIN3/EIL1 to regulate ethylene response. Many ERF family proteins play multiple roles in abiotic stress response; however, whether these proteins are involved in ethylene response remains largely unclear. We propose that if one ERF protein has one or a few of these features, it may be regarded as a component participating in regulation of ethylene pathway. First, it should affect ethylene response phenotypes in overexpressing or RNAi transgenic plants, e.g., hypocotyl growth or other measurable parameters. Second, the gene expression or protein levels of a given ERF may be altered by ethylene treatment. Third, the ERF may affect ethylene biosynthesis, ethylene signaling, and/or expression of ethylene-responsive genes. If one ERF has at least one of the above features and at the same time it affects stress response, then we may adopt that the ERF is involved in ethylene-regulated stress adaptation process.

Systematic analysis of the ERF family proteins would give a full picture of their roles in ethylene response and abiotic stress response.

Together, through the above analysis we find that ethylene signaling is required for salt tolerance. However, plant response to salt stress may depend on the homeostasis of ethylene and its receptors since the two has a negative relationship. Too much ethylene or receptors would disrupt the balance and plants may be very small with early flowering or has large rosette with late flowering. Plants need to adjust between these two extreme conditions for better survival under salt stress. Further identification of ethylene-regulated genes should facilitate the understanding of ethylene roles in salt tolerance and other stress responses.

Ethylene Cross Talk with Other Hormones

Ethylene regulates multiple developmental processes and a variety of stress responses. In most processes, ethylene interacts with other hormonal pathways at multiple biochemical levels to achieve its diverse functions. The cross talk between ethylene and other hormones has been reviewed elsewhere (Vandenbussche and Van Der Straeten 2007; Yoo et al. 2009; Zhao and Guo 2011). Here, we focus on its interplay with auxin and ABA.

Ethylene–Auxin

Ethylene and auxin have a close interplay in many developmental processes. Ethylene functions through modulating auxin biosynthesis, transport, and/or signaling. In a genetic screen for *wei* mutants, several genes encoding proteins for auxin synthesis have been identified. *WEI2*/*ASA1* and *WEI7*/*ASB1* genes encode the alpha and beta subunits of anthranilate synthase, a rate-limited enzyme of tryptophan biosynthesis, respectively. Ethylene treatment results in a significant induction of these two genes, which account for the accumulation of auxin in the tip of primary roots (Stepanova et al. 2005). The *WEI8*/*TAA1* gene encodes a tryptophan aminotransferase that functions in the indole-3-pyruvic acid (IPyA) branch of auxin synthesis pathway (Stepanova et al. 2008). Ethylene induces auxin production in root tip through upregulation of these *WEI* genes so as to achieve auxin-dependent and tissue-specific ethylene response. On the other hand, elevated levels of auxin also stimulate ethylene synthesis via upregulation of *ACS* transcripts (Tsuchisaka and Theologis 2004a; Wang et al. 2005). Strikingly, the amounts of these two phytohormones can be simultaneously coordinated by the VAS1 (for reversal of *sav3* (shade avoidance 3) phenotype) aminotransferase. VAS1 transfers amino from ethylene biosynthetic precursor Met to auxin biosynthetic intermediate IPyA to produce L-tryptophan and 2-oxo-4-methylthiobutyric acid (Zheng et al. 2013). This means that VAS1 inhibits both auxin and ethylene biosynthesis by decreasing the levels of IPyA and Met, respectively.

Isolation of auxin transport mutants *aux1* (Pickett et al. 1990) and *eir1*/*pin2* (Luschnig et al. 1998) in screens for reduced ethylene response mutants gives us a new insight into understanding the cross talk between ethylene and auxin. In *Arabidopsis* primary root, AUX1 and PIN2 facilitate auxin transport from root tip to elongation zone. Ethylene stimulates the expression of *PIN2* and *AUX1* in roots (Ruzicka et al. 2007). These findings suggest that ethylene-mediated root growth inhibition require AUX1- and PIN2-dependent auxin transport to the elongation zone. In addition to the effects on primary root, exogenously applied ACC can inhibit *Arabidopsis* lateral root development. Recent studies show that ACC treatment can enhance *PIN3* and *PIN7* expression, which elevate auxin transport and destroy localized accumulation of auxin needed for driving lateral root formation (Lewis et al. 2011). Ethylene-triggered changing of auxin transport is limited not only in root but also in apical hook. Exaggeration of the apical hook is part of triple response, and auxin influx carrier AUX1 is involved in this process (Vandenbussche et al. 2010). Another auxin transporter mutant *pin3* exhibits reduced ethylene sensitivity and never forms exaggerated apical hook. Further study showed that ethylene asymmetrically enhanced the lateral localization of PIN3 protein in the cortex cell membranes on the convex side, which may lead to asymmetrical auxin distribution and then exaggerated hook formation (Zadnikova et al. 2010).

Besides auxin synthesis and transport, the mutants that impair auxin perception (e.g., *tir1*) or signaling (e.g., *axr1*) also show reduced response to ethylene in *Arabidopsis* root (Alonso et al. 2003; Stepanova et al. 2007). By contrast, complete ethylene-insensitive mutants such as *ein2-5* show nearly normal response to exogenous auxin (Stepanova et al. 2007). This indicates that ethylene signaling pathway acts upstream of auxin pathway, which is further supported by the identification of *HOOKLESS1* (*HLS1*) gene. *HLS1* is involved in differential cell elongation in the *Arabidopsis* hypocotyl, and its mRNA levels increase when treated with ethylene but decrease in *ein2* mutant. Interestingly, the expression patterns of two primary auxin response genes *SAUR* and *AtAUX2-11* are altered in *hls1* mutant (Lehman et al. 1996). Auxin response factor ARF2 was identified as *hls1* suppressor. Application of ethylene can suppress accumulation of the ARF2 protein and this effect required HLS1 but independent on ethylene-modulated auxin concentration or distribution (Li et al. 2004).

Taken together, auxin synthesis, distribution, and signaling are required for ethylene-regulated growth, but ethylene signaling is not indispensable for auxin-regulated growth. To illustrate the complicated relationships between ethylene and auxin, there are still some issues that should be documented in the future research. Firstly, several genes (i.e., *WEI2*, *PIN3*, and *ACS*) are regulated by ethylene and auxin in transcriptional levels, but the corresponding transcription factors remain to be identified. Secondly, exogenously supplied ethylene promotes an increase in the levels of the DR5:GUS activity in the root, which required TIR-dependent auxin signing pathway. However, the DR5:GUS activity never reaches into the root cells of the fast elongation zone, where ethylene functions mainly (Stepanova et al. 2007). This raises a question whether a TIR-independent auxin signaling pathway is responsible for the ethylene-induced root inhibition.

Ethylene–ABA

ABA plays an import role in seed dormancy and germination, stomatal closure, and adaptive stress responses. ABA biosynthesis and signal transduction are described in Chap. 2. Ethylene and ABA signaling pathways have a close interplay, as allelic mutations of *ctr1* and *ein2* are recovered as enhancer and suppressor of *ABA insensitive1-1* (*abi1-1*), respectively (Beaudoin et al. 2000). Ethylene and ABA interact in both antagonistic and synergistic manners. The two hormones have an opposite effect on seed germination. Ethylene counteracts the inhibitory effects of ABA by repressing its accumulation and signal transduction. The ethylene-insensitive mutants *etr1-1* and *ein2* accumulate high levels of ABA, which are associated with upregulation of the ABA biosynthetic gene *NCED3* and/or downregulation of the catabolism-related gene *CYP707A2* (encoding ABA 8′-hydroxylase) (Beaudoin et al. 2000; Cheng et al. 2009; Ghassemian et al. 2000). Moreover, the ABA signaling component *ABI1* is downregulated in *etr1-1* (Cheng et al. 2009). In the case of wild type, however, the accumulated ethylene promotes seed germination by interfering with ABA signaling rather than affecting the seed ABA levels (Linkies et al. 2009). The contrasting interactions of ethylene and ABA are also reported in submerge-induced shoot elongation of semiaquatic plants. In deepwater rice and *Rumex* species, for example, the accumulated ethylene stimulates shoot elongation by inhibiting ABA biosynthesis via a reduction of *NCED*s expression and enhancing the ABA 8′-hydroxylase-mediated degradation (Benschop et al. 2005; Saika et al. 2007). In guard cell response, although both hormones induce stomatal closure, ethylene can antagonize ABA-induced stomatal closure by inhibiting the ABA signaling pathway (Tanaka et al. 2005; Desikan et al. 2006). This contrasting effect of ethylene and ABA is more obvious in older leaves or under soil drying conditions (Chen et al. 2013a).

Besides the antagonistic interactions, ethylene and ABA can also synergistically regulate a number of developmental processes. In tomato fruit ripening, both ethylene and ABA can promote this process, during which *LeNCED1*-mediated ABA accumulation at the breaker stage acts as a primary inducer for climacteric ethylene production and onset of ripening (Zhang et al. 2009b). In root growth, high levels of ethylene and ABA inhibit root elongation. Genetic evidences reveal that ABA signaling pathway acts upstream of ethylene signaling, as *etr1-1* and *ein2* root growth are insensitive to ABA, whereas the roots of *abi1* and ABA-deficient mutant *aba2* display normal ethylene response (Beaudoin et al. 2000; Cheng et al. 2009; Ghassemian et al. 2000). In abiotic stress responses, the cross talk between ethylene and ABA appears more complicated. The analysis of *ein2* mutant shows that EIN2 positively regulates salt and drought tolerance by enhancing ABA biosynthesis and inducing the expression of ABA-dependent stress-responsive genes (Wang et al. 2007). In contrast, characterization of *acs7* knockout mutant reveals that ACS7 acts as a negative regulator in salt and drought responses through repression of ABA accumulation and ABA-dependent stress-responsive genes (Dong et al. 2011). These divergent observations may be due to the different cross talk nodes in the signaling network of ethylene, ABA, and stress. In the case of *acs7* mutant, deficient

ACC synthesis possibly leads to an increase in the levels of polyamines that share common substrate, SAM, with ethylene. Polyamines in turn can promote ABA biosynthesis (Alcázar et al. 2010).

Collectively, ethylene and ABA interact extensively in the regulation of plant growth, development, and adaptive responses. However, their interplay is complicated, depending on biological process, tissue/organ, growth conditions, and species. Identification of the exact cross talk nodes will provide more insights into their interactions.

Biotechnological Manipulation of Ethylene Biosynthesis and Signaling in Agriculture

The importance of ethylene in the regulation of plant growth, flower development, organ senescence, fruit ripening, and adaptive responses makes it an agriculturally important hormone. Thus, ethylene biosynthesis, signal perception, and signaling cascades have become successful genetic and management targets for producing longer-lived flowers, reducing post-harvest losses, and improving crop production. Here we describe and discuss the achievements in biotechnological manipulation of ethylene biosynthesis and signaling in crop, fruit, and flower plants.

Fruit and Flower Plants

Three strategies have been employed to delay fruit ripening: (1) inhibition of ethylene biosynthesis, (2) inhibition of ethylene perception, and (3) interruption of ethylene signaling. Most of the studies have been conducted in tomato. Ethylene production can be inhibited at the level of SAM degradation, ACC synthesis, or ACC oxidation. SAM hydrolase is a bacteriophage enzyme that can convert SAM to MTA and homoserine. Ectopic expression of the SAM hydrolase gene in tomato plants under the control of ripening specific promoter E8 confers reduced ethylene production and delayed fruit ripening (Good et al. 1994). As with ACC synthesis, antisense suppression of *LeACS2* gene in tomato results in 99.5 % decrease in ethylene production and no fruit ripening unless addition of exogenous ethylene (Oeller et al. 1991). As an alternative strategy for reducing ACC contents, the gene encoding bacterial ACC deaminase is introduced into tomato plants to deplete endogenous ACC (Klee et al. 1991). ACC deaminase catalyzes the conversion of ACC into ammonia and α-ketobutyrate. The transgenic plants exhibit significant delays in fruit ripening; and reduction of ethylene synthesis does not cause any apparent vegetative phenotypic abnormalities (Klee et al. 1991). Disruption of ACC oxidation by silencing *ACO* genes has been extensively employed in extending fruit shelf life. Antisense suppression of *LeACO1* in tomato plants results in 97 % reduction in ethylene synthesis. The transgenic plants display extended shelf life as well as

delayed leaf senescence (Hamilton et al. 1990; John et al. 1995). Gene silencing by small antisense RNA is also successfully used in shutting down *LeACO1* gene expression (Han and Grierson 2002). RNAi suppression of *LeACO1* in tomato plants results in prominent effects; the transgenic fruits release only trace amounts of ethylene and have a prolonged shelf life of more than 120 days (Xiong et al. 2005). Inhibition of ethylene perception has been achieved by ectopic expression of the *Arabidopsis etr1-1* mutant receptor gene in tomato, conferring strong ethylene insensitivity and thus causing significant delay in fruit ripening and prolonged shelf life of more than 100 days (Wilkinson et al. 1997). Furthermore, a regulated state of ethylene insensitivity is achieved through the controlled expression of *etr1-1* gene using an inducible promoter (Gallie 2010). Interruption of ethylene signaling has been achieved by silencing the *LeERF1* in tomato. The transgenic plants expressing antisense *LeERF1* display reduced ethylene sensitivity and extended shelf life up to 60 days (Li et al. 2007). Besides tomato, similar strategies are also successfully used in other fruits such as melon and apple (Ayub et al. 1996; Dandekar et al. 2004). Although improvement of fruit shelf life has achieved a great success as described above, one side effect of these strategies is the compromised fruit quality (Guptaa et al. 2013). As an attempt to overcome this problem, simultaneously silencing three *ACS* homologs (*LeACS1A*, *LeACS2*, and *LeACS6*) by RNAi was achieved in tomato plants, resulting in dramatically reduced ethylene production and delayed fruit ripening with a longer shelf life. More importantly, the transgenic tomato exhibit improved fruit processing quality that is associated with increased levels of polyamines (Guptaa et al. 2013).

In floral plants, transgenic strategies similar to that used in fruit plants have been employed to produce longer-lived flowers. Antisense suppression of *ACO* genes in carnation (*Dianthus caryophyllus*) and torenia (*Torenia fournieri* Lind.) results in markedly delayed petal senescence (Savin et al. 1995; Aida et al. 1998). Transgenic petunia (*Petunia* x *hybrida*) plants harboring the *Arabidopsis etr1-1* gene exhibit 5 days delay in flower senescence as well as enhanced tolerance to pathogens (Wilkinson et al. 1997; Wang et al. 2013b). Similarly, heterologous expression of the *Arabidopsis etr1-1* gene in carnation confers 6–16 days delay in flower senescence and threefold increase in vase life (Bovy et al. 1999). Transgenic petunia plants expressing antisense *PhEIN2* gene exhibit reduced ethylene sensitivity and sixfold increase in flower longevity (Shibuya et al. 2004).

Major Crops

Rice

Rice is the world's most important food crop that feeds about half of the world's population. As a semiaquatic plant, rice adapts to hypoxia conditions through various acclimation responses, such as coleoptile elongation, adventitious root formation, aerenchyma development, and enhanced (submergence escape) or repressed

(submergence tolerance) shoot elongation. Ethylene plays a central role in these adaptive responses (Ma et al. 2010). In addition, ethylene also regulates many aspects of rice developmental processes such as germination, grain filling, leaf senescence, and yield formation (Ma et al. 2010). The genes for ethylene biosynthesis, perception, and signaling have been identified in rice (reviewed in Rzewuski and Sauter 2008; Ma et al. 2010). However, only a few of them have been functionally characterized. *OsACO1*-overexpressing rice plants and null mutants show longer and shorter culm length, respectively, indicating that alternation of ethylene biosynthesis can affect plant height that is one of the most important agronomic traits in rice breeding (Iwamoto et al. 2010). We have demonstrated that rice ethylene receptor OsETR2 has Ser/Thr kinase activity. *OsETR2*-overexpressing rice plants exhibit delayed flowering and increased accumulation of starch in stems (Wuriyanghan et al. 2009). Knockout *OsCTR2* results in delayed flowering time, reduced plant height, and increased tiller numbers (Wang et al. 2013a). *Osein2*/*mhz7* null mutation delays leaf senescence, and overexpression of *OsEIN2*/*MHZ7* confers reduced plant height and increased grain size (Ma et al. 2013). Transgenic rice plants overexpressing *OsEIL1* exhibit short root, coiled primary root, and slightly short shoot phenotypes (Mao et al. 2006). Bioinformatics analysis has predicted 139 *ERF* members in rice genome (Nakano et al. 2006). Most *OsERF* genes are induced by abiotic stress conditions; thus, modulation of abiotic stress response at the level of ERF has been extensively studied. Complete submergence caused by flooding is a major constraint to rice production in South and Southeast Asia (Xu et al. 2006). Submergence 1A (Sub1A) is an ERF that confers submergence tolerance by repressing shoot elongation during the inundation period so as to conserve carbohydrates and increase survival under flash flood conditions. Introgression of the *Sub1A-1* gene into intolerant variety results in enhanced submergence tolerance to the plants (Xu et al. 2006). Apart from regulating submergence response, *Sub1A* can also improve drought resistance and delay leaf senescence in rice (Fukao et al. 2011, 2012). As opposed to flooding tolerance strategy, the ERFs Snorkel1 and Snorkel2 (SK1 and SK2) trigger fast stem elongation of deepwater rice to allow the plant to rise above the water level. Introduction of the *SK* genes into non-deepwater rice enables it to become deepwater rice (Hattori et al. 2009). AP37 is an ERF that positively regulates rice drought tolerance. The transgenic rice plants expressing *OsCc1*:*AP37* show significantly enhanced drought tolerance in the field, which increase grain yield by 16–57 % over controls under severe drought conditions, yet exhibit no significant difference under normal growth conditions (Oh et al. 2009). However, an opposite effect of the same gene on drought response is reported in a recent study in which this gene is named as *OsERF3*. It is found that OsERF3 negatively regulates drought tolerance through its EAR motif, as the transgenic rice plants expressing *35S*:*OsERF3* are hypersensitive to drought stress, whereas overexpression of the mutated *OsERF3* gene with a null mutation in the EAR motif results in enhanced drought tolerance (Zhang et al. 2013). The inconsistent observations may be due to different genetic backgrounds or different stress conditions. Besides the effects in stress responses, rice ERFs also regulate some aspects of plant growth. OsEATB is an instance, which affects rice plant architecture and yield.

Overexpression of *OsEATB* in rice reduces plant height but promotes the branching potential of both tillers and spikelets, which are useful traits for breeding high-yielding crops (Qi et al. 2011).

Grain filling is an important physiological process that directly determines the grain weight. High levels of ethylene in grains inhibit endosperm cell division and grain filling in rice, while ABA can antagonize the negative effects of ethylene. Thus, a higher ratio of ABA to ethylene in rice spikelets is required to maintain a faster grain-filling rate (Yang et al. 2006). On the other hand, under drought conditions, an antagonistic interaction between ethylene and polyamines regulates rice grain filling in response to soil drying (Chen et al. 2013b). Although ethylene plays such an important role in rice grain filling, biotechnological applications of such knowledge have not been reported so far.

Wheat

Wheat (*Triticum aestivum*) is one of the most important grain crops of the world. Ethylene-related researches in wheat mainly focus on defense responses. TaEIL1 is wheat ortholog of *Arabidopsis* EIN3. Suppression of *TaEIL1* by VIGS in wheat leaves can enhance the resistance of plant to stripe rust fungus (Duan et al. 2013). This indicates that TaEIL1 can serve as an effective target for genetic improvement of wheat stripe rust tolerance. In addition, several pathogen-inducible *ERF* genes involved in defense responses are identified in wheat. *TaERF3* can activate defense response of wheat plants to *Blumeria graminis* and *Fusarium graminearum* (Zhang et al. 2007). *TaPIEP1* overexpressing wheat plants show obviously improved resistance to *Bipolaris sorokiniana*, which is associated with activation of some defense genes (Dong et al. 2010b). *TiERF1* is a pathogen-induced *ERF* conferring *Rhizoctonia cerealis* resistance in wheat wild relative *Thinopyrum intermedium*. Overexpression of *TiERF1* in susceptible wheat varieties enhances resistance to *R. cerealis* by activating pathogenesis-related (PR) genes in an ethylene-dependent pathway (Chen et al. 2008). In addition to defensive functions, some TaERFs are found to be responsible for regulating abiotic stress responses. For instance, *TaERF1* is involved in multiple stress responses. Overexpression of *TaERF1* enhances drought, cold, and salt tolerance in transgenic *Arabidopsis* (Xu et al. 2007). TaERF4 is a salinity-responsive ERF that functions as a transcription repressor. Heterologous expression of *TaERF4* in *Arabidopsis* confers hypersensitivity to salinity stress (Dong et al. 2012).

Maize

Maize (*Zea mays*) is an important cereal crop in the world after wheat and rice. Ethylene regulates diverse aspects of maize growth and development. The gene families for ethylene biosynthesis, receptors, EIN2, and EILs have been identified in maize (Gallie and Young 2004). The ethylene biosynthetic genes are functionally

characterized in more detail. The *ACS* gene family is composed of three members (i.e., *ZmACS2*, *ZmACS6*, and *ZmACS7*), and the *ACO* gene family is composed of four members (i.e., *ZmACO15*, *ZmACO20*, *ZmACO31*, and *ZmACO35*) (Gallie and Young 2004). These ethylene biosynthetic genes are differentially regulated during seed development and in maize roots in response to hypoxia (Gallie and Young 2004; Geisler-Lee et al. 2010). Characterization of the *ZmACS6* null mutant reveals that this gene plays a major role in maize leaf and root development, as *Zmacs6* mutant exhibits multiple phenotypes including delayed leaf senescence under normal growth conditions and inhibited drought-induced senescence, and increased root growth when largely unimpeded and reduced root growth in the soil (Young et al. 2004; Gallie et al. 2009). Heterologous expression of the mutated *ZmERS1b* or *ZmETR2b* gene harboring the *Arabidopsis etr1-1*-like dominant negative mutation confers ethylene insensitivity and delayed leaf senescence in the transgenic *Arabidopsis*, indicating functional conservation between the maize and *Arabidopsis* ethylene receptors (Chen and Gallie 2010). As for ZmEIN2, ZmEILs, and ZmERFs, their biological functions remain to be determined. Unfortunately, there is no report so far on ethylene-related biotechnological applications in maize, which may be due to the difficulty in genetic transformation of maize plants.

Legume

Legume plants include some important food and forage crops, such as soybean (*Glycine max*), peanut (*Arachis hypogaea*), peas (*Pisum sativum*), beans (*Phaseolus vulgaris*), *Medicago*, and *Lotus*. Ethylene plays an important role in root nodule development of most legumes. In *M. truncatula*, ethylene-insensitive mutant *MtSkl1*/*Mtein2* exhibits dramatically increased nodule number per plant, indicating that MtEIN2-midiated ethylene signaling negatively regulates legume symbiosis (Penmetsa and Cook 1997; Penmetsa et al. 2008). Consistently, ethylene-insensitive transgenic *L. japonicus* expressing the *Arabidopsis etr1-1* gene displays increased nodulation (Dasharath Lohar et al. 2009). However, unexpectedly, the ethylene-insensitive *L. japonicus* mutant *enigma*/*Ljein2* exhibits phenotypes lacking the expected hypernodulation response which is proposed to be bypassed by a duplicated copy of *LjEIN2* (Chan et al. 2013). In soybean, neither ethylene-insensitive mutations nor blocked ethylene signaling by Ag^+ treatment can affect nodule number, indicating that regulation of soybean nodulation is independent of ethylene signaling (Schmidt et al. 1999). In contrast, however, one report shows that treatment of soybean roots by ethylene or ACC can inhibit its nodulation (Caba et al. 1999). Collectively, ethylene plays mostly a negative role in regulation of nodulation, but the effects appear complicated in some legumes such as *Lotus* and soybean. Further efforts are needed to dissect the involvement of ethylene in nodulation of these species.

In summary, ethylene-related biotechnological applications have been successfully achieved in extending fruit shelf life and in producing longer-lived flowers. For fruits, reversible inhibition of ethylene effects is preferred, as fruit ripening is

eventually required. Thus manipulation of ethylene action at biosynthetic level is more acceptable in fruit plants. For floral plants, unlike fruits, complete block of ethylene signal transduction is a preferred strategy. In all cases, the use of tissue-specific or inducible promoters is recommended to overcome the side effects caused by alternated ethylene biosynthesis or signaling. In crop plants, biotic and abiotic stresses are a major constraint to agricultural productivity. As a stress hormone, ethylene enables plants to adapt to multiple stressful environments. Thus, ethylene-related researches in crops have mainly focused on plant stress adaptation. Alternation in ethylene biosynthesis or signaling mediated by the upper components (from receptors to EIN2) often leads to pleiotropic effects on plant growth and stress responses, some of which are undesirable. Alternatively, ERF can serve as an ideal target for transgenic manipulation of ethylene action for improvement of plant stress tolerance, owing to their specificity of individual members in regulating stress response. Successful application of this strategy largely depends upon further identification of the corresponding *ERF* genes.

Perspectives

Major advances in our understanding of the molecular mechanisms for ethylene biosynthesis, signaling, and interaction with other hormones have been achieved during the past two decades. Such knowledge has been successfully applied in plant genetic improvement (e.g., reducing post-harvest losses, delaying flower senescence, and improving stress tolerance). Nevertheless, many issues are still unresolved. (1) Ethylene biosynthetic pathway shares a common precursor/substrate SAM with a number of metabolic pathways such as polyamine synthesis and methylation reactions. Yet how these pathways influence each other is less obvious. Additionally, just because of this, when manipulating ethylene biosynthesis, interpretation of the results should be very cautious because either blocked or promoted ethylene biosynthetic pathway should conversely affect the SAM fluxes which in turn may influence the SAM-related metabolic pathways (an example is given in Guptaa et al. 2013). (2) The exact biochemical mechanisms of action of most of the signaling components remain unclear. For example, how ethylene binding affects receptor's activity, how ethylene receptors would transfer the signal to the CTR1, and how EIN2 C-terminus activates EIN3. Additionally, what the ethylene receptors will do during plant growth when ethylene is not available or at very low concentration. Moreover, since *ctr1-1* is still responsive to ethylene, what the remaining alternative components are. How ethylene would desensitize the ethylene response is also an open question. (3) Ethylene signaling pathway is established in *Arabidopsis*, and the main focus is on dicot plants. Little is known about the ethylene actions in monocot plants, although people have long believed that ethylene signaling mechanism is conversed between dicot and monocot plants. Emerging evidence suggests that monocot rice plants likely possess both conserved and diverged signaling mechanisms (Ma et al. 2013). Thus exploring the molecular mechanisms of

ethylene action in rice and/or other plants should lead to a more complete picture of the ethylene signaling. (4) Ethylene interacts extensively with other hormones and various developmental factors in regulating plant growth, development, and stress responses. However, the regulatory network is far from clear. Overall, addressing these issues will enable us more precisely manipulating ethylene actions in plant production.

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Gibberellin Implication in Plant Growth and Stress Responses

Eugenio G. Minguet, David Alabadí, and Miguel A. Blázquez

Abstract Hormones gibberellins (GAs) are a class of diterpenoid acids that control many aspects of plants' life, including both developmental processes and stress responses. Nowadays, we have a good understanding of how GA levels are regulated and how this information is translated into physiological responses, mainly through genetic and biochemical approaches carried out during the last two decades in rice and *Arabidopsis*. Here, we review the current knowledge of the GA pathway from GA metabolism to the downstream responses and pay special attention to the regulatory molecular mechanisms. GA biosynthesis starts in plastids, whereas its last steps, and also the GA inactivation, take place in the cytosol. Importantly, the expression of gene coding enzymes that catalyze limiting steps, for example, the soluble GA 20-oxidases, is usually regulated by environmental cues, making the GA level very sensitive to changes in the environment. The binding of the hormone to the GID1 receptor provokes the degradation of the master negative regulators in the pathway, the transcriptional regulators DELLA proteins, and GA-promoted responses proceed. The biochemical basis of the GID1-GA-DELLA regulatory module is well established, but how DELLA proteins regulate downstream events is a matter of current intensive research. In this regard, the regulation of transcription factors' activity through direct physical interaction seems to be an extended yet not unique mechanism of DELLA action. Finally, how all this wealth of information is being used with biotechnological purposes is also discussed.

Keywords Gibberellins • Metabolism • Signal transduction • DELLA • Growth • Stress

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Brief History of Gibberellin Research

Gibberellins (GAs) are a class of diterpenoid acids that regulate many aspects of plant growth and development including seed germination, stem elongation, leaf expansion, and flower and fruit development (Sun 2010). We have to go back in time until the beginning of the twentieth century to find the first steps that led to the discovery of GAs. Briefly, first investigations were carried out by Japanese pathologists studying a disease in rice called "bakanae" or "foolish seedlings" that caused considerable economic loss. Seedlings with the disease were slender and yellowish, and the disease had strongly diminished the grain production, whereas in many cases, the most affected seedlings died. In the 1920s the necrotroph fungus *Gibberella fujikuroi* was identified as the causative agent of the disease, when it was showed that treatment of rice and maize seedlings with cell-free medium where the fungus was grown caused the disease symptoms. In the next decade, "gibberellin" was coined as the name of the active substance from the fungus causing the disease, and two active crystalline forms were isolated and named gibberellins A and B. In the 1950s, large-scale fermentation procedures allowed two laboratories, in the United States and in the United Kingdom, to isolate independently a new form of active GA, called gibberellic acid (GA_3). Importantly, the structural studies defined GA_3 as a tetracyclic-dihydroxy-lactonic acid. The original gibberellin A preparation was determined to be a mixture of GA_1, GA_2, and GA_3, with the latter being the major component. The studies of effects of GA_3 in plants and fungi were parallel with the discovery of the extended natural occurrence of these substances in many plants. GA-like substances were identified mainly from developing seeds, shoots, or fruits, and shortly later GA_1 was purified from seeds of several *Phaseolus* species. Nowadays, 126 GAs have been identified in plant and fungi, most of which are nonactive metabolic intermediates in the production of the active forms GA_1, GA_3, GA_4, and GA_7.

Gibberellin Metabolism

Biosynthesis and Catabolism

Currently, we have a good understanding of the GA metabolic pathway. A combination of the biochemical and molecular approaches that led to the purification of some enzymes and their genes in species, such as pumpkin, using classic forward genetics performed mainly in *Arabidopsis* and rice, has allowed the discovery of the main players involved in the GA biosynthetic and catabolic pathways (Fig. 1).

The first stage in the GA biosynthesis pathway takes place in plastids and starts with the synthesis of *ent*-kaurene from geranylgeranyl diphosphate (GGDP), a common precursor for diterpenoids, chlorophylls, or carotenoids (Lichtenthaler 1999). Most of the GGDP devoted for the GA biosynthesis is provided by the

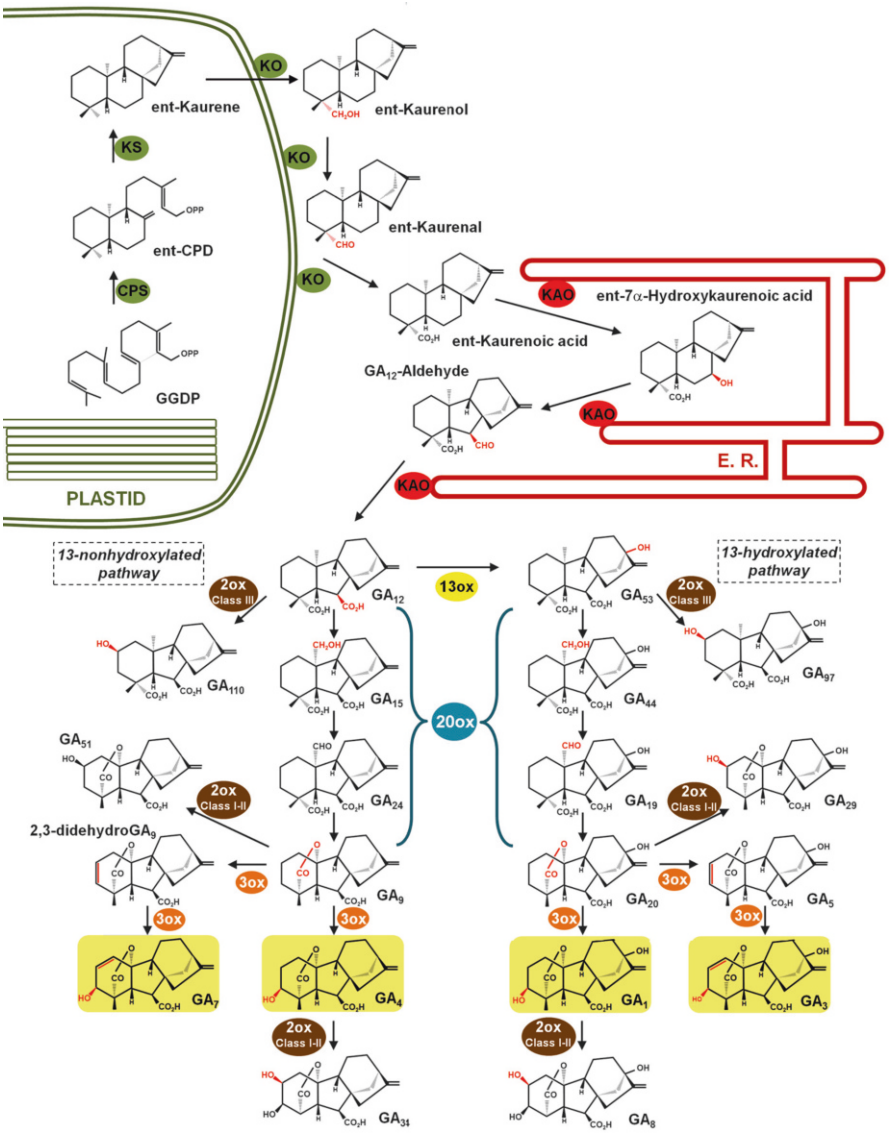

Fig. 1 The GA metabolic pathway. *CPS ent*-copalyl diphosphate synthase, *KO ent*-kaurene oxidase, *KAO ent*-kaurenoic acid oxidase, *13ox* GA 13-oxidase, *20ox* GA 20-oxidase, *3ox* GA 3-oxidase, *2ox* GA 2-oxidase. Active GAs are highlighted in *yellow*. Modifications in GA molecules due to the preceding enzymatic activity appear in *red. E.R.* endoplasmic reticulum

methylerythritol phosphate pathway in the plastid, although there is also a minor contribution from the cytoplasmic mevalonate pathway (Kasahara et al. 2002). Two terpene synthases participate in the conversion of GGDP to *ent*-kaurene: *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) (Sun and Kamiya 1994;

Saito et al. 1995; Yamaguchi et al. 1998b). These two steps were defined genetically with the GA-sensitive, severe dwarf *Arabidopsis* mutants *ga1* and *ga2* (Koornneef and Van der Veen 1980). CPS and KS are both encoded by a single gene in *Arabidopsis* as in many plant species, thus explaining the strong phenotype conferred by the null alleles. The expression pattern of *CPS* is cell-type specific in *Arabidopsis* with very low levels of transcript throughout development and high expression associated to active growing tissues (Silverstone et al. 1997a). A similar expression pattern has been described for *KS* gene but with the overall amount of transcript being higher than that of *CPS* (Silverstone et al. 1997a; Yamaguchi et al. 1998b), suggesting that the expression and location of *CPS* control the synthesis of *ent*-kaurene, what is supported by the dramatic increase in *ent*-kaurene accumulation in *Arabidopsis* lines overexpressing *CPS*, whereas no changes are detected in lines overexpressing *KS* (Fleet et al. 2003). Interestingly, overexpression of either *CPS* or *KS* genes in transgenic *Arabidopsis* lines does not result in increased levels of GAs, indicating that these two steps are not limiting (Fleet et al. 2003).

In the next stage, *ent*-kaurene is converted to GA_{12} by the consecutive action of two cytochrome P450 monooxygenases: the *ent*-kaurene oxidase (KO) catalyzes the conversion of *ent*-kaurene to *ent*-kauronic acid (Helliwell et al. 1998), which is subsequently converted to GA_{12} by an *ent*-kauronic acid oxidase (KAO) (Helliwell et al. 2001a). The step catalyzed by KO was defined genetically with the GA-sensitive dwarf mutant *ga3* (Koornneef and Van der Veen 1980). Transient expression experiments of green fluorescent protein fusions indicate that KO is mainly present in the cytosolic side of the outer membrane of the plastid, whereas KAO is located in the endoplasmic reticulum (ER) (Helliwell et al. 2001b). KO is encoded by a single gene in most species whereas KAO is encoded by two gene copies in some species, such as *Arabidopsis* (Yamaguchi 2008). In this species, both *AtKAO1* and *AtKAO2* are expressed in all tissues examined (Helliwell et al. 2001a) whereas some specificity has been found for the expression of these genes in pea, for instance, *PsKAO2* is detected only in seeds, thus explaining the normal seed development in the dwarf mutant *na*, which is defective in PsKAO1 (Davidson et al. 2003).

At this point, GA_{53} is synthesized by 13-hydroxylation of GA_{12}, a reaction that splits the pathway in two, the non-13-hydroxylated and 13-hydroxylated pathways committed to the synthesis of GA_4/GA_7 and GA_1/GA_3, respectively. GA_1 is present in rice and many other plants as the most abundant bioactive GA, but in *Arabidopsis* and several *Cucurbitaceae* species, GA_4 is the predominant bioactive GA. Interestingly, the affinity to the GA receptor GIBBERELLIN-INSENSITIVE DWARF1 (GID1) for GA_1 is lower than for GA_4 (see next sections) (Ueguchi-Tanaka et al. 2005; Nakajima et al. 2006). The presence of two pathways leading to the biosynthesis of active GAs is intriguing. Moreover, the fact that the gene or genes coding for enzymes that catalyze the 13-hydroxylation of GA_{12} have been unknown for many years has hampered the functional, genetic analysis of the relative relevance of each pathway. Remarkably, it has been demonstrated very recently that two *CYTOCHROME P450* (*CYP*) genes in rice, *CYP714B1* and *CYP714B2*, encode enzymes with the long sought GA 13-hydroxylation activity (Magome et al. 2013). Mutant rice plants deficient in GA 13-hydroxylation, *cyp714b1 cyp714b2*, have increased levels of 13-H GAs whereas those of 13-OH GAs were decreased,

indicating these two genes perform a major role in the GA 13-hydroxylation pathway in rice. In agreement with this, 13-OH GA levels were increased when any of these genes were overexpressed in *Arabidopsis* plants. Importantly, the uppermost internode at the heading stage of the *cyp714b1 cyp714b2* mutant rice was more elongated than the wild type, whereas the overexpression of any of the genes produced semidwarf *Arabidopsis* plants, despite levels of GA_1 were increased by 10-fold (Magome et al. 2013). These results suggest that the presence of the 13-hydroxylation pathway might provide the plant with a mechanism to finely regulate the relative levels of GA_4 and GA_1 as a way to control the strength of the response, given the different affinities of each GA species for the GID1 receptor. For instance, induction of GA 13-hydroxylation activity triggered by an environmental cue in a certain tissue would attenuate the response, compared to a situation in which the only active pathway was the non-13-hydroxylation. Detailed phenotypic characterization of mutant plants of other species lacking the GA 13-hydroxylation activity, as well as the expression profiling of their genes is necessary to understand the physiological relevance of each pathway.

In the third stage, the pathway reaches the synthesis of bioactive GAs by two parallel chains of oxidative reactions on carbons 20 and 3 and is catalyzed by two 2-oxoglutarate-dependent dioxygenases (2ODD): GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox), respectively. GA20ox catalyzes sequential oxidations that convert $GA_{12/53}$ into $GA_{9/20}$ (Lange et al. 1994), whereas GA3ox adds a 3β-OH group to synthesize the bioactive $GA_{4/1}$ (Williams et al. 1998). GA20ox and GA3ox are usually encoded by small families of genes, for instance, there are five and four genes encoding for GA20ox and GA3ox in *Arabidopsis*, respectively (Hedden and Phillips 2000), that are differentially regulated by developmental and environmental signals (see below). These two steps were defined genetically with the *Arabidopsis* GA-sensitive semidwarf mutants *ga4* and *ga5* (Koornneef and Van der Veen 1980) that encode GA3ox1 (Chiang et al. 1995) and GA20ox1 (Xu et al. 1995), respectively. Contrary to what was observed after overexpressing genes coding for enzymes catalyzing the first steps in the pathway (Fleet et al. 2003), a typical GA overdose phenotype was observed when GA20ox genes were overexpressed in transgenic plants of several species (Huang et al. 1998; Coles et al. 1999; Vidal et al. 2001; Fagoaga et al. 2007; Gallego-Giraldo et al. 2008; Garcia-Hurtado et al. 2012). However, overexpression of *GA3ox* genes either in poplar or tobacco did not produce major morphological changes and did not affect GA_4/GA_1 levels (Israelsson et al. 2004; Gallego-Giraldo et al. 2008), indicating that 20-oxidation rather than 3-oxidation is the limiting step for the synthesis of bioactive GA.

Phenotypes of the different mutants in these genes are in accordance with their expression patterns. For instance, *GA20ox1*, *GA20ox2*, and *GA20ox3* are the most highly expressed genes in many tissues examined, with *GA20ox1* showing the highest expression in stems consistent with the semidwarf phenotype of the *ga5*, which is defective in GA20ox1 activity (Rieu et al. 2008b). Further genetic analyses have demonstrated that GA20ox1, GA20ox2, and GA20ox3 are broadly the more prominent activities in *Arabidopsis* and that plants lacking the three of them are severe dwarfs very similar to *ga1* mutants (Plackett et al. 2012).

In the case of GA3ox, *GA3ox1* and *GA3ox2* are expressed both in vegetative and reproductive tissues (Mitchum et al. 2006), whereas *GA3ox3* and *GA3ox4* are expressed mainly in the latter (Mitchum et al. 2006; Hu et al. 2008). Again, both the pattern and the strength of the expression correlate well with the phenotypes of the corresponding mutants. For example, the most highly expressed gene in stems is *GA3ox1* correlating with semidwarf phenotype of *ga4* plants, lacking GA3ox1 activity, whereas the double *ga3ox1 ga3ox2* is similar to the *ga1* mutant indicating that both are the relevant GA3ox activities controlling vegetative development (Mitchum et al. 2006; Hu et al. 2008). Similarly, clear floral defects are displayed in the triple mutant *ga3ox1 ga3ox3 ga3ox4* (Hu et al. 2008).

The levels of active GAs depend not only on the flow through the biosynthetic pathway but also on different mechanisms that inactivate the active GAs and their precursors (Fig. 1). The major and best-characterized deactivation pathway is the 2β-hydroxylation, which is catalyzed by the GA 2-oxidase (GA2ox), a class of 2ODD that were first characterized at the molecular level from a runner bean (Thomas et al. 1999). GA2ox are organized in classes I, II, and III based in their phylogenetic relationship. GA2ox of classes I and II use C_{19}-GAs as substrates, i.e., GA_9, GA_{20}, GA_4, GA_1, and GA_7, while GA2ox of class III use C_{20}-GAs, i.e., GA_{12} and GA_{53} (Thomas et al. 1999). Nonetheless, a GA2ox in cucumber has been described recently that can use both C_{19} and C_{20} GAs as substrate (Pimenta Lange et al. 2013), which makes the functional distinction less clear. Again, these enzymes are encoded by small gene families in several species, for example, there are six and seven genes coding for them in rice and *Arabidopsis*, respectively (Yamaguchi 2008). When overexpressed in transgenic plants cause severe dwarfism (Schomburg et al. 2003), and in agreement, the converse phenotype is observed in plants in which the GA 2-oxidase activity is genetically compromised in pea and *Arabidopsis* (Martin et al. 1999; Rieu et al. 2008a). In particular, *Arabidopsis* mutant plants defective in the five class I and II *GA2ox* genes present additional phenotypes other than extreme elongation, such as striking defects in pistil and fruit development (Schomburg et al. 2003).

Deactivation can also be done by epoxidation of the 16,17-double bond of non-13-hydroxylated GAs, a reaction catalyzed by a P450 (CYP714D1) identified in the tall rice mutant *elongated uppermost internode* (*eui*) (Zhu et al. 2006). The GAs 16,17-dihydrodiols are found in many plant species indicating that it can be a general deactivation mechanism. Indeed, genetic characterization of the two *Arabidopsis* orthologs of EUI has shown that this deactivation mechanism also contributes to the regulation of development by GAs in this species (Zhang et al. 2011a). Moreover, it has been described that methylation of the C6 carboxyl group of GAs by GAMT1 (GIBBERELLIN METHYLTRANSFERASE1) and GAMT2 also contributes to GA inactivation in *Arabidopsis* (Varbanova et al. 2007). Overexpression of these genes produced a GA deficiency phenotype, whereas the double mutant showed less inhibition of germination than the wild type in the presence of an inhibitor of GA synthesis, in accordance with their predominant expression in developing and germinating seeds. This deactivation pathway might be present in more plant species given that heterologous ectopic expression of *Arabidopsis* GAMT1 in tobacco and petunia caused dwarfism (Varbanova et al. 2007). Nonetheless, further investigations are

needed to determine how extended and relevant the GA-methylation mechanism is. Finally, GAs can also be conjugated with sugars but little is known about their functional relevance and several possibilities have been suggested: they might serve as storage of GAs; it might represent an additional way to deactivate GAs; or they might have a biological function (Piotrowska and Bajguz 2011). Identification of GA-glycosyl transferases will clarify the importance of these conjugates in plant development.

Regulation of Gibberellin Metabolism

The broad implication of GAs in plant development is strictly associated to tight regulation of their metabolism by multiple environmental and endogenous factors, ranging from light and temperature to other hormones including feedback control by GAs themselves. As explained below, most of this regulation is exerted via transcriptional control.

GA homeostasis is achieved through feedback and feedforward mechanisms acting mainly on *GA20ox*, *GA3ox*, and *GA2ox* genes, but not on the genes encoding CPS, KS, and KO (Hedden and Phillips 2000; Yamaguchi 2008). In *Arabidopsis*, expression of *GA20ox1*, *GA20ox2*, *GA20ox3*, and *GA3ox1* is downregulated by GA treatment whereas *GA2ox1* and *GA2ox2* are upregulated (Phillips et al. 1995; Xu et al. 1995; Thomas et al. 1999; Matsushita et al. 2007). However, *GA3ox2*, *GA3ox4*, *GA20ox4*, and *GA20ox5* do not show the regulation by the feedback mechanism, at least under these physiological circumstances (Matsushita et al. 2007; Rieu et al. 2008b), indicating that there may be developmental or environmental situations in which feedback regulation needs to be uncoupled from other signals. The molecular mechanism that directs this regulation has not been completely identified, but it definitely involves GA signaling elements including the soluble GA receptor encoded by *GID1* and the DELLA proteins (see below). For instance, loss of DELLA function causes reduced levels of *GA3ox1* expression (Dill and Sun 2001; King et al. 2001a), while mutants defective in the GID1 receptor show increased expression of *GA20ox* genes (Ueguchi-Tanaka et al. 2005).

Two transcription factors have been identified with a putative role in the execution of feedback regulation. *REPRESSION OF SHOOT GROWTH* (*RSG*) encodes a tobacco basic leucine zipper (bZIP) transcriptional activator (Fukazawa et al. 2000). Expression of a dominant negative version of *RSG* provokes dwarfism in tobacco and prevents feedback regulation by GAs (Ishida et al. 2004). Wild-type RSG is normally translocated into the nucleus when GA levels are low, in a process regulated by 14-3-3 proteins, which suggests a possible connection between these proteins and GA function. In addition, the AT-hook protein encoded by *AGF1* in *Arabidopsis* has been shown to bind a *cis* element in the *GA3ox1* promoter required for feedback regulation (Matsushita et al. 2007), although it is unknown how this putative transcription factor would mediate GA control of gene expression.

Recently, an attempt has been made to integrate current knowledge of feedback regulation of GA metabolism in a mathematical model to investigate the relevance of the different loops (Middleton et al. 2012). Interestingly, the model highlights the

importance of the feedback regulation of *GA20ox*, while the other individual feedback loops have only minor contributions, at least in roots, to GA homeostasis.

Other hormones have also been proposed to exert part of their action through the modulation of GA levels in different tissues. Given the multiple interactions between the different hormone pathways, it is difficult, in many cases, to establish the major mechanism for the interaction (i.e., whether a hormone primarily regulates GA signaling and this causes an indirect effect on GA metabolism through feedback regulation or whether this hormone regulates GA metabolism directly). The least controversial case is the regulation of GA metabolism by auxin. It has been convincingly shown that auxin modulates the expression of several GA metabolism genes resulting in a net increase of bioactive GAs. For instance, it has been shown that auxin is necessary to reach appropriate GA levels in elongating pea internodes (Ross et al. 2000). Reduction of auxin levels by decapitation (removal of the apical bud) also reduced the levels of GA_1 which can be reversed by IAA application, which correlates with an induction of *PsGA3ox1* and a repression of *PsGA2ox1* (O'Neill and Ross 2002). Similar correlations have also been observed in tobacco and barley (Wolbang and Ross 2001; Wolbang et al. 2004). In *Arabidopsis* seedlings auxins also induce the expression of *GA20ox1* and *Ga20ox2* and also of certain *GA2ox* genes (Frigerio et al. 2006). This apparent paradox reflects tissue-specific differences of auxin response, which is supported by the observation that the induction of the expression of *GA20ox2* and *GA2ox2* occurred in separate seedling organs. Interestingly, the regulation of GA metabolism by auxins does not require DELLA proteins, indicating that it does not occur through the interaction with GA feedback regulation (Frigerio et al. 2006; O'Neill et al. 2010).

Light is a major environmental factor that affects plant development. Many of the processes regulated by GAs are also affected by light, and in fact it has been shown for some of them that the regulation by light occurs through the modulation of GA metabolism. For instance, seed germination requires light perception through phytochromes A and B (Shinomura et al. 1996), and GA-deficient mutants are impaired in germination (Koornneef and Van der Veen 1980). The same result is obtained in wild-type plants with inhibitors of GA biosynthesis, such as paclobutrazol (PAC) and uniconazole (Nambara et al. 1991; Jacobsen and Olszewski 1993), indicating that de novo synthesis of GAs is needed during seed germination. It has been demonstrated that light induces the expression of *GA3ox* genes in seeds, while *GA2ox* gene expression is reduced thereby increasing GA levels (Toyomasu et al. 1998; Yamaguchi et al. 1998a; Oh et al. 2006; Seo et al. 2006). This regulation depends on the phytochromes, which induce the degradation of PHYTOCHROME-INTERACTING FACTOR3-LIKE5 (PIL5), a basic helix–loop–helix (bHLH) transcription factor that inhibits germination by repressing GA biosynthesis (Oh et al. 2006). Germination repression induced by overexpression of PIL5 can be rescued by GA application and light-independent germination of *pil5* is inhibited with PAC treatment. Interestingly PIL5 does not bind directly to the promoters of GA metabolism genes, but it binds to the promoters of at least two of DELLA genes, encoding RGA (REPRESSOR OF *ga1-3*) and GAI (GA INSENSITIVE) (Oh et al. 2007).

After germination, light irradiation switches from inducing to inhibiting the accumulation of bioactive GAs, still with the participation of phytochromes and cryptochromes (Gil and García-Martínez 2000; O'Neill et al. 2000). As in the case of germination, this regulation is exerted through coherent transcriptional changes in the GA metabolism genes (Reid et al. 2002; Folta et al. 2003; Zhao et al. 2007). High levels of GAs in etiolated seedlings have been found essential to maintain the repression of the photomorphogenic program (Alabadí et al. 2004), and rapid upregulation of GA2ox genes is the most likely cause of the drop in GA levels that occur upon illumination and that promote growth cessation and the initiation of photomorphogenesis (Alabadí et al. 2004, 2008; Achard et al. 2007).

Photoperiodic control of stem elongation is also mediated by GAs. Studies with different rosette plants in shifts between noninductive short days and long days have revealed that stem elongation is accompanied by an increase in the concentration of active GAs caused by induction of *GA20ox* gene expression (Lee and Zeevaart 2002, 2007). The physiological relevance of these changes is supported by the inhibition of long-day promotion of stem elongation by the application of GA biosynthesis inhibitors (Zeevaart et al. 1993). Regarding flowering, the involvement of GAs does not seem to be through a universal mechanism. While photoperiod seems to induce flowering in certain grasses like *Lolium* through the activation of GA activity, other plants like *Arabidopsis* require GAs for flowering under noninductive conditions, but their participation is minor under inductive photoperiods. Moreover, GAs inhibit, instead of promote, flowering in another set of plant species including *Citrus* (Guardiola et al. 1982). In the case of *Lolium*, the application of certain GA molecules is as efficient as a single long-day pulse to induce flowering (Evans et al. 1990), GAs applied to intact leaves are transported to the apex and promote flowering (King et al. 2001b), and long-day treatments induce a twofold increase in GA content in the apex (King et al. 2003). In the absence of environmental factors that promote flowering, *Arabidopsis* maintains the transition to the reproductive phase via the GA pathway. This is supported by the lack of flowering of the GA-deficient mutant *ga1* under photoperiodic conditions of day-length shorter than 10 h (Wilson et al. 1992). Moreover, overexpression of GA biosynthesis genes results in early flowering (Coles et al. 1999), and there is a gradual increase in GA levels in short-day growing plants approaching flowering (Eriksson et al. 2006), with *GA20ox2* being the main control point for GA biosynthesis regarding flowering (Rieu et al. 2008b). Under inductive conditions, GA biosynthesis also plays a role in floral induction, and *TEMPRANILLO* (*TEM*) genes encode transcription factors that directly regulate the expression of *GA3ox1* and *GA3ox2* genes (Osnato et al. 2012).

GA biosynthesis is also regulated by temperature. Cold stratification of imbibed seeds induces germination in many plant species. In *Arabidopsis* this cold treatment results in an increase of bioactive GAs through upregulation of *GA20ox2* and *GA3ox1* and downregulation of *GA2ox2* (Yamauchi et al. 2004). Regulation of bioactive GAs also occurs at high temperatures. At high temperatures germination is inhibited in *Arabidopsis* (thermoinhibition) to avoid seed germination in summer and initiate development in the correct season. Experimental data suggest that this process is controlled by abscisic acid (ABA) through mainly downregulating the

GA20ox and *GA3ox* gene expression (Toh et al. 2008). Upregulation of *GA3ox1* by higher temperatures has also been reported in lettuce in bolting stem elongation (Fukuda et al. 2009) and in *Arabidopsis* hypocotyls (Stavang et al. 2009).

Another condition under which the regulation GA metabolism is biologically relevant is the exposure of plants to different stress factors. In this situation, plants arrest growth as part of their defense program (Vettakkorumakankav et al. 1999). Stress-induced growth cessation occurs to a large extent through the decrease in GA levels, as indicated by the observation that overexpression of *DWARF AND DELAYED FOWERING1* (*DDF1*), encoding an AP2 transcription factor of the dehydration-responsive element binding protein/C-repeat binding factor (DREB1/CBF) subfamily involved in stress responses (Mitchum et al. 2006), exposure to cold, and *CBF1* overexpressors (Achard et al. 2008a), generates dwarf *Arabidopsis* plants, mainly by reducing levels of bioactive GAs that are more tolerant to salt and cold stress, respectively. Transcriptomic analyses have revealed upregulation of *GA2ox7* by DDF1, which can bind in vitro DRE-like motifs of *GA2ox7* promoter (Magome et al. 2008). Five additional *GA2ox* genes were upregulated under high-salinity stress, indicating additional regulation of bioactive GAs independently of DDF1.

The Gibberellin Signaling Pathway

As in the case of the elucidation of the GA metabolic pathway, genetic analyses carried out in *Arabidopsis* and rice have been fundamental to identify the core components of the GA signaling pathway, basically through the isolation and characterization of dwarf, GA-insensitive mutants. The components that form the basic skeleton of the pathway are the GA receptor GID1 (Ueguchi-Tanaka et al. 2005), the transcriptional regulators DELLA proteins (Peng et al. 1997), and the F-box proteins GID2/SLEEPY1 (SLY1) (McGinnis et al. 2003; Sasaki et al. 2003). In essence, binding of GAs to the GID1 receptor allows its interaction with DELLA proteins, which are the negative regulators in the pathway. Once this tertiary complex is formed, DELLAs are ubiquitinated and degraded by the 26S proteasome, a process mediated by the interaction of DELLAs with GID2/SLY1, thus releasing the brake on GA responses imposed by their activity (Daviere and Achard 2013).

In the next sections, we will review in detail the current knowledge of how the GA signal is translated through these elements into physiological responses.

DELLA Proteins: The Transcriptional Regulators That Repress GA Signaling

The DELLA Gene Family

The founder member of the DELLA family of transcriptional regulators was the *Arabidopsis* GAI (Peng et al. 1997). *GAI* was originally isolated in *Arabidopsis* as

a semidominant, GA-insensitive, and dwarf mutant, *gai-1* (Koornneef et al. 1985). Mutant plants showed the morphological features typically caused by GA deficiency: reduced stature, dark-green color, and compactness, among others. However, two features in *gai-1* indicated that this mutant was not impaired in the GA metabolism: (1) the insensitivity to the hormone and (2) the accumulation of high levels of active GAs (Talón et al. 1990), the latter indicating that it affected the feedback mechanism that normally operates to control the GA homeostasis (Hedden and Phillips 2000). All these evidences together pointed out that this mutation hit in a protein with a central, negative role in either GA perception or signaling (Peng et al. 1997). However, it was not until the isolation of a null allele of *GAI*, *gai-t6*, when it was unambiguously shown that the GAI protein performs a negative role in GA signaling, since the mutation conferred certain GA-independent growth: *gai-t6* plants were partially resistant to the growth-restraint effect of the GA biosynthesis inhibitor PAC (Peng et al. 1997). This ability of *gai-t6* was shared with the newly identified recessive alleles of another locus, *RGA* (Silverstone et al. 1997b), that were identified based on their ability to suppress, to a certain extent, the dwarf phenotype of the GA-deficient mutant *ga1-3*.

The molecular lesion in *gai-t6* was caused by the insertion of a *DS* transposon within the *GAI* locus in *gai-1* mutant plants, which reversed their dwarfism (Peng and Harberd 1993; Peng et al. 1997). The transposon tagged the mutant locus, allowing Peng and co-workers to uncover the molecular identity of *GAI* and thus the molecular lesion causing the *gai-1* phenotypes (Peng et al. 1997). It encodes a protein of 532 amino acids in its wild-type version, whereas it presents an in-frame deletion of 17 amino acids close to the N-end terminus in gai-1. Authors proposed that these 17 amino acids were responsible of either perceiving the GA itself, i.e., acting as a receptor, or making the protein responsive—indirectly—to the hormone. Interestingly, the name of the family was coined based on five amino acids, D-E-L-L-A, present within this region and that are highly conserved. GAI was the only member of the family for a short time. With the molecular cloning of the *RGA* locus, another member joined the DELLA family (Silverstone et al. 1998). Both proteins show 83 % of identity at the amino acid level, and this is reflected in that they perform highly redundant functions in the plant. For instance, genetic removal of GAI and RGA functions acts synergistically to restore the wild-type growth ability to the stem of the GA-deficient mutant *ga1-3* in the *ga1-3 gai-t6 rga-2* triple mutant (Dill and Sun 2001; King et al. 2001a). The completion of the *Arabidopsis* genome sequencing allowed the identification of three additional members of the family, *RGA-like1* (*RGL1*), *RGL2*, and *RGL3* (Lee et al. 2002). All these proteins act as paralogs and the redundancy showed by GAI and RGA is extended, to a certain extent, to the other DELLAs.

The molecular cloning of *GAI* in *Arabidopsis* paved the way to identify DELLA orthologs in other species. This way, it was soon unmasked that mutations in the *la cry* mutant of pea, *slender* in rice, *slender1* in barley, or *procera* in tomato affected their respective DELLA genes (Ikeda et al. 2001; Chandler et al. 2002; Jasinski et al. 2008; Weston et al. 2008). Remarkably, it was also shown that the wheat varieties introduced in the 1960s and 1970s and that were the base of the so-called green revolution due to their shorter stature and higher grain production carried molecular

lesions in one of the two wheat DELLA genes, *Reduced height 1* (*Rht-1*) (Peng et al. 1999a), similar to the one found in the *Arabidopsis gai-1*. Similar mutations were identified as the cause of the dwarf phenotype of the *d8* mutant in maize (Peng et al. 1999a) and, interestingly, also of the conversion of tendrils into inflorescences in the dwarf grapevine variety Pinot Meunier that increases considerably the fruit production (Boss and Thomas 2002).

The availability of sequence information of an ever increasing number of species has revealed that the number of *DELLA* genes in different species is quite variable, ranging from five genes in *Arabidopsis*, for example, to only one in rice, maize, or tomato (Peng et al. 1999a; Ikeda et al. 2001; Lee et al. 2002; Martí et al. 2007; Jasinski et al. 2008). The presence of more than one *DELLA* gene in many species has likely aroused during evolution due to events of gene duplication and posterior subfunctionalization of the different copies. At least, this seems to be the case in *Arabidopsis*. In this species, two DELLA proteins whose mutations cause quite different phenotypes in the plant, RGA and RGL2, are able to perform each other's role in promoter swapping experiments, i.e., RGL2 complements the lack of RGA when expressed under the *RGA* promoter in an *rga* null mutant background and vice versa (Gallego-Bartolomé et al. 2010). Importantly, expression profiles of the five *Arabidopsis DELLA* genes over more than 100 publicly available microarray experiments grouped with a topology very similar to that reproducing the phylogenetic relationship between the corresponding DELLA proteins, suggesting that the subfunctionalization between DELLAs is mainly due to different expression profiles of the corresponding genes, rather than to differences in the proteins themselves (Gallego-Bartolomé et al. 2010).

Sequence Features of DELLA Proteins

Comparison of the first DELLA sequences—GAI and RGA—to the available protein databases did not provide a clear-cut view of their possible biochemical function but a few clues that suggested that these proteins most likely act as transcriptional regulators (Peng et al. 1997; Silverstone et al. 1998). These two proteins, together with SCARECROW (SCR) (Di Laurenzio et al. 1996), are the founder members of a family of plant-specific transcriptional regulators named GRAS (from *G*AI, *R*GA, and *S*CR) (Pysh et al. 1999). Proteins belonging to this family have been found in many species, with 33 and 60 members in *Arabidopsis* and rice, respectively (Tian et al. 2004; Lee et al. 2008; Tong et al. 2009). The C-terminal two thirds in all members of this family, known as GRAS domain, are quite similar and encompass a few characteristic sequence motifs in the following order: leucine heptad repeat 1 (LHR1), VHIID, LHR2, PFRYE, and SAW (Pysh et al. 1999). The presence of the LHRs, usually involved in protein–protein interactions, a putative nuclear localization signal, and an SH2-like domain—encompassing the PFRYE and SAW motifs and found in the metazoan STAT factors—strengthened the idea that these proteins might function as transcriptional regulators (Richards et al. 2000).

Remarkably, the N-terminal part of the DELLA proteins, known as DELLA domain, makes them different from the other members of the GRAS family. Besides the abovementioned DELLA motif, two other sequence features are conserved: the TVHYNP and a polymeric Ser/Thr/Val. As we discuss below, the TVHYNP and DELLA motifs perform a similar role mediating the interaction of the DELLA protein with the GID1 receptor, whereas the polymeric Ser/Thr/Val seems to be important for the putative regulation of the protein by phosphorylation. Besides, DELLAs and some other GRAS proteins contain the motif LXXLL in the GRAS domain (Peng et al. 1997). This motif mediates the binding of transcriptional co-activators to the nuclear receptors in animals (Heery et al. 1997), suggesting that it might perform a role in transcriptional regulation in plants as well.

GA Regulation of DELLA Proteins and the Role of Their Conserved Domains

The first insights supporting the possible mode of action of DELLA proteins came when it was shown that DELLA fusions to fluorescent proteins were nuclear in *Arabidopsis*, rice, and barley as sequence analysis predicted (Silverstone et al. 1998; Gubler et al. 2002; Itoh et al. 2002). More importantly, same analyses demonstrated that the accumulation of the protein in the nucleus was dependent upon the levels of GAs, in such a way that DELLAs accumulated when GA levels were low, whereas they disappeared when GA levels were high (Silverstone et al. 2001; Gubler et al. 2002; Itoh et al. 2002; Hussain et al. 2005). In fact, treatments as short as 30 min with the hormone were enough to provoke a reduction in their levels. Remarkably, the GA-induced destabilization of DELLAs is a process dependent upon the activity of the 26S proteasome, as first demonstrated for the barley SLN1 and the rice SLR1 (Fu et al. 2002; Sasaki et al. 2003). These results pointed out that DELLAs are destabilized in response to the hormone, which agreed the idea, supported by genetic analyses, that DELLAs are the negative regulators in the pathway.

The gai-1 protein was insensitive to the GA signal, owing to the deletion within the DELLA domain (Peng et al. 1997). In an elegant approach, Dill and co-workers showed that a mutant version of RGA, rga-Δ17, and equivalent to gai-1 caused dwarfism when expressed in transgenic *Arabidopsis* plants (Dill et al. 2001). But more importantly, this mutation made the protein to be resistant to the destabilizing effects of GAs: it stayed in the nucleus independently of the levels of the hormone, thus continuously repressing GA-regulated processes. Therefore, these results indicated that the DELLA domain was critical for the GA-induced destabilization of the protein. The importance of the DELLA domain for the destabilization of the protein was confirmed with other dwarfing mutations affecting this particular domain. For instance, a single amino acid change within this domain in the barley *Sln1d* stabilized the protein in barley (Gubler et al. 2002) and also when expressed in transgenic *Arabidopsis* plants (Willige et al. 2007). Similarly, deletion mutants affecting the DELLA, the TVHYNP, or both motifs equivalent to the ones present

in the "green revolution" dwarfing alleles of wheat and maize—Rht, d8-1, and d8-mp—and similar mutant versions of SLR1 and RGL2 were all stabilized when expressed in *Arabidopsis*, rice, and tobacco BY2 cells, respectively (Itoh et al. 2002; Hussain et al. 2005; Willige et al. 2007). As expected, dwarf or semidwarf phenotypes were obtained. Moreover, expression of a deletion mutant of SLR1 that lacks the polymeric Ser/Thr/Val caused GA-responsive, severe dwarf rice plants, suggesting that this is an important regulatory region that normally attenuates DELLA repressive activity but that it is not required for GA responsiveness (Itoh et al. 2002).

The importance of other conserved motifs for the activity of DELLA proteins has been demonstrated through the identification of point mutations in several alleles from different species. For instance, *rga-1* in *Arabidopsis*; *slr1-2*, *slr1-3*, and *slr1-4* in rice; and *sln1c* in barley all generate a premature stop codon within the SAW motif (Silverstone et al. 1998; Ikeda et al. 2001; Chandler et al. 2002), thus producing a truncated polypeptide lacking a few amino acids at the very end of the protein, as demonstrated for rga-1 and sln1c (Gubler et al. 2002; Dill et al. 2004). Moreover, deletion of the Asn^{562} that lies within the SAW motif in rga-22 causes a similar phenotype (Dill et al. 2004). Similarly, the recessive *procera* mutation in tomato and *rga-2* of *Arabidopsis* caused an amino acid change at a conserved position within the VHIID and PFRYE motifs, respectively (Silverstone et al. 1998; Jasinski et al. 2008). The recessive nature of these alleles indicates that the VHIID, PFRYE, and SAW motifs are important for the repressive activity of DELLAs.

The F-Box Proteins GID2/SLY1 Mediate the GA-Induced Degradation of DELLAs

The pathway that defines the degradation of DELLA proteins by the 26S proteasome was identified genetically, with the isolation and characterization of two recessive, GA-insensitive mutants, *sly1* in *Arabidopsis* and *gid2* in rice, that caused dwarfism among other GA-related phenotypes, such as impairment of the GA induction of α-amylase gene expression in rice (Steber et al. 1998; Sasaki et al. 2003). The fact that these mutations were recessive was remarkable, since the only ones causing similar phenotypes were the semidominant alleles of the negative regulators DELLA proteins, suggesting that in this case the mutations likely hit in a novel protein performing a positive role in the pathway.

GID2/SLY1 Encodes F-Box Proteins That Interact with DELLAs in Response to GAs

The positional cloning of the genes affected by *sly1* and *gid2* revealed that indeed this was the case, since both mutations affected homologous genes coding for a small, plant-specific novel F-box protein (McGinnis et al. 2003; Sasaki et al. 2003). Proteins having an F-box form part of the multi-protein SCF-type E3 ubiquitin

ligases, which are in charge of attaching a polyubiquitin chain to the protein target previous to its degradation by the 26S proteasome (Lechner et al. 2006). Three other subunits form part of the SCF complex: S PHASE KINASE-ASSOCIATED PROTEIN1 (SKP1), RING BOX1 (RBX1), and CULLIN1 (CUL1). The F-box protein interacts with the target and therefore provides specificity to the complex, whereas SKP1 and CUL1 perform scaffold functions, and RBX1 catalyzes the attachment of ubiquitin moieties. The F-box itself attaches the F-box protein to the complex through its interaction with SKP1, whereas the recognition of the target proteins occurs through a domain usually at the C-terminal of the F-box. In fact, co-immunoprecipitation analyses showed that SLY1 and GID2 associate in vivo with CUL1 and with members of the SKP1 family (Fu et al. 2004; Gomi et al. 2004), indicating that they indeed form part of SCF complexes in the plant.

Comparison of the SLY1 and GID2 with their homologs from other plant species revealed the presence of two other conserved domains—GGF and LSL—present in the putative target recognition region (McGinnis et al. 2003). The importance of these two domains is supported by the fact that the *gid2* and *sly1* alleles all affect the C-terminal part of the protein (McGinnis et al. 2003; Sasaki et al. 2003), and deleted versions of GID2 lacking any of the two domains did not complement the *gid2* phenotype (Gomi et al. 2004), suggesting that they might impair target recognition. The most obvious candidates to be targeted for degradation by GID2/SLY1 were the DELLA proteins. Indeed, SLR1 and RGA over-accumulated in *gid2* and *sly1* mutants, respectively, and this accumulation was not ameliorated by GA treatment (McGinnis et al. 2003; Sasaki et al. 2003), suggesting that the dwarf phenotype of the mutants was a consequence of the accumulated DELLA proteins. This was demonstrated genetically, as null alleles of *gai* and *rga* in *Arabidopsis*, or *slr1* in rice, reverted the dwarf phenotype of *sly1* and *gid2*, respectively (McGinnis et al. 2003; Sasaki et al. 2003; Dill et al. 2004; Fu et al. 2004).

The biochemical evidences supporting the idea that DELLAs are targeted for degradation by GID2/SLY1 through physical interaction came independently from three labs working in *Arabidopsis* and rice. First, it was shown that SLY1 and GID2 are nuclear proteins, like DELLAs, therefore sharing the intracellular localization (Dill et al. 2004; Gomi et al. 2004). Second, the interaction between both proteins was shown by different means, such as pulldowns in vitro and in vivo (Dill et al. 2004; Fu et al. 2004; Gomi et al. 2004) and yeast two-hybrid (Y2H) assays (Dill et al. 2004; Fu et al. 2004). The importance of the LSL domain of SLY1 for the interaction was demonstrated by Y2H, showing that both the sly1-10 mutant protein lacking the last eight amino acids and a deleted version lacking the whole LSL motif were unable to interact with RGA and GAI (Dill et al. 2004; Fu et al. 2004). Conversely, deletion analysis of GAI showed that the interaction with SLY1 occurs through the GRAS domain, and accordingly, the rga-1 mutant protein that lacks the last 67 amino acids of the protein affecting this domain accumulates in the plant and is resistant to GA-induced degradation (Dill et al. 2004).

The model was reinforced with the molecular characterization of the *gai revertant2* (*gar2*) mutant of *Arabidopsis*, identified as a dominant suppressor of the dwarf phenotype of *gai-1* (Wilson and Somerville 1995). Remarkably, the *gar2* mutation

caused reduced accumulation of the dominant gai-1 and rga-Δ17 proteins, suggesting that the GAR2 function was closely related to DELLAs (Dill et al. 2004; Fu et al. 2004). The *gar2* mutation resulted to be a new allele of *SLY1*, identified independently at Nicholas Harberd's and Tai-ping Sun's laboratories through positional cloning (Fu et al. 2004) and by a candidate approach (Dill et al. 2004), respectively. The mutant protein, SLY1$^{gar2\text{-}1}$, carries a Glu-to-Lys amino acid change within the LSL motif, at position 138 that is highly conserved. Importantly, and in agreement with the idea that this motif is critical for the interaction with DELLAs, Y2H and pulldown assays demonstrated that the SLY1$^{gar2\text{-}1}$ protein is able to interact more strongly than the wild-type version with its targets, thus providing an explanation for its dominant phenotype (Dill et al. 2004; Fu et al. 2004).

In *Arabidopsis*, there is a SLY1 homolog called SNEEZY (SNE)/SLY2, showing 33 % homology at the amino acid level (Fu et al. 2004; Strader et al. 2004; Ariizumi et al. 2011). Genetic analyses of *sne* and *sne sly* mutations indicate that SNE/SLY2 participates in the GA signaling pathway, although performing a less prominent role than SLY1. The *sne* mutant did not show any evident phenotype, whereas this mutation enhanced the phenotypes of a null *sly* allele (Ariizumi and Steber 2011). Moreover, expression of SNE/SLY2 in a *sly1* mutant background was able to suppress, to a certain extent, its phenotypes, and having the same domain requirements with SLY1 suggests that both proteins perform the same biochemical function (Ariizumi et al. 2011). In fact, SNE/SLY2 interacts in vivo with CUL1 and therefore forms part of an SCF complex (Ariizumi et al. 2011). Nonetheless, certain substrate specificity differentiates both proteins, since SLY1 but not SNE was able to interact with the DELLA protein RGL2 (Ariizumi et al. 2011).

Phosphorylation of DELLAs and Their GA-Induced Degradation

Studies in *gid2* also showed that two forms of SLR1 accumulated in the mutant, being the form with the lower electrophoretic mobility phosphorylated (Sasaki et al. 2003). In fact, treatments with inhibitors of either protein kinases or protein phosphatases prevented degradation of the barley SLN1 in response to GAs (Fu et al. 2002). Moreover, the *Arabidopsis* gai-1 accumulates as a phosphorylated protein as well, and it interacts more efficiently with SLY1$^{gar2\text{-}1}$ than the non-phosphorylated version (Fu et al. 2004), whereas only the phosphorylated SLR1 was able to bind GID2 in vitro (Gomi et al. 2004). All these results were in line with the accepted idea that proteins targeted for degradation by the 26S proteasome have to be modified posttranslationally, phosphorylated in this case.

Posterior studies, though, did not support a direct role of DELLA phosphorylation in its GA-induced degradation. For instance, RGL2 was found to be phosphorylated in the plant, and treatments with inhibitors of either Ser/Thr protein phosphatases or Tyr protein kinases prevented its degradation in response to GAs in BY2 tobacco cells (Hussain et al. 2005, 2007). Moreover, site-directed mutagenesis of several candidates Ser, Thr, and Tyr residues indeed generated GA-resistant versions of RGL2, but that lost most of their repressive activity, making authors to

suggest that this effect was likely due to conformational defects caused by the amino acid changes rather than an alteration in the phosphorylation status of the protein (Hussain et al. 2005, 2007). Wang and co-workers also found that phosphatase inhibitors prevented degradation of RGA in cell-free degradation assays (Wang et al. 2009). Same authors, however, stressed the importance of interpreting these results with caution, since inhibitors might be affecting the phosphorylation status of a regulatory element needed for GA-induced degradation of DELLAs, rather than the phosphorylation of the DELLA itself. In the same line, studies carried out in rice calli demonstrated that both phosphorylated and non-phosphorylated versions of SLR1 are degraded with the same kinetics in response to GAs, indicating that this modification is irrelevant for the degradation of the protein (Itoh et al. 2005).

Besides these fuzzy results, genetic analyses in rice shed some light on the role of phosphorylation in DELLA activity, with the characterization of the *el1* mutant that hit the gene coding for casein kinase I (*CKI*) (Dai and Xue 2010). *el1* mutants had enhanced GA signaling, suggesting that CKI activity was needed to suppress it, and indeed SLR1 was more efficiently degraded in response to GAs in the *el1* mutant than in the wild type. Remarkably, authors showed that SLR1 interacts physically with and is phosphorylated by CKI, being this phosphorylation important to keep SLR1 activity in vivo. Nonetheless, it is not clear from this work if the enhanced degradation of SLR1 in the mutant is also a direct consequence of the lack of phosphorylation of SLR1 by CKI or if it is an indirect consequence. Whether the basal phosphorylation of SLR1, formerly manifested in *gid2* mutants (Sasaki et al. 2003), is due to CKI activity and whether DELLAs from other species are also targets of CKIs await further investigations.

GID1 Is a GA Receptor That Promotes GA-Dependent Interaction of DELLA with GID2/SLY1

Despite the identification of GID2/SLY1 represented an important step forward in our understanding of the GA signaling pathway mechanism, there were still several important questions to solve. For instance, how the hormone is perceived and how does this fact relate to the DELLA degradation by the 26S proteasome.

Identification of a GA Receptor

Genetics had the key again, and answers came from the characterization and positional cloning of a GA-insensitive and dwarf mutant of rice, *gid1* (Ueguchi-Tanaka et al. 2005). In this mutant all known GA responses are affected. For instance, leaf elongation and α-amylase induction were totally impaired; plants were male sterile and in addition over-accumulated active GAs as a consequence of altered feedback regulation of the GA metabolic pathway. All these phenotypes were shared with *gid2* mutants, and similarly, SLR1 over-accumulated in *gid1* as well. In agreement

with the idea that the excess of SLR1 was the cause of its phenotypes, *slr1* was completely epistatic over *gid1*. Interestingly, SLR1 accumulation and dwarfism in *gid1* were more similar to the GA-deficient mutant *cps* than to *gid2*, which accumulated more SLR1 but whose dwarfism was less severe (Ueguchi-Tanaka et al. 2005). Ueguchi-Tanaka and co-workers suggested that in some way, GAs can reach SLR1 in *gid2* mutants and reduce its activity (see below), whereas this cannot occur in *cps* since it is GA deficient, neither in *gid1*, which should therefore affect GA perception.

In principle, identification of the *GID1* locus did not provide clues about its function. It encodes a soluble protein present in the cytoplasm and in the nucleus and with homology to hormone-sensitive lipases (HSL) (Ueguchi-Tanaka et al. 2005). Nonetheless, one of the three key amino acids in the catalytic center of the enzyme is not conserved in GID1, and indeed it failed in enzymatic assays typical for this sort of proteins, indicating that its function in the plant was very likely different. Since then, GID1 orthologs have been identified in many species. For instance, the *Arabidopsis* genome contains three genes encoding GA receptors, *GID1a*, *GID1b*, and *GID1c* (Griffiths et al. 2006; Nakajima et al. 2006; Willige et al. 2007), that were able to complement the *gid1* mutation when expressed in rice (Nakajima et al. 2006). The three proteins showed overlapping roles in the GA pathway along the life cycle of the plant (Griffiths et al. 2006; Iuchi et al. 2007; Willige et al. 2007). In fact, the single loss-of-function mutants do not show apparently any GA-related defects, thus explaining why they were not identified in forward genetic screens, whereas defects start to appear in double mutant combinations, especially in the *gid1a gid1c* that is dwarf and has lost the apical dominance, consistent with the low expression of *GID1b* in inflorescence stems (Suzuki et al. 2009). Remarkably, all known GA responses are impaired in the triple *gid1a gid1b gid1c* (Griffiths et al. 2006; Iuchi et al. 2007; Willige et al. 2007), paralleling the situation caused by the *gid1* mutant in rice. It was demonstrated genetically that defects in the triple mutant were caused by overaccumulation of DELLAs, as *rga* and *gai* null alleles were epistatic over the *gid1* mutations (Griffiths et al. 2006; Willige et al. 2007). Regarding the transcriptional regulation of these genes, it is worth mentioning that their expression is subjected to negative feedback regulation by GAs in a DELLA-dependent manner (Griffiths et al. 2006; Iuchi et al. 2007), as mentioned above for genes in the GA biosynthetic pathway. Moreover, they are also under the control of the circadian clock, being this regulation important to control of cyclic processes such as elongation growth (Arana et al. 2011). Contrary to their regulation by GAs, the regulation by the circadian clock seems to be independent of DELLA activity.

In a seminal work, Ueguchi-Tanaka and co-workers demonstrated unambiguously that the GID1 protein from rice was indeed a GA receptor (Ueguchi-Tanaka et al. 2005), as shown later for the *Arabidopsis* orthologs (Nakajima et al. 2006). By means of classical biochemical approaches, these authors showed that the GID1 proteins were able to bind with high-affinity and high-specificity active GAs, such as GA_4, GA_1, and GA_3. Authors also showed that the association–dissociation between GID1 and the GA was very fast—around 5 min—being a critical feature also shared with mammalian soluble receptors that is important to respond very rapidly to small changes in hormone concentrations (Ueguchi-Tanaka et al. 2005).

Importantly, proteins carrying strong *gid1* alleles were unable to bind GAs whereas the weak alleles did not impair completely the binding of the hormone, correlating with phenotypes of mutant plants (Ueguchi-Tanaka et al. 2005; Hirano et al. 2010).

GID1 Interacts with DELLAs in a GA-Dependent Manner and Promotes Their Degradation

The question of how GAs are perceived was finally answered. The next standing question was how this fact relates to the degradation of DELLAs in response to GAs. Ueguchi-Tanaka and co-workers opted for the simplest explanation: GID1 loaded with GA might translate directly the GA signal to SLR1 by physical interaction (Ueguchi-Tanaka et al. 2005). And this was indeed the case; by means of Y2H assays they demonstrated the GA-dependent interaction between GID1 and SLR1. The interaction was confirmed for all pairs of *Arabidopsis* GID1-DELLA; nonetheless, the dependence on GAs for the interaction was not so clear, since GID1b showed certain ability to interact with DELLAs even in the absence of the hormone (Griffiths et al. 2006; Nakajima et al. 2006; Yamamoto et al. 2010). The different affinities of the *Arabidopsis* GID1 receptors for the different DELLAs together with their particular spatial and temporal expression patterns contribute to define the role of each gene in controlling a particular process in the plant, as well as to understand the particular responsiveness of that process to the hormone (Suzuki et al. 2009).

From a biochemical point of view, the GID1-GA-SLR1 three-way interaction could be reconstituted in vitro, indicating that the formation of the complex does not require any additional element (Ueguchi-Tanaka et al. 2007); and importantly, it was also shown in vitro that the presence of the DELLA protein increased the affinity of GID1 for the GA over 100-fold (Nakajima et al. 2006), whereas the GA binding to GID1 was stabilized (Ueguchi-Tanaka et al. 2007), which suggests that the complex has evolved to rapidly establish the interaction with DELLA in the presence of the hormone. The GA-dependent interaction was confirmed in vivo by co-immunoprecipitation assays both in *Arabidopsis* and rice (Griffiths et al. 2006; Ueguchi-Tanaka et al. 2007) and also by bimolecular fluorescence complementation (BiFC) in leaves of *Nicotiana benthamiana* for the rice partners (Ueguchi-Tanaka et al. 2007).

The dominant versions of DELLA proteins GAI, RGA, and SLR1 lacking the DELLA motif failed to interact with GID1 receptors in the presence of GAs in Y2H and BiFC assays (Griffiths et al. 2006; Ueguchi-Tanaka et al. 2007). These results indicated that the formation of the GID1-GA-DELLA complex might be relevant for the degradation of DELLAs in response to the hormone, since the dominant versions of DELLAs are GA insensitive (Dill et al. 2001; Itoh et al. 2002). Importantly, Griffiths and co-workers demonstrated in an elegant approach that the formation of the GID1-GA-DELLA complex enhances dramatically the ability of SLY1 to interact with DELLA in Y3H assays (Fig. 2) (Griffiths et al. 2006). In fact, SLR1 can even be degraded in response to GAs in the yeast when the GID1-GA-SLR1-GID2 complex forms (Hirano et al. 2010). The formation of this complex was further confirmed in vivo (Hirano et al. 2010; Ariizumi et al. 2011). This was a remarkable

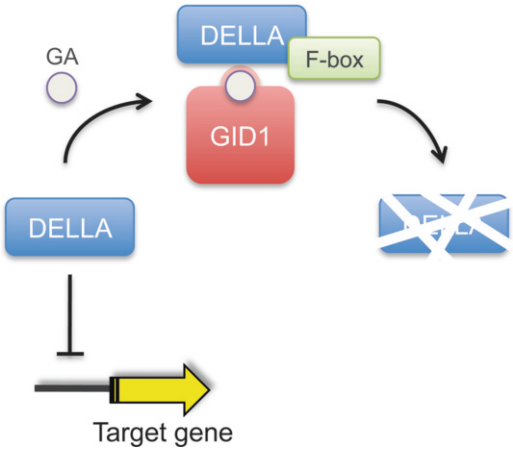

Fig. 2 Scheme of the GA signaling pathway. When GA levels are low, DELLAs accumulate and regulate transcription of target genes. On the contrary, when hormone levels increase, the GA-loaded GID1 receptor is able to interact with the DELLA protein, thus facilitating its ubiquitination and degradation mediated by the F-box protein SLY1

result, since it provided for the first time biochemical evidences linking the perception of the hormone with the degradation of DELLAs, a necessary step for GA responses to proceed.

With these results in hand, the Dr Makoto Matsuoka's laboratory started a tour de force to unmask the molecular determinants in the three proteins—GID1, SLR1, and GID2—that conferred them the ability to form the complex in the presence of GAs (Ueguchi-Tanaka et al. 2007; Hirano et al. 2010). For that purpose, authors prepared dozens of mutant GID1, GID2, and SLR1 proteins in which conserved amino acids were changed to Ala and assayed their ability to interact with the other partners in Y2H and Y3H assays in the presence or absence of GAs. These approaches rendered relevant details of the molecular mechanism of the DELLA degradation in response to GAs: (1) there is a good overlap between the regions of GID1 needed for both GA binding and SLR1 binding, confirming that binding of a GA is a requisite for interacting with SLR1; (2) GGF and LSL domains in GID2 mediate interaction with SLR1, confirming and extending genetic analysis; (3) only changes in GID1 amino acids important for GID1-SLR1 interaction prevent interaction of SLR1 with GID2 in Y3H assays, suggesting that GID1 does not interact directly with GID2; (4) the VHIID and LHR2 domains in SLR1 seem to be important for interaction with GID2, although the C-terminal part of the VHIID mediates interaction with GID1 as well; and (5) the PFRYE and SAW domains participate in stabilizing the interaction with GID1, besides their role in the repressive activity of the SLR1 protein. Again, these results confirm and extend previous results obtained with the genetic analysis, as explained in previous sections.

Structure of the GID1 Receptor

As mentioned above, GID1 receptors are similar to HSL. In general, the secondary structure of proteins belonging to the HSL family seems to be conserved.

Comparison of the predicted secondary structure of GID1 with the actual structure of a *Archaeoglobus fulgidus* esterase (AFEST) of the HSL family allowed predicting that the GID1 structure is formed by an α/β hydrolase fold and an N-terminal region that forms a lid, being both features typical in this family (Ueguchi-Tanaka et al. 2007). Moreover, localization of residues of GID1 that mediate GA and SLR1 binding on its predicted secondary structure showed that they clustered around the substrate binding pocket and lid region of HSLs, suggesting that those regions have evolved in GID1 to bind the hormone and the DELLA protein (Ueguchi-Tanaka et al. 2007).

These predictions were faithfully confirmed when the quaternary structures of the *Arabidopsis* GID1a-GA-GAI (Murase et al. 2008) and rice GID1-GA (Shimada et al. 2008) crystallized complexes were deciphered by X-ray analyses. Regarding the interaction with the active GA, these studies showed that an Ser and an Asp in the substrate binding pocket make contacts with the C6 carboxyl group of the GA_4. These two residues are two of the three conserved residues in the catalytic center of HSLs. The other residue in these proteins is a His that is substituted by Val in GID1 proteins, and that makes contact with the γ-lactone of the GA. In addition to these contacts, the GA seems to be stabilized in the binding pocket also through interactions with amino acids located at the lid region. In fact, GID1 proteins in which a single amino acid in the lid that makes contacts with the GA was mutated to Ala showed reduced ability to bind the hormone in vitro (Shimada et al. 2008).

Remarkably, Murase and co-workers solved the structure of the GID1a-GA bound to the DELLA domain of GAI and could confirm and extend the sequence requirements for the interaction with the receptor, which include the DELLA and VHYNP motifs (Murase et al. 2008). These authors propose that the N-terminal lid of the receptor acquires the conformation able to interact with the DELLA protein upon the binding of the GA, then allowing the unstructured DELLA domain to fold properly and to bind the GID-GA complex. Indeed, the DELLA domain seems to be unstructured in solution (Murase et al. 2008; Sun et al. 2010). Finally, this complex formation might confer a conformational change in the GRAS domain of the DELLA protein that enhances its affinity for GID2/SLY1 (Murase et al. 2008). Indeed, the GRAS domain is important to mediate interaction not only with GID2/SLY1 but also with GID1, since a mutant allele of *SLR1*, *Slr1-d4*, that has a missense mutation at the very end of the GRAS domain stabilizes the protein by preventing interaction with the receptor and causes a semidwarf phenotype (Hirano et al. 2010).

DELLAs Are Inactivated by the GID1-GA Complex Previous to Their Degradation

Is the GA-induced degradation of DELLAs the only mechanism to derepress GA signaling? Several experimental observations pointed out that GA signaling might occur in the absence of DELLA degradation (Ariizumi et al. 2008; Ueguchi-Tanaka

et al. 2008). First, *gid2*/*sly1* mutants accumulated more DELLA than *gid1* or GA-deficient mutants, despite the *gid2*/*sly1* phenotype was less severe. Second, the *gid2*/*sly1* phenotype was alleviated by overexpressing GID1, without affecting the amount of DELLAs; this rescue was dependent upon the presence of GAs and on the DELLA motif. Therefore, DELLAs in *gid2*/*sly1* are not fully active, and GA signaling is partially functional in these mutants, causing, for instance, an increase in the expression of *DELLA* genes. Importantly, these phenotypes can be explained by a model in which DELLAs are inactivated through the interaction with the GID1-GA complex (Ariizumi et al. 2008; Ueguchi-Tanaka et al. 2008). For instance, treatment of *gid2*/*sly1* mutants with inhibitors of GA biosynthesis aggravates their dwarf phenotype while reducing DELLA levels: the reduction in GA levels prevents the formation of the GID1-GA complex that in turn results in more active DELLAs that repress both growth and the expression of their own genes. This mechanism would ensure a first, rapid inactivation of DELLAs in the presence of the receptor and GAs in advance to their degradation by the 26S proteasome. For instance, this could be important under physiological circumstances where the $SCF^{GID2/SLY1}$ pathway could be limiting.

Evolution of the GA-GID1-DELLA Regulatory Mechanism

What is the origin of this regulatory module is an interesting question in terms of evolutionary biology. Sequence comparisons in different species have shown the presence of clear GA-GID1-DELLA components in seed plants (Vandenbussche et al. 2007). The same study showed that there were no candidates in *Cyanidioschyzon merolae* (red algae) nor in *Chlamydomonas reinhardtii* (green algae), while related sequences were identified in *Physcomitrella patens* (*Pp*; a moss) and *Selaginella moellendorffii* (*Sm*; a spikemoss). Detailed analysis of candidates for GID1s and DELLAs from *Pp*, *Sm*, and *Selaginella kraussiana* (*Sk*) has revealed interesting differences in the GID1-DELLA interaction and in the dependence of this interaction upon GAs (Hirano et al. 2007; Yasumura et al. 2007). The PpDELLA, which lacks the conserved DELLA motif, was not able to establish interactions with any GID1 protein, either from *Pp*, *Sk*, or *Arabidopsis. Selaginella* and *Arabidopsis* DELLAs and GID1s were able to interact each other, and the interaction was enhanced by GAs (Yasumura et al. 2007). Interestingly, expression of a *PpDELLA* in the *Arabidopsis* triple mutant *gai-t6 rga24 ga1-3* was equally effective as *SkDELLA* or *RGA*, suggesting that PpDELLA is able to interact with the right protein partners in *Arabidopsis* to restrain growth (Yasumura et al. 2007). All these results suggest that GID1 and DELLA proteins were present in the basal land plants but likely without connection functional between them or with GAs (Hirano et al. 2007; Yasumura et al. 2007; Engstrom 2011). After bryophyte diversification, the GID1-DELLA interaction was acquired and it became susceptible to GA regulation, which agrees with the lack of clear growth responses to GAs in *Pp*.

Regulation of Downstream Processes by DELLA Proteins

In the previous sections, we have reviewed the core GA signaling that transduces the information "contained" in the GA level into the inactivation/degradation of the negative regulators DELLA proteins (Fig. 2). To understand how this is translated into physiological responses, we need to understand at the molecular level how DELLAs regulate downstream events. Numerous and varied evidences gathered during the last years support a role for these proteins as transcriptional regulators that modulate the transcriptome in response to changes in GA levels. Evidences are as follows: (1) transient activation of DELLA proteins provokes rapid changes in the transcriptome; (2) DELLA proteins are able to activate transcription; and (3) DELLA proteins interact physically with numerous transcriptional regulators. In this section, we will review in detail these evidences and the resulting molecular mechanisms that explain how DELLA proteins repress GA responses.

DELLA Proteins Provoke Changes in the Transcriptome

Microarray analyses aimed at the identification of early gene targets of GAI and RGA demonstrated that these two proteins can alter very rapidly the transcriptome upon activation, in agreement with the idea that they act as transcriptional regulators (Zentella et al. 2007; Gallego-Bartolomé et al. 2011a). Authors expressed the dominant versions rga-Δ17 and gai-1 under the control of inducible promoters and found 475 and 148 genes whose expression was altered at least 1.5-fold within the first 4 h after induction, respectively. In both cases, more genes were up- than downregulated, 336 vs. 139 genes in the case of rga-Δ17 (Zentella et al. 2007) and 90 vs. 58 genes in response to gai-1 (Gallego-Bartolomé et al. 2011a).

As expected for bona fide transcriptional regulators, these two DELLA proteins had the ability to regulate gene expression directly. This was supported by two lines of evidence. First, RGA was able to interact in vivo with the promoters of some of its target genes, as demonstrated by chromatin immunoprecipitation analysis (Zentella et al. 2007). All genes tested were upregulated. Nonetheless, RGA is able to sit at the promoters of downregulated genes as well (Park et al. 2013), consistent with the finding that the expression of many RGA targets is reduced after induction of the DELLA protein (Zentella et al. 2007). This was a remarkable result, since it indicated that DELLAs can act as *cis*-acting transcriptional regulators on target genes, either up- or downregulated. The lack of any recognizable DNA binding motif within the DELLA sequence suggests that they do bind to chromatin through the interaction with other proteins.

Second, gai-1 was able to both up- and downregulate the transcription of target genes in the absence of protein synthesis (Gallego-Bartolomé et al. 2011a, b, c). This was demonstrated by using a transgenic line that expresses a translational fusion between gai-1 and the receptor domain of the rat glucocorticoid receptor, which endows the fusion protein with the ability to move from the cytosol,

where it accumulates, to the nucleus after treatment with the synthetic steroid dexamethasone (Gallego-Bartolomé et al. 2011c). The combination of dexamethasone and cycloheximide treatments allowed demonstrating protein synthesis-independent changes in gene expression for many GAI targets, indicating that GAI can regulate directly gene expression, both positively and negatively. *GID1a* and *GID1b* genes were found as direct targets by both experimental approaches. The regulation of other direct targets is compatible with DELLAs sitting at their promoters and also with alternative mechanisms, such as sequestration of transcription factors (see below).

DELLA Proteins Have Transcriptional Activation Activity

Early studies showed that the rice SLR1 was able to activate transcription of reporter genes by itself (Ogawa et al. 2000). In these transcriptional assays, performed in spinach leaves, SLR1 was fused to the DNA binding domain of the yeast GAL4 transcription factor that allowed recruiting the fusion protein to the engineered target promoter containing GAL4-binding sites. Deletion analyses identified the N-terminal DELLA domain of SLR1 as responsible for the transcriptional activation ability. This ability of the full-length protein, and of the DELLA domain, is manifested in heterologous systems as well, such as yeast (Hirano et al. 2012), indicating that DELLAs might interact with and activate conserved elements of the basal transcriptional machinery. The ability to activate transcription was inhibited upon interaction with GID1, both in spinach leaves and in yeast (Hirano et al. 2012), which is consistent with the capacity of the receptor to inactivate the DELLA protein by interaction previously to its degradation (Ariizumi et al. 2008; Ueguchi-Tanaka et al. 2008). A direct correlation between the transcriptional activation activity of different SLR1 deleted versions in yeast and spinach leaves and their ability to suppress growth in rice plants was established, suggesting that this activity is necessary to regulate negatively GA signaling, at least the branch that restrains growth (Hirano et al. 2012).

"Sociology" of DELLA Proteins: Preferred Interaction with Transcription Factors

The fact that DELLAs are able to sit at promoters of certain target genes and have intrinsic gene activation capacity does give us hints about them as transcriptional regulators but does not tell us much about the molecular mechanism by which they regulate gene expression. Based in DELLA's protein sequence, they most likely do not bind to DNA. Therefore, they rely in the interaction with other proteins to exert their transcriptional regulation activity, included binding to promoters. In this scenario, the identification of DELLA-interacting proteins, i.e., to know their "sociology," seems key to understand from a mechanistic point of view how DELLAs regulate gene expression.

An increasing number of novel DELLA interactors have been identified during the last years, mainly in *Arabidopsis*, being most of them bona fide transcription factors. The transcription factors belong to different families, being those of the bHLH family the most abundant. For instance, the bHLHs PIF3 and PIF4 were the first ones identified (de Lucas et al. 2008; Feng et al. 2008). These two transcription factors promote elongation growth and their levels are negatively regulated by light (Al-Sady et al. 2006; Nozue et al. 2007), while their DNA binding ability is inhibited upon the interaction with DELLAs. Thus, these results provided a molecular mechanism that explains (1) how GAs regulate elongation growth and (2) the interaction between GA and light signaling. Similarly, the identification of other bHLH proteins that interact with DELLAs has clarified the molecular mechanism through which GAs regulate certain physiological processes. For instance, the interaction with PIF5 is relevant for the regulation of apical hook development (Gallego-Bartolomé et al. 2011b), the interaction with ALCATRAZ (ALC) mediates in the regulation of the fruit patterning (Arnaud et al. 2010), and the interaction with MYC2 is important to regulate the synthesis of volatile terpenes in joint action with jasmonate (JA) signaling (Hong et al. 2012). In addition, DELLAs also interact with PIF1/PIL5 and SPATULA (SPT), although the relevance of these interactions has not been demonstrated (Gallego-Bartolomé et al. 2010).

DELLA interactors belonging to other families of transcription factors can also be found. For example, DELLAs interact with BZR1/BES1 (Bai et al. 2012; Gallego-Bartolomé et al. 2012; Li et al. 2012) and with EIN3 (An et al. 2012), which mediate genomic responses to brassinosteroids and ethylene, respectively. In both cases the interaction defines cross-regulatory nodes between these hormone pathways that are important to control, at least, elongation growth -BZR1/BES1- and apical hook development -EIN3. Moreover, DELLAs also interact with SPL9, being this important to the control of floral transition by GAs (Yu et al. 2012). These three proteins belong to plant-specific families of transcription factors.

All these results are remarkable, since they allow for the first time understanding the chain of events that go from changes in GA levels to the modification in the transcriptome through direct interaction with bona fide transcription factors. Importantly, a common theme found in all these interactions is that the transcription factor is inhibited upon DELLA binding, i.e., DELLAs sequester the transcription factor into an inactive complex that prevents its binding to the target promoter (Fig. 3a).

Besides these transcription factors, DELLAs also interact with proteins that regulate transcription but that do not bind DNA. For instance, interaction with the JASMONATE-ZIM-DOMAIN (JAZ) transcriptional regulators defines another cross-regulatory point with the JA signaling pathway (Fig. 3b) (Hou et al. 2010; Wild et al. 2012). JAZ proteins are the negative regulators of JA-induced gene expression by interacting with MYC2 and other transcription factors, whereas the hormone promotes JAZ degradation (Chini et al. 2007; Thines et al. 2007; Fernandez-Calvo et al. 2011). DELLA interaction with JAZ relieves MYC2 from the JAZ-mediated repression, being this is important to the proper response to necrotroph pathogens, for instance (Wild et al. 2012). Other transcriptional regulators such as SCARECROW-LIKE3 (SCL3) (Zhang et al. 2011b) and

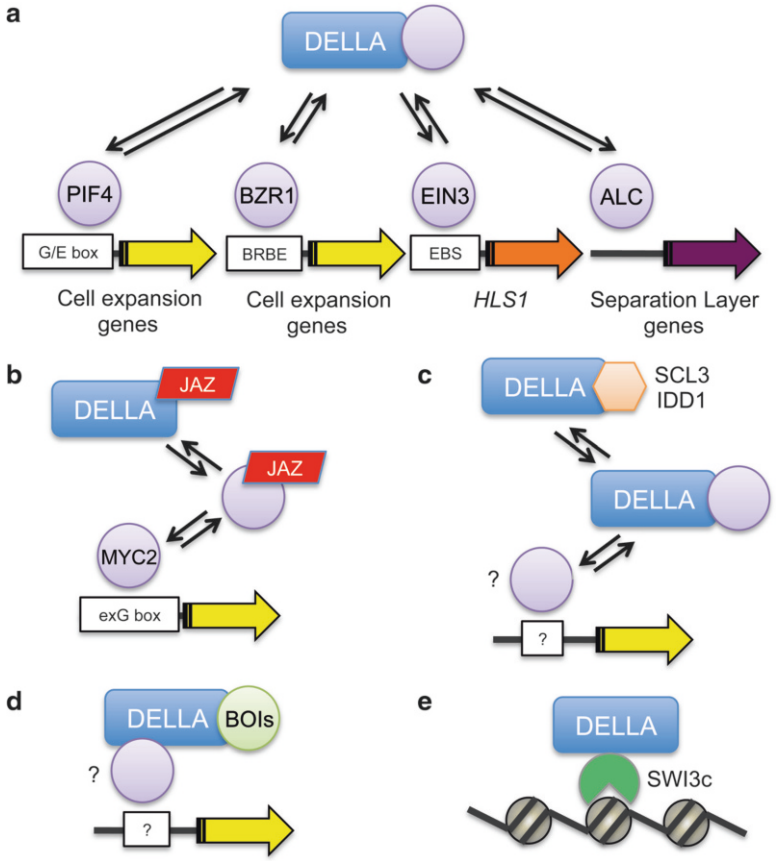

Fig. 3 Mechanisms by which DELLA proteins control transcription. (**a**) DELLAs inhibit the DNA binding activity of transcription factors upon interaction (PIF4, BZR1, EIN3, and ALC). (**b**) DELLAs inhibit the activity of non-DNA binders, transcriptional regulators (JAZ) that have consequences in other transcription factors' activity (MYC2). (**c**) DELLAs interact with non-DNA binders, transcriptional regulators (SCL3 and IDD1) as part of transcriptional complexes at target promoters. (**d**) DELLA activity is modulated by the interaction with other transcriptional regulators (BOIs). (**e**) DELLAs might modulate chromatin structure by interacting with chromatin remodelers (SWI3C). *Question mark*, unknown proteins and *cis*-elements; *white box*, relevant *cis*-elements; *big arrows*, target genes; *grey circles*, nucleosomes

INDETERMINATE DOMAIN1 (IDD1) (Feurtado et al. 2011) seem to attenuate DELLA activity in the context of the GA regulation of growth and germination, likely by preventing its interaction with transcription factors (Fig. 3c).

All above described interactions occur away from the chromatin. However, DELLAs have been found in the context of promoters. Interestingly, the interaction of DELLAs with the RING finger proteins BOTRYTIS SUSCEPTIBLE1 INTERACTORs (BOIs) seems to be maintained while both proteins are part of

transcriptional complexes bound to chromatin (Fig. 3d) (Park et al. 2013). Genetic and molecular analyses support the requirement of BOIs for DELLAs activity regulating several aspects of plant physiology, such as growth or the regulation of flowering. The identity of the proteins that target the DELLA-BOI complex to the chromatin is unknown.

The protein SWITCH SUBUNIT3C (SWI3C), which is part of the SWI/SNF chromatin remodeler complex, is able to interact with at least two DELLA proteins in *Arabidopsis*, RGL2 and RGL3, and its activity seems to be required for some DELLA functions, including regulation of GA biosynthesis (Sarnowska et al. 2013). This interaction, if proven to be relevant in vivo, might represent another layer of transcriptional regulation exerted by DELLAs, in this case by modulating the accessibility of transcriptional regulators to certain promoters (Fig. 3e).

Non-genomic Responses Regulated by DELLA Proteins

The different mechanisms described above involve transcriptional regulation. Nonetheless, the identification of prefoldin 5 (PFD5) and PFD3 as DELLA-interacting proteins provided the first clues of a non-genomic role for DELLAs in the control of plant growth (Locascio et al. 2013). These two proteins are part of the PFD complex formed by six subunits (PFD1–6). It is conserved from yeast to humans and functions as a chaperone in the cytosol, being tubulins its main client proteins (Vainberg et al. 1998). Remarkably, the whole PFD complex localizes to the nucleus upon interaction with DELLA. This has immediate consequences in the cytosolic function of PFD, and the amount of properly folded α/β-tubulins heterodimers drops, being this the most likely cause of the disorganization of microtubules that prevents anisotropic growth. Thus, the microtubule organization is indirectly regulated by GA levels through the interaction DELLA-PFD. The regulation of the cytosolic function of PFD by DELLAs seems to operate on a daily basis, allowing the maximum growth rate of seedlings to occur at the end of the night (Arana et al. 2011; Locascio et al. 2013).

Interestingly, a role for the yeast PFD complex in the nucleus has been recently described, showing that it participates in transcription elongation (Millán-Zambrano et al. 2013). Given the conservation of the PFD, a similar role for the plant counterpart could be envisioned.

SPINDLY: The Black Sheep in GA Signaling?

At present, we have a good understanding of how GA signaling proceeds, from the perception of the hormone to the degradation of DELLAs and in some cases to the regulation of gene expression. Nonetheless, is there any major question in the signaling pathway left or any piece to fit in the puzzle yet? The answer is yes and it is related to the protein SPINDLY (SPY).

spy mutants were first identified in *Arabidopsis* based in their ability to germinate in the presence of the GA biosynthesis inhibitor PAC (Jacobsen and Olszewski 1993). Phenotypic analyses of the mutant showed that it resembled wild-type plants that have been repeatedly treated with GAs, for instance, they had long hypocotyls, light green color, or early flowering. This mutation was able to cause a major reversion to the phenotypes of GA-deficient mutants (Jacobsen and Olszewski 1993; Silverstone et al. 1997b), suggesting that it enhanced GA signaling. And in agreement with this, *spy* mutants also suppressed phenotypes of *gai-1* and *rga-Δ17* (Wilson and Somerville 1995; Peng et al. 1999b; Silverstone et al. 2007). Similarly, RNAi transgenic rice with low transcript levels of *Oryza sativa SPY* (*OsSPY*) suppressed the dwarf phenotypes of GA-deficient and GA-insensitive mutants (Shimada et al. 2006), and functional assays with *Hordeum vulgare* SPY (HvSPY) showed that it was able to inhibit the GA induction of α-amylase in barley aleurone cells (Robertson et al. 1998). The recessive nature of *spy* mutations and the extent of the GA-independent growth and development they caused suggested that SPY performs a major, negative role in the GA signaling pathway.

The *SPY* locus encodes a protein with similarity to animal *O*-linked *N*-acetylglucosamine (GlcNAc) transferases (OGTs) (Jacobsen et al. 1996). These proteins transfer GlcNAc to Thr or Ser residues of target proteins, being this modification important to regulate their activity and/or their subcellular localization. Interestingly, SPY is located both in the nucleus and cytosol (Swain et al. 2002). Usually, its target proteins are also modified by phosphorylation of the same or adjacent residues, and in some cases both modifications influence each other (Hurtado-Guerrero et al. 2008). SPY, like OGTs, has tetratricopeptide repeats (TPRs; ten in this case) and a catalytic domain at its N-terminal and C-terminal halves, respectively. Phenotypic analyses of several *spy* alleles demonstrate that TPRs 6, 8, and 9 as well as the catalytic domain participate in the regulation of GA signaling (Silverstone et al. 2007). TPRs are believed to function as interfaces for protein–protein interaction, suggesting that these particular TPRs might be involved in the interaction with targets relevant for GA signaling. The most obvious targets in the GA pathway to be regulated and activated by SPY are DELLA proteins. These proteins accumulate more in *spy* mutants than in the wild type, whereas their localization is not affected (Silverstone et al. 2007), suggesting that DELLAs are less active in the *spy* background. These results are consistent with the hypothesis that modification of DELLAs by SPY is a requisite for their activity. Nonetheless, this attractive hypothesis has been challenged by studies showing that a SPY version that is being continuously excluded from the nucleus, where DELLAs reside, is able to suppress GA responses (Maymon et al. 2009), suggesting that SPY and DELLA activities would regulate GA signaling through different pathways. In any case, further experimental evidences are needed to clarify the role of SPY in GA signaling, being particularly relevant to define its biochemical function, i.e., if it has OGT activity, and to identify its target proteins.

In silico analysis identified a *SPY* homolog in *Arabidopsis*, called *SECRET AGENT* (*SEC*) (Hartweck et al. 2002). SEC does not seem to be involved in GA signaling, as SPY. *sec* mutations do not cause any obvious GA-related phenotype

and do not suppress the GA-deficient phenotype of *ga1* when mutations are combined (Hartweck et al. 2006). However, embryo lethality is obtained when combined with *spy* alleles (Hartweck et al. 2002, 2006), suggesting that both proteins have redundant roles, at least to control embryo development.

SPY is not fully dedicated to GA signaling. Detailed phenotypic analyses of *spy* alleles showed that they had phenotypes not related to GAs, for instance, defects in flower phyllotaxis in the inflorescence stem (Swain et al. 2001), or in some cytokinin responses (Gan et al. 2007; Maymon et al. 2009; Steiner et al. 2012). In particular, SPY regulates cytokinin responses in leaves and flowers through the physical interaction with the transcription factors TCP14 (TEOSINTE BRANCHED, CYCLOIDEA AND PCF14) and TCP15, whose activity is enhanced upon SPY binding (Steiner et al. 2012). Remarkably, both TCPs were GlcNAc modified by the SPY paralog SEC in assays performed in bacteria, suggesting that SPY could also perform this biochemical function in the plant (Steiner et al. 2012). Similarly, SPY interaction with the clock protein GIGANTEA (GI) mediates in circadian clock function, having impact in certain aspects of photomorphogenesis such as hypocotyl elongation and also in the regulation of flowering time; it is unknown if GI is GlcNAc-modified in vivo by SPY (Tseng et al. 2004). The involvement of SPY in other pathways has also been observed in rice and barley. For instance, OsSPY and HvSPY are involved in the regulation of brassinosteroid and ABA pathways, respectively (Robertson et al. 1998; Shimada et al. 2006).

Gibberellins as Targets for Biotechnological Applications

The use of GAs and GA biosynthesis inhibitors has been a common approach for the modification of agronomically important traits related to plant development in the past 60 years. In this section we will first review the extensive characterization of GA-related mutants from the perspective of potential field applications and provide a few examples of successful biotechnological modifications targeting GA metabolism and GA signaling.

Among all the traits affected in GA-deficient mutants, the most evident alteration refers to the size of almost all plant organs. This effect is common to all higher plant species, probably reflecting an ancestral role for endogenous GAs in the control of plant growth rate, and it is particularly relevant for those organs with rapid elongated growth, such as the stems of legumes and Brassicaceae. For instance, the *le* mutation that impairs the 3-oxidation of GA_{20} to the bioactive GA_1 results in dwarf shoots but close to normal roots and leaves (Yaxley et al. 2001). Dwarfism induced by GA deficiency can also be achieved through irrigation with GA biosynthesis inhibitors in field conditions. In fact, the triazole PAC that inhibits *ent*-kaurene oxidase is extensively used as a plant growth regulator in many species including cereals, vegetables, fruit trees, and ornamentals (Rademacher 2000).

Interestingly, endogenous GA levels seem to be limiting for growth in most tissues, as manifested by the slender phenotypes of plants defective in the 2-oxidases

that inactivate GAs (Martin et al. 1999). This opens the possibility to the modification of the expression of GA biosynthesis and inactivation as a tool to alter GA levels and, consequently, plant size and architecture. The validity of this approach was first tested in *Arabidopsis*, showing that overexpression of GA 20-oxidase genes under the control of a constitutive promoter would render taller plants mimicking the effect of continuous supply of GA_3 (Huang et al. 1998; Coles et al. 1999; Oikawa et al. 2004). And it has been successfully applied to several crops and woody plants, with the only limitation of the availability of technology to produce transgenic plants. This is the case of potato (Carrera et al. 2000), citrus trees (Fagoaga et al. 2007), or hybrid aspen (Eriksson et al. 2000), in which growth rate was increased through the overexpression of GA 20-oxidase genes, and the architecture was changed towards more slender plants. Alternatively, overexpression of 2-oxidase genes seems to be a good strategy to reduce active GA levels and restrict growth, as demonstrated in several monocots (Sakamoto et al. 2001) and dicots (Busov et al. 2003; Schomburg et al. 2003). In fact, unbiased selection of compact varieties of plum have been eventually identified as naturally occurring overexpressors of a GA 2-oxidase gene (El-Sharkawy et al. 2012), indicating the relevance of GA levels in the determination of plant stature across higher plants.

However, the fact that GAs regulate a vast array of developmental processes very often converts biotechnological manipulation of GA metabolism into a double-edged sword. In fact, enhanced production of 2-oxidase has been reported to produce not only more compact rice plants (a desirable trait in some cases) but also a strong reduction in flowering and in grain yield (Sakamoto et al. 2003). In this particular case, the substitution of the constitutive actin promoter by that of a GA 3-oxidase gene expressed only at the site of GA production in shoots resulted in dwarf plants with normal reproduction (Sakamoto et al. 2003). Therefore, more complex strategies need to be implemented, such as the confinement of overexpressed genes to certain tissues, as in the previous example, or the conditional induction of transgene expression through localized application of specific chemicals (Curtis et al. 2005).

The identification of DELLA proteins as the main target for GA regulation at the molecular level has also shifted the focus of biotechnological applications towards the use of these signaling elements, especially because of the existence of naturally occurring dominant alleles for the corresponding genes, whose use can be extended to any cultivated plant species. The GA-insensitive alleles of *DELLA* genes have a leading role in the Green Revolution that increased agriculture production around 1960. Spontaneous dwarf wheat varieties originating in Japan were used in breeding programs with more temperate cultivars resulting in high-yielding semidwarf varieties (Khush 2001; Hedden 2003). The responsible *Rht* allele in wheat was later identified as an ortholog of *GAI* and the maize *d8* genes, harboring a mutation in the DELLA domain (Peng et al. 1999a; Pearce et al. 2011), and also in classical semidwarf rice varieties extensively used in agriculture (Asano et al. 2009). The semidwarfism caused by dominant DELLA alleles is particularly attractive because it is accompanied by traits that increase the harvest index, such as a reduction in lodging. But, more importantly, these alleles have also been linked to enhanced disease

resistance in wheat and in barley against necrotrophic pathogens (Saville et al. 2012), in tune with previous findings in *Arabidopsis* (Navarro et al. 2008). Moreover, the involvement of DELLAs in the response to various abiotic stress factors, such as cold (Achard et al. 2008a), drought (Claeys et al. 2012), or shade (Djakovic-Petrovic et al. 2007; Gallego-Bartolomé et al. 2011c), and also in the reduction of reactive oxygen species produced in adverse conditions (Achard et al. 2008b) has increased the potential value of these DELLA alleles in crop improvement. In support of this potential use, wheat cultivars with dwarfing *Rht* alleles have also been reported to exhibit differential responses to potassium deprivation (Moriconi et al. 2012).

Apart from grasses, the cultivation of other agronomically important species can also be benefitted by the manipulation of DELLA activity, with an impact not only in the plant stature but also in branching, flowering time, the production of seedless fruits, and wood production, among other traits. A spontaneous mutation in tomato, named *procera*, later identified as a loss-of-function allele in the single *DELLA* gene in this species, causes very severe changes in plant architecture, such as a reduction in leaflet number in the dissected leaves (Jasinski et al. 2008) and the suppression of axillary bud development (Bassel et al. 2008), supporting a positive role of DELLAs in branching. This activity has indeed been used to alter plant architecture by expressing antisense or dominant alleles of the tomato or *Arabidopsis DELLA* genes, with the additional outcome that elimination of DELLA activity rendered parthenocarpic fruit with smaller size and elongated shape (Martí et al. 2007).

In woody plants, modification of DELLA activity has also been used as a successful approach to improve plant performance. For instance, it has been possible to produce more compact apple trees with fewer nodes by ectopically expressing the heterologous *Arabidopsis gai-1* allele (Zhu et al. 2008). And in hybrid aspen, DELLA-dominant alleles cause the formation of shorter shoots (presumably through the reduction of carbon flux in leaves towards lignin biosynthesis and a shift to the allocation of secondary storage and defense metabolites), but an increase in root growth (proposed to happen as a consequence of increased respiration) (Busov et al. 2006). Moreover, the observations that the genes encoding the aspen GID1 receptors and DELLA proteins are strongly expressed in xylem cells and that GA levels are high around the cambial region (Israelsson et al. 2005) suggest a possible role for GAs in the control of fiber production and wood quality. The prospect that engineering of DELLA activity in the cambium can change wood properties in forest plantations is also supported by the enhanced fiber production achieved by suppression of GA 2-oxidase activity in tobacco plants (Dayan et al. 2010) or by GA 20-oxidase overexpression in hybrid aspen (Eriksson et al. 2000), although the situation can be complicated by the fact that GAs seem to be required in two distinct wood formation processes that have tissue-specific signaling pathways: xylogenesis, mediated by GA signaling in the cambium, and fiber elongation in the developing xylem (Mauriat and Moritz 2009).

Flowering time and other traits associated to the early stages of reproductive development are also a very likely biotechnological target through the modification of DELLA activity. In *Arabidopsis*, DELLAs have been shown to have a role in the transition to flowering (Blázquez et al. 1998; Galvao et al. 2012; Yu et al. 2012)

and in floral development (Achard et al. 2004; Yu et al. 2004; Hou et al. 2008). Accordingly, overexpression of a rose *DELLA* gene in *Pelargonium* has been found to produce not only more compact plants, which is a desirable trait in ornamental species, but also a delay or even suppression of flowering (Hamama et al. 2012). On the other hand, a naturally occurring DELLA-dominant mutation in Pinot noir cultivar of grapevine that happened to be expressed only in the L1 layer was found responsible for the enhanced flowering without affecting berry size (Boss and Thomas 2002).

The fact that more refined results were obtained with mutant DELLA versions being expressed in certain layers of chimeric plants indicates that more subtle approaches are necessary to modify the desired aspects of plant development, rather than ectopic overexpression of the genes of interest. In this respect, it is important to remark that both GA biosynthesis and GA signaling are cell-type specific, as indicated by several recent reports. For instance, it has been shown that localized expression of the dominant *gai-1D* allele exclusively in the endodermis is sufficient to restrict growth of the whole root (Úbeda-Tomás et al. 2008; Ubeda-Tomas et al. 2009). Despite these results being obtained with a dominant, gain-of-function allele, the results very likely reflect a physiological control of root growth because GAs accumulate specifically in elongating endodermal cells (Shani et al. 2013). Moreover, GA accumulation has been shown to occur asymmetrically in roots undergoing gravitropic reorientation, with an asymmetric effect on the degradation of DELLA proteins (Lofke et al. 2013). These observations suggest that there are spatial restrictions for GA action, an aspect from which biotechnological applications can take advantage.

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Brassinosteroids Implicated in Growth and Stress Responses

Andrzej Bajguz and Alicja Piotrowska-Niczyporuk

Abstract Brassinosteroids (BRs) are steroidal hormones essential for plant growth and development. They are implicated in plant responses to abiotic environmental stresses such as low and high temperature, drought, salt, infection, pesticides, and heavy metals. BR-regulated stress response is a result of a complex sequence of biochemical reactions such as activation or suppression of key enzymatic reactions, induction of protein synthesis, and the production of various chemical defence compounds. However, the molecular mechanism of BR-induced plant abiotic stress tolerance remains poorly understood. The BR signalling is initiated by a ligand-induced kinase activation followed by receptor oligomerisation. The signal transduction in the cell is mediated through phosphorylation and transcription factors which directly bind to promoters of BR-responsive genes to regulate their expression. BRs that are biosynthesised using sterols as precursors are structurally similar to the cholesterol-derived, human steroid hormones and insect moulting hormones. The biosynthetic pathway of BRs is divided into multiple subunits. Depending on C-22 hydroxylation at campesterol, the BR pathway is further divided into the early and late C-22 oxidation pathways. Similarly, the C-6 position can be oxidised at campestanol or later at 6-deoxocathasterone stage, and thus these are called the early and late C-6 oxidation pathways, respectively. The pathways of BR biosynthesis in plants are well studied. Nevertheless, in order to understand properly the role of BRs during plant development under stress conditions, it seems essential to summarise the experimental data, focusing on the biosynthesis and signal transduction.

Keywords Biosynthesis • Brassinosteroid • Plant stress tolerance • Signal transduction

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Introduction

Brassinosteroids (BRs), a group of plant hormones, have been found in a wide range of organisms from lower to higher plants. BRs have been detected at low concentrations in all plant organs such as pollen, anthers, seeds, leaves, stems, roots, flowers, and grain as well as unicellular green algae, pteridophytes, and bryophytes. Thus, it is conceivable that BRs are ubiquitous in the plant kingdom. They also occur in the insect and crown galls of *Castanea crenata*, *Distylium racemosum*, or *Catharanthus roseus*. These plants have higher levels of BRs than the normal tissues. Furthermore, young growing tissues contain higher levels of BRs than mature tissues. Pollen and immature seeds are the richest sources of BRs, while shoots and leaves usually have lower amounts. However, precise spatial and subcellular distribution of BRs still remains unknown (Bajguz and Tretyn 2003).

BRs are characterised by their polyhydroxylated sterol structure. They were first isolated and purified from *Brassica napus* pollen in 1979. The chemical structure of brassinolide (BL), the first BR, and that of the second compound, castasterone (CS), discovered in 1982, was found to be similar to that of ecdysone, the insect moulting steroid hormone (ecdysteroids), and mammalian steroids (e.g. estrogens, androgens, mineralocorticoids, and glucocorticoids). So far, more than 70 BL-related compounds have been identified from plants. Natural BRs have 5α-cholestane skeleton, and their structural variations come from the kind and orientation of oxygenated functions in A ring and B ring. They are divided into free (64) and conjugated (5) compounds. Among the 70 different BRs, BL was shown to possess the greatest growth-promoting activity. CS only exhibits about 10 % of the activity of BL. Other BRs are mainly intermediates of the BL biosynthetic pathway or inactivated products that resulted from various BR catabolic reactions. As inferred from the chemical structure of BL, it was hypothesised that active BRs should possess the following structural requirements. First, the A and B rings must be in the *trans* configuration, which is determined by an α hydrogen at C-5. Second, the B ring should contain a 6-oxo or a 6-oxo-7-oxa group. Third, the hydroxyl groups at C-2 and C-3 in ring A should be *cis* α-oriented. Fourth, the *cis* α-oriented hydroxyl groups at the C-22, C-23, and the C-24 positions should be occupied by either α-oriented methyl or ethyl groups (Fig. 1) (Bajguz and Tretyn 2003).

BRs function in multiple developmental stages, including regulation of gene expression, cell division and expansion, differentiation, programmed cell death, and homeostasis. BRs are implicated in physiological and biochemical response in plants, like vascular differential, stem elongation, leaf bending, epinasty, pollen tube growth, root inhibition, induction of ethylene biosynthesis, activation of proton pumps, photosynthesis, regulation of gene expression, and nucleic acid and protein synthesis (Hayat et al. 2010b). BRs also play a significant role in amelioration of various environmental stresses. More recently, interactions of BRs with other plant hormones, such as abscisic acid (ABA), auxins, cytokinins, gibberellins, and ethylene, have also been found to play a major role in plant stress alleviation. Furthermore, ability of BRs to boost antioxidant system of plants is extensively used to confer

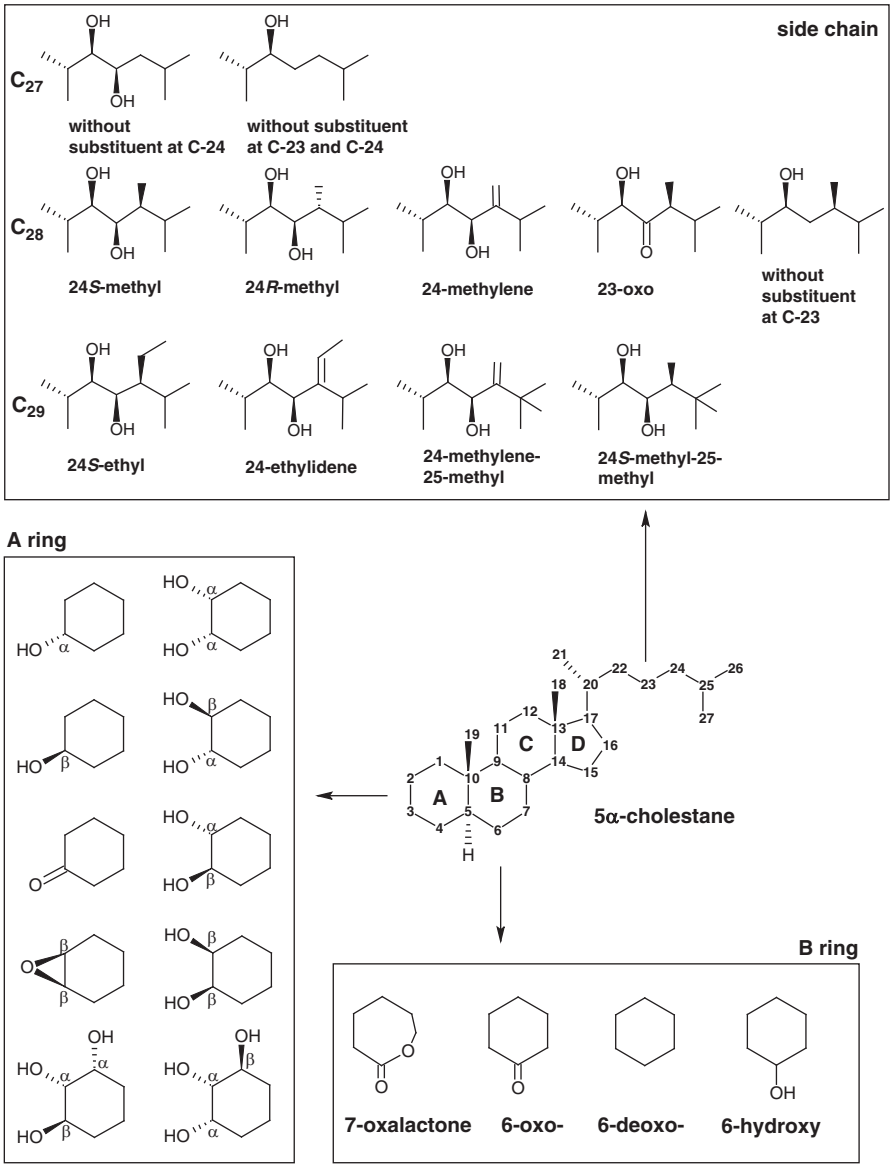

Fig. 1 Structural variations in brassinosteroids (adopted from Bajguz and Tretyn (2003))

resistance in plants against a variety of abiotic stresses, such as drought, heavy metal, pesticides, salinity, and thermal. Although much has been learned about their roles in plant development, the mechanisms by which BRs control stress responses and regulate stress responsive gene expression in plants are not fully acknowledged. Since BRs crosstalk with other plant hormones, it is likely that the stress tolerance

conferring ability of BRs lies in part in their interactions and stimulation of other stress hormones. BRs are not only implicated in plant response to abiotic and biotic stresses but also have medicinal applications (Bajguz and Hayat 2009). At present, our knowledge of the effects of BRs in animals or human is still rather fragmentary. However, it is known that BRs have an anabolic action, anticancer, and antiproliferative properties. BRs have also antiviral activities against herpes simplex viruses type I and II, arenaviruses, measles viruses, and vesicular stomatitis virus. BRs may prove to be promising leads for the development of new generation of drugs, especially against cancer or viral infection (Bajguz et al. 2013).

Brassinosteroid Biosynthesis

Campesterol, one of the major plant sterols, is the precursor of BRs, which is primarily derived from isopentenyl diphosphate (IPP). Sterols are synthesised via the non-mevalonate pathway in lower plants or the mevalonate pathway of isoprenoid metabolism in higher plants. In plants, IPP, the precursor of isoprenoids, is synthesised from acetyl-CoA via mevalonic acid (mevalonate pathway) or by pyruvate and glyceraldehyde 3-phosphate (non-mevalonate pathway). Isoprenoids are synthesised in all living organisms in at least one of two pathways. Plants synthesise isoprenoids by both the mevalonate pathway and the non-mevalonate pathway, segregating these pathways into different compartments: the non-mevalonate pathway synthesises IPP and dimethylallyl diphosphate in plastids, whereas the mevalonate pathway synthesises cytosolic IPP. The non-mevalonate pathway exists in eubacteria, algae (*Chlorella*, *Chlamydomonas*, and *Scenedesmus*) and higher plants (*Lemna* and *Wolffia*) (Bajguz and Asami 2004, 2005; Bajguz 2005; Choe 2006; Zhao and Li 2012).

The major pathway for BR biosynthesis has been established in *Catharanthus roseus* and *Arabidopsis thaliana* by conversion experiments using applied isotope-labelled BR intermediates. In this pathway, campesterol (the precursor of C_{28} BRs) is converted to campestanol (Fig. 2), which is then converted into two biologically active BRs (castasterone and brassinolide) via two parallel pathways, the early and late C-6 oxidation pathways. These oxidative steps are performed by cytochrome P450-type monooxygenases belonging to the closely related CYP85 and CYP90 families. While most of these enzymes were originally identified in *Arabidopsis*, several of their orthologs were soon recognised in other species, e.g. maize, rice, and tomato. BR biosynthesis mutants have defects in cytochrome P450 monooxygenases (P450s or CYPs) (Choe 2006). Enzymes of the BR biosynthetic pathway are summarised in Table 1.

Although metabolic experiments with labelled C_{27} BRs have not yet been performed, the natural occurrence of C_{27} BRs in plant tissues, e.g. tomato and *Arabidopsis* (6-deoxo-28-norcathasterone, 6-deoxo-28-norteasterone, 6-deoxo-28-nortyphasterol, 6-deoxo-28-norcastasterone, and 28-norcastasterone) suggests an in

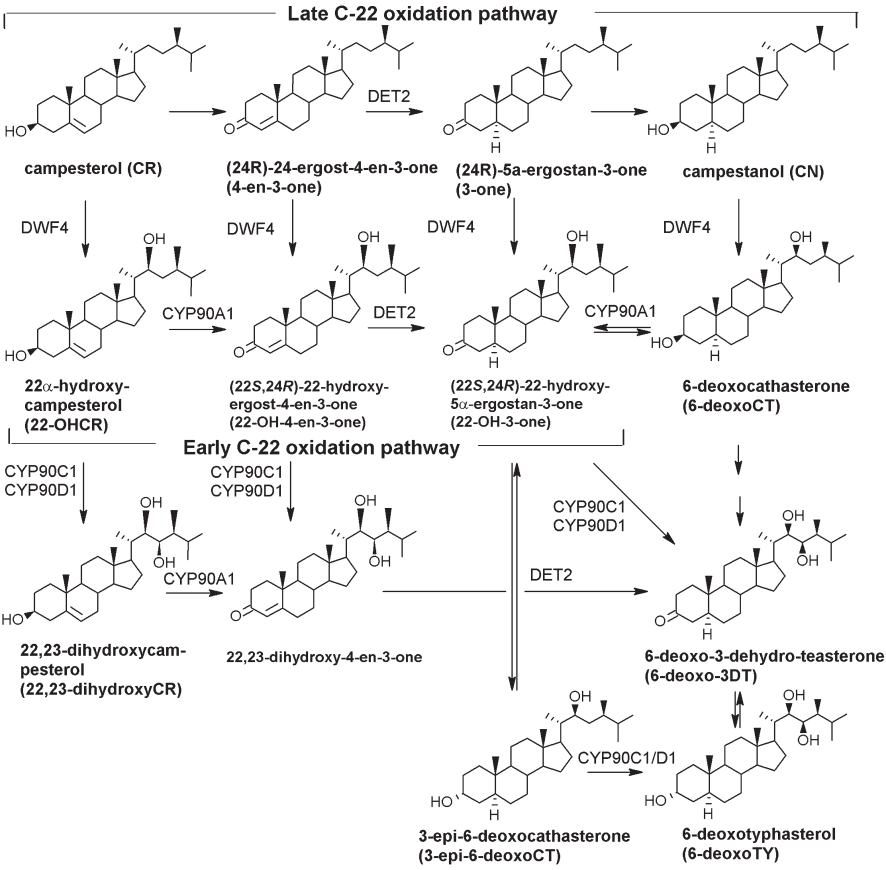

Fig. 2 C-22 oxidation pathways of sterols and their connection established in brassinosteroid biosynthesis in *Arabidopsis thaliana* (adopted from Bajguz (2005), Ye et al. (2011), Zhao and Li (2012))

vivo biosynthetic sequence of 28-nor-22-OH-campesterol → 28-nor-22-OH-4-en-3-one → 28-nor-22-OH-3-one → 6-deoxo-28-norcathasterone. Based on these findings, a biosynthetic pathway of C_{27} BRs has been suggested: cholestanol → 6-deoxo-28-norcathasterone (6-deoxo-28-norCT) → 6-deoxo-28-norteasterone (6-deoxo-28-norTE) → 6-deoxo-28-nor-3-dehydroteasterone (6-deoxo-28-nor-3DT) → 6-deoxo-28-nortyphasterol (6-deoxo-28-norTY) → 6-deoxo-28-norcastasterone (6-deoxo-28-norCS) → 28-norcastasterone (28-norCS) in tomato seedlings. In addition, the cell-free enzyme extract of tomato seedlings catalysed the conversion of cholesterol to cholestanol and 6-deoxo-28-norTE to 28-norCS via 6-deoxo-28-nor-3DT, 6-deoxo-28-norTY, and 6-deoxo-28-norCS. The reactions, named the late C-6 oxidation pathway for C_{27} BRs, have been demonstrated in Figs. 2 and 3 (Fujioka and Yokota 2003; Kim et al. 2004, 2005, 2008; Choe 2006; Choudhary et al. 2012; Joo et al. 2012).

Table 1 Enzymes of the brassinosteroid biosynthetic pathway in *Arabidopsis thaliana* (Schneider 2002; Choe 2006; Ye et al. 2011; Zhao and Li 2012)

Enzyme name	Description	Site of action
CYP85A1	BR C-6 oxidase	22-dihydroxyCR to 22,23-dihydroxy-4-en-3-one
CYP85A1, A2	BR C-6 oxidase	6-deoxoTE to TE, 6-deoxo-3DT to 3DT, 6-deoxoTY to TY, 6-deoxoCS to CS
CYP85A2	BR C-6 oxidase	6-deoxo-28-norTE to 28-norTE, 6-deoxo-28-nor-3DT to 28-nor-3DT, 6-deoxo-28-norTY to 28-norTY, 6-deoxo-28-norCS to 28-norCS, CS to BL
CYP90A1/CPD	Putative BR hydroxylase	22-OHCR to 22-OH-4-en-3-one, 22-OH-3-one to 6-deoxoCT
CYP90B1/DWF4	Steroid C-22 hydroxylase	CR to 22-OHCR, (24*R*)-24-ergost-4-en-3-one to 22-OH-4-en-3-one, (24*R*)-5α-ergostan-3-one to 22-OH-3-one, CN to 6-deoxoCT, 6-oxoCN to CT
CYP90C1/ROT3	BR C-23 hydroxylase	22-OHCR to 22-dihydroxyCR, 22-OH-4-en-3-one to 22-23-dihydroxy-4-en-3-one, 22-OH-3-one to 6-deoxo-3DT, 6-deoxoCT to 6-deoxoTE, CT to TE, 3-epi-6-deoxoCT to 6-deoxoTY
CYP90D1	BR C-23 hydroxylase	
DET2	Steroid-5α-hydroxylase	(24*R*)-24-ergost-4-en-3-one to (24*R*)-5α-ergostan-3-one, 22-OH-4-en-3-one to 22-OH-3-one, 22,23-dihydroxy-4-en-3-one to 6-deoxo-3DT

The *Arabidopsis dwarf4* (*dwf4*), *constitutive photomorphogenesis and dwarfism* (*cpd*) mutants are, through phenotypic rescue experiments using BR intermediates, thought to be blocked in the hydroxylation of C-22 and C-23, respectively. The *dwarf* (*d*) tomato mutant represents a new locus with the *Dwarf* gene (*D*) encoding a P450. It has been classified as CYP85 with high homology to CPD and DWF4. The tomato mutant *dumpy* (*dpy*) has been suggested to be the equivalent of *cpd*. DWARF acts as a C-6 oxidase, catalysing multiple C-6 oxidation reactions including 6-deoxoteasterone (6-deoxoTE) to teasterone (TE), 6-deoxo-3-dehydroteasterone (6-deoxo-3DT) to 3-dehydroteasterone (3-DT), 6-deoxotyphasterol (6-deoxoTY) to typhasterol (TY), and 6-deoxocastasterone (CS) to CS. Most of these reactions were confirmed in yeast using DWARF or its ortholog CYP85A1 (BR6ox1) from *Arabidopsis*. It is the key step linking the late C-6 oxidation pathway to the early C-6 oxidation pathway. The double mutant of *CYP85A1* and *CYP85A2 (BR6ox2)* displays a severe BR-defective phenotype, while the *CYP85A1* null mutant does not show any altered phenotypes and *CYP85A2* only exhibits subtle defective phenotypes (Kim et al. 2005). CYP85A2 catalyses those steps of C-6 oxidation overlapping with CYP85A1, but it is worth noting that only CYP85A2 (and not CYP85A1) is responsible for the Baeyer–Villiger oxidation step converting CS to BL (Clouse and Feldmann 1999; Bishop and Yokota 2001; Shimada et al. 2001; Bishop 2003, 2007; Fujioka and Yokota 2003; Müssig and Altmann 2003; Kim et al. 2005; Choudhary et al. 2012).

Arabidopsis de-etiolated2 (*det2*) was first identified as a mutant with a de-etiolated seedling phenotype when grown in the dark. Recessive mutation of *DET2*

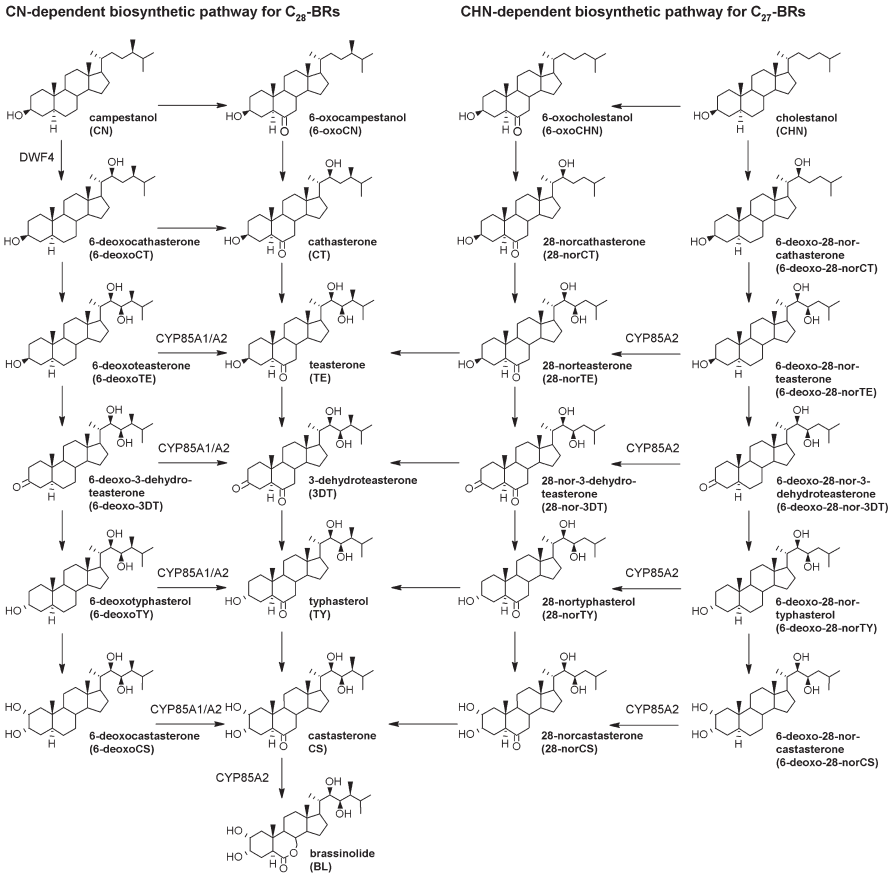

Fig. 3 Biosynthetic pathways for C_{27}- and C_{28}-brassinosteroids in *Arabidopsis thaliana* (adopted from Choe (2006), Ye et al. (2011), Joo et al. (2012), Zhao and Li (2012))

exhibits a typical BR-deficient phenotype including severe dwarfism, dark green colour, delayed flowering, reduced male fertility, and constitutive photomorphogenesis in the dark (Li et al. 1996). Biochemical analyses indicated that DET2 is involved in converting (24*R*)-ergost-4-en-3-one (4-en-3-one) to (24*R*)-5α-ergost-3-one (3-one), which is the second step in the BR-specific biosynthesis pathway (Fujioka et al. 1997; Noguchi et al. 1999a, b). Subsequently, a new subpathway via early C-22 oxidation was found, and in the *det2* mutant, the step converting 22-OH-4-en-3-one to (22*S*, 24*R*)-22-hydroxy-5α-ergost-3-one (22-OH-3-one) is blocked (Fujioka et al. 2002). *det2* mutants have also been identified in other plant species such as pea (*lk*), tomato, and *Pharbitis nil* (Suzuki et al. 2003; Nomura et al. 2004, 2005). DET2 is probably the only known non-P450 catalytic enzyme of the BR-specific biosynthesis pathway.

A T-DNA-tagged dwarfed mutant *dwf4* can only be rescued by BRs but not by other phytohormones (Azpiroz et al. 1998). Feeding experiments have suggested

that DWF4 may contribute to multiple C-22 hydroxylation steps in the BR biosynthetic pathway because only 22α-hydroxylated BRs can rescue the *dwf4* defective phenotypes (Choe et al. 1998). In the early C-22 oxidation pathway, DWF4 was found to catalyse steps like campesterol (CR) to 22-OHCR, 4-en-3-one to 22-OH-4-en-3-one, and 3-one to 22-OH-3-one (Fujioka et al. 2002). In tomato, these steps are catalysed by CYP724B2 and CYP90B3, both of which share a high sequence identity with DWF4 from *Arabidopsis* and rice (Ohnishi et al. 2006b).

In *Arabidopsis* seedlings, *CPD*/*CYP90A1* and *CYP85A2* transcripts were detected mainly in shoots, *ROTUNDIFOLIA3* (*ROT3*)/*CYP90C1* and *CYP90D1* transcripts preferentially in roots, while *DET2* and *DWF4*/*CYP90B1* mRNAs were found in comparable amounts in both the seedling parts (Bancoş et al. 2002). Similar partitioning of the orthologous *CYP90A9*, *CYP90A10*, *CYP85A1*, *CYP85A6*, *CYP90D7*, *LK*, and *CYP90B8* transcripts was observed in pea seedlings (Nomura et al. 2007). The enzyme encoded by the *CPD* (At5g05690) gene was shown to be required for the synthesis of C-23-hydroxylated BRs (Szekeres et al. 1996); gene construct was highly active in expanding rosette leaves, particularly in the adaxial parenchimatic tissues, axillary leaves, and sepals.

Of the *ROT3* (*At4g36380*) and *CYP90D1* (*At3g13730*) genes, which encode functionally redundant C-23 hydroxylases (Ohnishi et al. 2006a), only the expression of the *ROT3* was studied with a *GUS* fusion construct. Early analyses have suggested that ROT3 and its homologue CYP90D1 catalyse different steps in the BR biosynthetic pathway. In young plants, it was found ubiquitous and almost equal in all vegetative organs. *CYP85A1* (*At5g38970*) encodes the C-6 oxidase, and *CYP85A2* (At3g30180) the C-6 oxidase and BL synthase that produce the bioactive BR forms CS, or CS and also BL, respectively (Shimada et al. 2001, 2003; Kim et al. 2005; Nomura et al. 2005). A very similar expression pattern was observed with *Dwarf*, the *CYP85A1* gene of tomato, which was also most active in meristematic regions and developing organs (Montoya et al. 2005). A quantitative comparison of mRNA levels in organs of mature *Arabidopsis* indicated that each of the BR biosynthetic P450 genes has a unique organ-specific expression pattern (Shimada et al. 2003).

Inhibitors of the biosynthesis and metabolism of BRs have complementary roles in the analysis of the functions of BRs in plants to BR-deficient mutants. The P450 inhibitors, clotrimazole and ketoconazole, have been found to suppress the 25-hydroxylation of 24-epiBL (24-epibrassinolide) and BL in tomato cell suspension cultures, indicating that the 25-hydroxylation is catalysed by a P450 enzyme. Recently, the first specific BR biosynthesis inhibitor, brassinazole (Brz), has been synthesised. The application of Brz, a triazole derivative, to plants resulted in growth inhibition or dwarfism but exogenous brassinolide reversed the negative effect. *Arabidopsis* seedlings treated by Brz show a typical BR-deficient mutant phenotype similar to those of *det2* and *cpd*. Brz blocks the conversion of campestanol to 6-deoxoCT, 6-deoxoCT to 6-deoxoTE, 6-oxocampestanol to cathasterone (CT), and CT to TE in BR biosynthetic pathways (Asami and Yoshida 1999; Asami et al. 2003).

The cell cultures produced representatives of C_{28} BRs, such as CT, TE, 3-DT, TY, CS, and BL. The levels of BRs in cell cultures of *C. roseus* have been found to

be comparable to those of BR-rich plant tissues such as pollen and immature seeds. The occurrence of 6-deoxoBRs such as 6-deoxoCS, 6-deoxoTE, and 6-deoxoTY in several plants suggested that the parallel or/and alternative BR biosynthetic route exists. This late C-6 oxidation pathway for C_{28} BRs in *A. thaliana*, *C. roseus*, *L. esculentum*, *Chlorella vulgaris*, and *Marchantia polymorpha* has been investigated. The conversion of 6-deoxoCS to CS via 6α-hydroxyCS has been found in *A. thaliana*. In addition to the early and late C-6 oxidation pathways of C_{28} BRs, cross-links between both branches also exist. The two pathways converge at CS, which ultimately leads to the biosynthesis of BL. Conversion of CS to BL is the final biosynthetic step of BRs. Unfortunately, the biosynthesis of C_{29} BRs is still unclear. An early C-22 oxidation branch, also called the CN-independent pathway, was demonstrated to occur alongside the previously reported CR to CN pathway, and it could be the dominant upstream BR biosynthesis pathway (Fig. 2). Campestanol plays an important intermediate in the BRs biosynthetic pathway. The biosynthetic sequence between campesterol and campestanol leads to completion of the carbon skeleton including trans stereochemistry of the A/B ring junction. The following conversions, campesterol → (24*R*)-ergost-4-en-3β-ol (4-en-3β-ol) → (24*R*)-ergost-4-en-3-one (4-en-3-one) → (24*R*)-5α-ergostan-3-one (3-one) → campestanol, named the late C-22 oxidation pathway, led to 6-deoxoCT. On the other hand, the conversion of campesterol to 6-deoxoCT via intermediates such as (22*S*)-22-hydroxycamesterol, (22*S*,24*R*)-22-hydroxyergost-4-en-3-one (22-OH-4-en-3-one), and (22*S*,24*R*)-22-hydroxy-5α-ergostan-3-one (22-OH-3-one) is now generally accepted as the early C-22 oxidation pathway. Furthermore, the conversion of (22*S*,24*R*)-22-hydroxy-5α-ergostan-3-one to 3-epi-6-deoxocathasterone also exists. Recently, Ohnishi et al. (2006a) reported C-23 hydroxylation shortcuts, leading (22*S*, 24*R*)-22-hydroxy-5-ergost-3-one (22-OH-3-one) and 3-*epi*-6-deoxocathasterone (3-epi-6-deoxoCT) to be directly converted to 3-dehydro-6-deoxoteasterone (6-deoxo-3DT) and 6-deoxotyphasterol (6-deoxoTY), respectively. In addition, the existence of high levels of 6-deoxoCT and 6-deoxoCS in different species analysed suggests that the late C-6 oxidation pathway probably is the predominant BR biosynthesis branch (Fig. 3) (Nomura et al. 2001; Bishop and Yokota 2001; Schneider 2002; Fujioka and Yokota 2003; Choe 2006; Bajguz 2009b; Choudhary et al. 2012).

Brassinosteroids and Abiotic Stress

Brassinosteroids are steroidal plant hormones implicated in the promotion of plant growth and development. One of the most interesting influences of BRs is their ability to confer resistance to plants against various abiotic stress (Fig. 4) (Bajguz and Hayat 2009; Hayat et al. 2010b). Plant responses to different types of stresses are associated with generation of reactive oxygen species (ROS), suggesting that ROS may function as a common signal in signalling pathways of plant stress responses. It was shown that exogenous application of BRs is involved in plant response to oxidative stress (Bajguz 2011). For example, when maize (*Zea mays*) seedlings

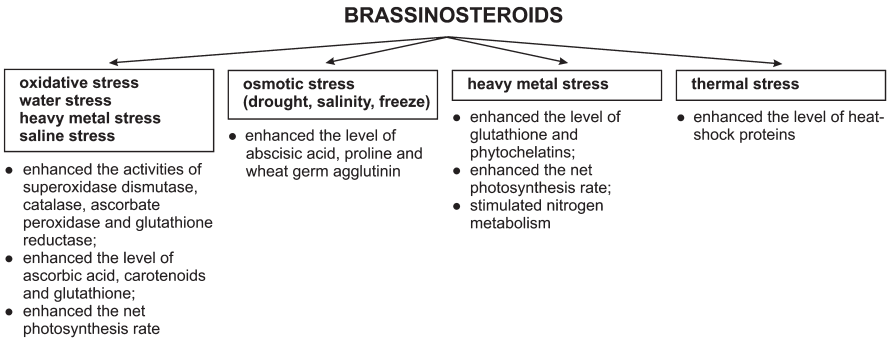

Fig. 4 Effects of brassinosteroids on plants exposed or subjected to abiotic stresses (adopted from Bajguz and Hayat (2009))

treated with BL were subjected to water stress, the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) as well as ascorbic acid and carotenoid contents increased (Li et al. 1998). Rice seedlings exposed to saline stress and treated with BR showed a significant increase in the activities of CAT, SOD, glutathione reductase (GR), and a slight increase in APX (Núñez et al. 2003). *C. vulgaris* responds to heavy metals (cadmium, copper, and lead) by inducing several antioxidants, including several enzymatic systems and the synthesis of low-molecular-weight compounds, such as phytochelatins (PCs). Treatment with BL was effective in increasing the activity of antioxidant enzymes (CAT, GR, and APX) and the content of ascorbic acid, carotenoids, and glutathione (Bajguz 2011). The influence of 24-epiBL on some enzymatic antioxidants in tomato leaf disc under high (40 °C) temperature was reported (Mazorra et al. 2002). Studies on cucumber (*Cucumis sativus*) indicate that BR levels are positively correlated with the tolerance to photooxidative and cold stresses and resistance to *cucumber mosaic virus*. The BR treatment enhanced NADPH oxidase activity and elevated H_2O_2 levels in apoplast. BR-induced H_2O_2 accumulation was accompanied by increased tolerance to oxidative stress (Xia et al. 2009). However, it is still unclear whether BRs directly or indirectly modulate the responses of plants to oxidative stress.

Drought-, salinity-, and freeze-induced dehydration constitute direct osmotic stresses, whereas chilling and hypoxia can indirectly cause osmotic stress via effects on water uptake and loss. Water-stress-induced decline in root nodulation is associated with increase in ABA and decline in cytokinin contents in the nodulated roots (Kang et al. 2009). BRs have the potential to improve root nodulation and pod yield in the irrigated and water-stressed plants, an effect that could be mediated through an influence on cytokinin content in the nodulated roots of *Phaseolus vulgaris*. BR application also resulted in the enhancement of seedling growth, which was evident in terms of seedling length, seedling fresh, and dry weights of sorghum (*Sorghum vulgare*) under osmotic stress (Vardhini and Rao 2003; Upreti and Murti 2004). Similar results have been shown in sugar-beet plants under drought stress, in which a reduction of taproot weight was correlated to stress severity. Treatment with BR

fully compensated for the reduction in biomass caused by mild drought stress. On the other hand, increases in biomass was correlated with increases in acid invertase activity in young leaves, which could have likely provided more assimilates to the plant due to their larger sizes (Schilling et al. 1991). Furthermore, osmotic stress resulted in a considerable reduction in the protein contents in all the three varieties of sorghum. However, BRs not only restored but also stimulated the level of protein and free proline (Vardhini and Rao 2003). 28-homobrassinolide (28-homoBL) also had a stimulatory effect on the growth of drought-tolerant and drought-susceptible wheat (*Triticum aestivum*) varieties under stress conditions. Application of 28-homoBL resulted in increased relative water content, nitrate reductase activity, chlorophyll content, and photosynthesis under both conditions. It also improved membrane stabilisation. These beneficial effects resulted in higher leaf area, biomass production, grain yield, and yield-related parameters in the stress-treated plants. Results obtained by Fariduddin et al. (2009a) indicate that BRs may alleviate drought stress through activation of enzymatic antioxidant system such as CAT, APX, and SOD as well as stimulation of photosynthesis process in *Brassica juncea* plants. In drought-stressed *Chorispora bungeana* plants, BRs inhibited lipid peroxidation, measured in terms of malondialdehyde content, and stimulated antioxidant enzyme activity, chlorophyll content, and photosynthesis. These results suggested that BRs could improve plant growth under drought stress (Li et al. 2012).

Water stress led to oxidative damage. BR treatment of *Zea mays* leaves increased the content of ABA and upregulated the expression of the ABA biosynthetic gene in maize leaves. Moreover, BR treatment induced increases in the generation of nitric oxide (NO) in mesophyll cells of maize leaves, and treatment with the NO donor sodium nitroprusside up-regulated the content of ABA and the expression of ABA biosynthetic gene in maize leaves. These results suggest that BR-induced NO production and NO-activated ABA biosynthesis are important mechanisms for BR-enhanced water stress tolerance in leaves of maize plants (Zhang et al. 2011).

High concentrations of all metals in environment, including those essential for growth and metabolism, exert toxic effects on the metabolic pathways of plants. Plant responds to heavy metal toxicity in different ways, such as by enhancement of the content of PCs, upregulation of antioxidants, accumulation of compatible solutes, accumulation of low-molecular-weight metabolites, and changes in the ABA, auxin, cytokinin, and gibberellin levels. However, BRs are not involved by synthesising de novo in response of algal growth under heavy metal stress but might interact via increasing the contents of other plant hormones (e.g. auxin, cytokinin, and ABA) (Atici et al. 2005; Hsu and Kao 2003; Sharma and Kumar 2002; Bajguz 2011). A recent study indicated that in *C. vulgaris* cultures treated with heavy metals, the endogenous level of BL was very similar to that of control. This finding suggests that the activation of BR biosynthesis is not essential for the growth and development of *C. vulgaris* cultures in response to heavy metal stress (Bajguz 2011). BRs stimulate the synthesis of PCs that are directly involved in detoxification of heavy metals in *C. vulgaris* cells treated with lead. The stimulatory activity of BRs on PC synthesis was arranged in the following order: brassinolide (BL) > 24-epiBL > 28-homoBL > castasterone (CS) > 24-epiCS > 28-homoCS (Bajguz 2002).

The cultures of *C. vulgaris* treated with BRs and heavy metals show a lower bioaccumulation of heavy metals than the cultures treated with metals alone. Application of BRs to *C. vulgaris* cultures reduced the impact of heavy metal stress on growth; prevented chlorophyll, sugar, and protein loss; as well as stimulated the activity of enzymatic and nonenzymatic antioxidant system (Bajguz 2000, 2002, 2010). BRs also reduced the content of cadmium in the seedlings of winter rape (Janeczko et al. 2005) and copper in Indian mustard (Sharma and Bhardwaj 2007). BRs eliminate the toxic effect of cadmium on photochemical pathways in rape cotyledons, mainly by diminishing the damage in reaction centres and O_2 evolving complexes as well as maintaining efficient photosynthetic electron transport (Janeczko et al. 2005). Moreover, Bilkisu et al. (2003) reported that BL during aluminium-related stress stimulated growth of *Phaseolus aureus*. It was shown that changes in the metal content were influenced by 24-epiBL and were dependent on the stage of plant development when the seeds were treated. The application of BRs also improved the performance of mustard (Hayat et al. 2007a), chickpea (Hasan et al. 2008), and tomato (Hayat et al. 2010a) subjected to cadmium stress and also of mung bean (Ali et al. 2008) and mustard (Alam et al. 2007) to aluminium and nickel, respectively. Hasan et al. (2008) reported that BRs enhanced activity of the antioxidant enzymes (CAT, SOD, peroxidase) and proline content in chickpea, which resulted in the improvement of nodulation, nitrogen fixation, and pigment composition, as well as carbonic anhydrase and nitrate reductase activities. A similar pattern of response together with an elevation in the photosynthesis was observed in the plants of mustard and tomato exposed to cadmium through nutrient medium (Hayat et al. 2007a, 2010a, b). The plants treated with 24-epiBL or 28-homoBL showed significantly enhanced growth, photosynthesis, antioxidant enzyme activities, and proline content in aluminium-stressed mung bean plants (Ali et al. 2008) and in *Brassica juncea* that was exposed to different levels of copper (Fariduddin et al. 2009b). In another independent study, the activities of the CAT, peroxidase, carbonic anhydrase, and nitrate reductase enzymes were found to exhibit a significant enhancement by BL treatment in mustard plants grown under nickel stress (Alam et al. 2007). Additionally, these BL-treated and nickel-stressed plants exhibited an elevation in the relative water content and photosynthetic performance. *Raphanus sativus* treated with 24-epiBL in combination with copper enhanced level of phytohormones such as indole-3-acetic acid (IAA) and ABA as well as polyamine contents which may be involved in plant adaptation to the stress factors (Choudhary et al. 2010).

BRs have been reported to alleviate salinity stress on seed germination and seedling growth in many plants. The application of 24-epiBL resulted in substantial improvement in the seed germination and seedling growth of *Eucalyptus camaldulensis* under saline stress (Sasse et al. 1995). BRs removed the salinity-induced inhibition of seed germination and seedling growth in case of rice (*Oryza sativa*). BRs also restored the level of chlorophylls and increased nitrate reductase activity under salt stress. The activity of this enzyme plays a pivotal role in the supply of nitrogen and the growth and productivity of plants, especially in cereals (Anuradha and Rao 2003). The 28-homoBL-treated plants also possessed higher seed yield in

comparison to the plants subjected to NaCl stress, at harvest. Similarly, the spray of 28-homoBL to the foliage or supply through roots of *B. juncea* plants generated from the seeds soaked in NaCl enhanced the growth, nucleic acid content, ethylene, and seed yield (Hayat et al. 2007b).

BRs may also induce tolerance to temperature stress in many plants. For example, leaf spraying of BRs on the rice seedlings at the 4th leaf stage increased plant height and the fresh weights of tops and roots under chilling stress (Fujii and Saka 2001). Extreme temperatures (7 and 34 °C) increased stress symptoms, i.e. necrotic areas on the leaves of bananas. However, in plants treated with a trihydroxylated spirotane, an analogue of BR, the effects of thermal stress were significantly reduced (González-Olmedo et al. 2005). Cool temperature affected leaf emergence with a significant reduction in their number, but application of BR analogue had marked positive effect. Plant height was also significantly reduced by both temperature extremes, whereas the application of BR analogue was effective only in plants exposed to the warmer temperature (González-Olmedo et al. 2005). Application of 24-epiBL minimally increased freezing tolerance of brome grass (*Bromus inermis*) cells by 3–5 °C but markedly enhanced cell viability following exposure to high (40–45 °C)-temperature stress (Wilen et al. 1995).

Treatment of *B. napus* and tomato seedlings with 24-epiBL led to an increase in the basic thermotolerance associated with the higher accumulation of four major classes of heat-shock proteins (hsps): hsp100, hsp90, hsp70, and low-molecular-weight hsps. The higher level of hsps in 24-epiBL-treated seedlings did not correlate with hsp mRNA levels during the recovery period. This finding suggests that 24-epiBL treatment limits the loss of some of the components of the translational apparatus during a prolonged heat stress and increases the level of expression of some of the components of the translational machinery during recovery. The higher hsp synthesis during heat stress resulted in a more rapid resumption of cellular protein synthesis following heat stress and a higher survival rate (Dhaubhadel et al. 1999, 2002). 24-epiBL also induced the expression of mitochondrial small hsps in tomato leaves. BR-treated tomato plants had better photosynthetic efficiency. Significantly higher in vitro pollen germination, enhanced pollen tube growth, and low pollen bursting have been observed in the presence of 24-epiBL at 35 °C, a temperature high enough to induce heat-stress symptoms in tomato, indicating a possible role of BRs during plant growth and reproduction. The beneficial effect of BR application was also observed in fruit yield, which was increased during heat-stressed conditions. This increase in fruit yield was mainly due to increase in fruit number by 24-epiBL application (Singh and Shono 2005).

The exogenously applied BL can also stimulate ABA content in *C. vulgaris* cultures subjected to short-term heat stress (30–40 °C). In parallel, under these conditions treatment with BL resulted in growth levels very similar to those of control cell cultures (nontreated). BL had no significant effect on the content of chlorophyll or sugar in *C. vulgaris* cells. Only a slight effect of BL on the protein content was observed. Under normal growth conditions (25 °C), BL showed a minor increase in the ABA content in *C. vulgaris* cells (Bajguz 2009a).

Signal Transduction of Brassinosteroids

Brassinosteroid Receptor

Recently by developing genetics, genomics, proteomics, and many other approaches performed mainly in *A. thaliana*, a model of BRs signal transduction pathway has been established. The process is commenced by the perception of the hormone ligand by the cell membrane-associated receptor complex, which initiates a relay mediated by phosphorylation/dephosphorylation cascade leading to changes in target gene expression (Gruszka 2013).

BRs are perceived by a plasma-membrane-localised leucine-rich-repeat (LRR) receptor-like kinase (RLK), standing for brassinosteroid-insensitive 1 (BRI1) (Li 2011). BRI1 was isolated and cloned following the identification of a large number of recessive mutant alleles on a single locus (Clouse et al. 1996; Li and Chory 1997). Recent structural studies have confirmed the role of BRI1 as a plasma membrane receptor for BRs (She et al. 2013). BRI1 protein possesses three major domains with unique function in BR perception and receptor activation: a large extracellular domain, a small transmembrane domain, and an intracellular kinase domain (Fig. 5). The extracellular domain of BRI1 contains an amino *N*-terminal signal peptide, a leucine-zipper motif, 24 LRRs, and an island domain located between the 20th and 21st LRRs (Vert et al. 2005; Yang et al. 2011). Further dissection of the extracellular domain of BRI1 revealed a minimal BR-binding region consisting of a 70-amino-acid island domain and its carboxyl *C*-terminal flanking LRR21, which together define a novel steroid-protein-binding element (Kinoshita et al. 2005). The intracellular domain can be further divided into a small intracellular juxtamembrane region (JM), a kinase catalytic domain, and a *C*-terminal tail. The JM domain is required for transducing signal from the outside to the inside of a cell (Wang et al. 2005). Experiments performed on *A. thaliana* plants indicated several Ser/Thr phosphorylation sites within the catalytic domain critical for BR signalling, which include Thr-1049, Ser-1044, and Thr-1045. BRI1 kinase with mutation of Ser-1049A or Ser-1044A/Thr-1045A completely lost its activity in vitro, and transgenic plants carrying these mutated BRI1 also failed to rescue the dwarf phenotype of *bri1-5* (Wang et al. 2005; Yang et al. 2011; Hao et al. 2013).

Given that BRI1 forms homodimer in the absence and presence of BRs, it was proposed that an auto-regulatory mechanism is involved in the activation of BRI1. Without BRs, BRI1 homodimer is kept at quiescent state by its *C*-terminal tail. BR binding induces the conformational change of its kinase domain, and subsequent auto-phosphorylation at a number of sites, including several Ser/Thr residues in the *C*-terminal tail to release its auto-inhibition (Wang et al. 2005). In addition, a specific negative regulator, called BKI1 (BRI1 kinase inhibitor 1), is also required to keep BRI1 at low and basal activity by preventing the interaction of BRI1 with other positive regulators (Wang and Chory 2006; Gruszka 2013).

In the BR receptor complex, besides BRI1, another receptor kinase BAK1 (Fig. 5) (LRR-RK BRI1-associated receptor-like kinase) was also reported to be

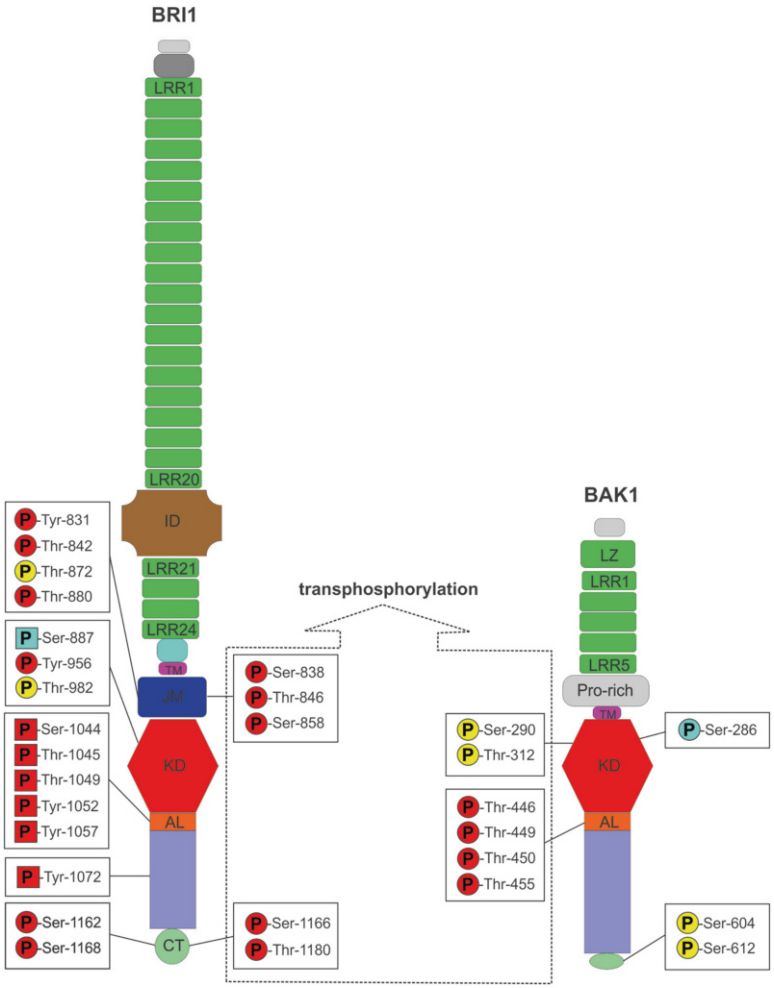

Fig. 5 The structure of BRI1 and BAK1. *LRR* leucine-rich repeats, *ID* island domain, *TM* single-pass transmembrane region, *JM* juxtamembrane region, *KD* kinase domain, *CT* C-terminal region, *LZ* leucine zippers, *pro-rich* proline-rich region, *AL* activation loop of kinases. The putative signal peptide region has been shown as a black box and unassigned regions have been shown as *grey boxes*. The confirmed phosphorylation sites have been marked with *circles*, and putative phosphorylation sites have been marked with *squares* containing the *letter P*. The activation phosphorylation sites have been shown in red, inhibitory sites in *blue*, and residues without significant effect on the kinase activity or not examined experimentally in *yellow* (adopted from Kim and Wang (2010))

required in the activation of BRI1. BRI1 and BAK1 can interact with each other through their kinase domains (Wang et al. 2005). After BR perception by the extracellular domain of BRI1, the kinase domain of BRI1 first phosphorylates and partially activates BAK1, then BAK1 in turn transphosphorylates BRI1 to further enhance the kinase activity of each other (Wang et al. 2008). Before BR binding,

BRI1 is kept inactive by auto-inhibition of its *C*-terminal region and by a negative regulator BKI1. Upon BR perception, BRs induce a conformational change of the intracellular domain of BRI1 playing role as Ser/Thr kinases to autophosphorylate its *C*-terminal tail and phosphorylate BKI1 to release their inhibition on BRI1 activity (Wang and Chory 2006). The pre-activated BRI1 will recruit BAK1 to its proximity to enhance each other's kinase activity via transphosphorylation and to form a fully activated receptor complex (Oh et al. 2009; Hao et al. 2013).

In addition to its critical role in BR signalling for plant growth, BAK1 has been discovered to impact plant MICROBIAL ASSOCIATED MOLECULAR PATTERN (MAMP)-/PATHOGEN-ASSOCIATED MOLECULAR PATTERN (PAMP)-triggered immunity (PTI) through the formation of heterodimers with other pattern-recognition receptors (PRRs) such as flagellin-sensing 2 (FLS2) in a BR-independent manner. Therefore, BAK1 plays key roles in multiple independent pathways by enhancing the signalling output of distinct LRR-RLKs that bind different ligands (Chinchilla et al. 2007).

Substrates of BRI1 Kinase

One of BRI1's substrates is BKI1. BKI1 acts as a negative regulator of BR signalling as indicated by overexpression of BKI1 causing a *bri1*-like dwarf phenotype and inhibiting BR-signalling outputs (Fig. 6) (Wang and Chory 2006). In vitro pull-down assays revealed that the interaction between BRI1 and BAK1 was severely reduced by additional BKI1 protein, suggesting that BKI1 inhibit BR signalling by preventing positive regulators, such as BAK1, from accessing BRI1. Interestingly, BR treatment can rapidly induce the dissociation of BKI1 from plasma membrane, and this process is dependent on a kinase-active BRI1. BKI1 can be phosphorylated by BRI1 kinase, which may lead to the dissociation of BKI1 from BRI1 and plasma membrane through unknown mechanisms (Wang and Chory 2006).

Another BRI1 substrate is polypeptide transthyretin-like protein (TTL) (Nam and Li 2002). TTL is a tetrameric, bifunctional protein with decarboxylase and hydrolase activity, which is phosphorylated by BRI1 and functions as a negative regulator of BR signalling. The exact role of TTL in regulation of this process is not known; however, it has been recently reported that TTL binds kinase-active BRI1 with higher affinity than kinase-inactive BRI1, indicating that TTL may inhibit BRI1 signalling after its activation (Gruszka 2013).

A proteomic analysis led to the identification of other components of the BR receptor complex—BR-signalling kinases (BSKs) belonging to the subfamily of the receptor-like cytoplasmic kinases (RLCK-XII) and functioning as positive regulators of BR signalling. The members of BSK family transmit the signal between membrane-bound receptor complex and cytoplasmic regulators of BR signalling. Two paralogous proteins, BSK1 and BSK3, interact directly with BRI1 in the absence of BR, whereas upon the ligand binding to BRI1, this kinase phosphorylates BSK1 on Ser-230, inducing its activation and release from the

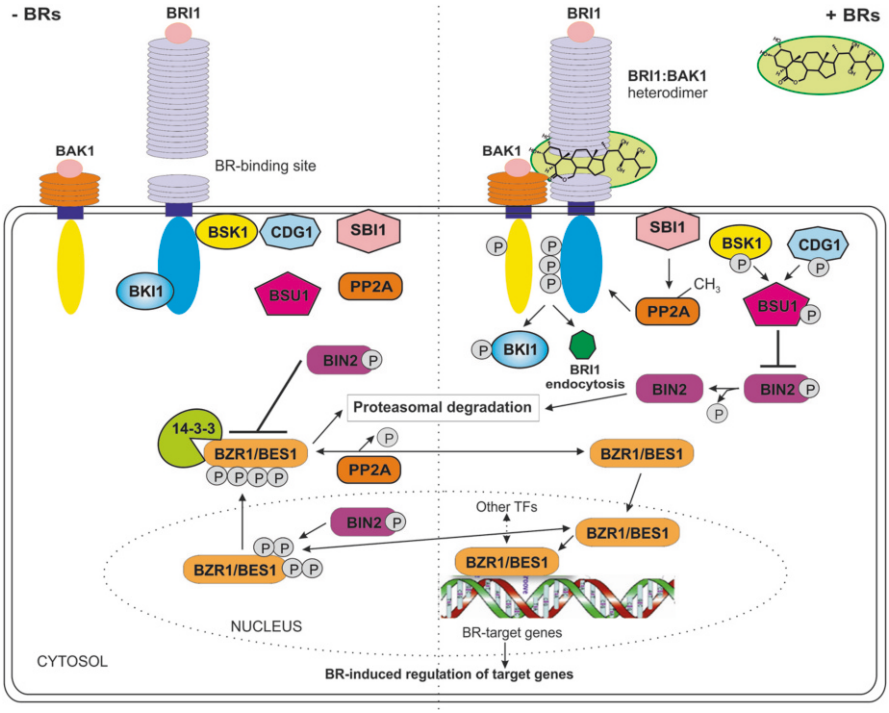

Fig. 6 The model of brassinosteroid signalling in plants (adopted from Bajguz et al. (2013)). Brassinosteroid (BR) signal is perceived by BR-insensitive1 (BRI1) which is a plasma membrane localised leucine-rich repeat (LRR) receptor-like kinase. In the absence of BRs, BRI1 is inactive as a homodimer, due to its binding with the negative regulator BRI1 KINASE INHIBITOR 1 (BKI1) through its cytoplasmic domain. In the presence of BRs, BR binding activates BRI1 kinase activity, through association with its co-receptor kinase BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1)/SOMATIC EMBRYOGENESIS RECEPTOR KINASE3 (SERK3) and phosphorylation of BKI1, leading to the disassociation of BKI1 from the plasma membrane. Activated BRI1 phosphorylates the receptor-like cytoplasmic kinases (RLCKs), BR SIGNALLING KINASE1 (BSK1) and CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1), which then activate a phosphatase, BRI1-SUPPRESSOR 1 (BSU1). BSU1 positively regulates BR signalling by dephosphorylating the negative regulator brassinosteroid-insensitive 2 (BIN2). This process facilitates accumulation of unphosphorylated brassinazole-resistant 1 (BZR1) and bri1-EMS-Suppressor 1 (BES1) in the nucleus. BES1 binds to E-box by interacting with BIM1 or MYB30 (TFs) to promote target gene expression. BZR1 could also bind to E-box and BES1 to BRRE, so the functions of the family members may overlap. These are key TFs activating the BR-signalling pathway in plants. Protein phosphatase 2A dephosphorylates BZR1 and also BRI1 in mediating BR signalling. BRI1 degradation depends on PP2A-mediated dephosphorylation that is specified by methylation of the phosphatase, thus leading to the termination of BR signalling

receptor complex. The activated BSK1 interacts with BRI1-supressor1 (BSU1) phosphatase promoting its interaction with the main negative regulator of BR signalling pathway—brassinosteroid-insensitive 2 (BIN2) (Fig. 6) (Tang et al. 2008; Gruszka 2013).

Downstream Events of Brassinosteroid Signalling

A crucial role in BR signalling is played by the serine-threonine kinase BIN2, which is another negative regulator of BR signalling, phosphorylating and thus inhibiting transcription factors regulating expression of target genes (Fig. 6) (Vert and Chory 2006; Yan et al. 2009). The *Arabidopsis BIN2* belongs to a multigene family encoding glycogen synthase kinase 3 (GSK3). GSK3-encoding genes are present in all land plants and in algae, and protists, raising questions about possible ancestral functions in eukaryotes. Studies have revealed that plant GSK3 proteins are actively implicated in hormonal signalling networks during development (e.g. development of generative organs) and as well as in biotic and abiotic stress responses (salinity stress and wounding). BIN2 is encoded by a member of the subfamily of ten related genes—*Arabidopsis* shaggy-like kinases (ASKs) (Vert and Chory 2006). The level of BIN2 protein can be regulated by BR signal likely through a proteasome-mediated protein degradation system, because the exogenously applied BRs can lead to a reduction of BIN2 proteins, and treatment with a proteasome inhibitor, MG132, can promote the accumulation of BIN2 (Peng et al. 2010). In the absence of BR, BIN2 autophosphorylates on Tyr-200 residue, which is required for its kinase activity. BIN2 kinase activity is suppressed by dephosphorylation of the Tyr-200 residue after perception of BR molecule by the BRI1-BAK1/SERK3 receptor complex and initiation of the signalling cascade. BIN2 activity is directly inhibited by BSU1 phosphatase, which dephosphorylates the Tyr-200 residue of BIN2 kinase (Ye et al. 2011; Hao et al. 2013).

A protein phosphatase, BSU1 (BRI1 suppressor protein 1), is a constitutively nuclear-localised Ser/Thr phosphatase (Mora-Garcia et al. 2004; Ryu et al. 2010). BSU1 plays a crucial role in positive regulation of BR signalling by repressing the activity of BIN2 kinase (Fig. 6). BSU1 contains *N*-terminal Kelch-repeat domain and *C*-terminal phosphatase domain and shows basal level of BIN2-binding and dephosphorylation. Activated BSU1 interacts with BIN2 kinase and inactivates it through dephosphorylation of Tyr-200, which is crucial residue for BIN2 activity. BSU1 phosphatase is localised in both the cytoplasm and nucleus; however, it was reported that BR response is mediated mainly by the cytoplasmic fraction of this enzyme. On the contrary, BIN2, which is the direct target of BSU1 phosphatase, operates mainly in the nucleus (Ryu et al. 2010). Therefore, BR perception can activate BRI1, BSKs, and BSU1 to inactive BIN2, resulting in the activation of downstream transcription factors (Kim and Wang 2010).

A Class of Brassinosteroid-Activated Transcription Factors and Their Regulation

The expression of many BR-responsive genes is directly regulated by a class of plant-specific transcription factors including BES1 (BRI1-EMS-suppressor 1), BZR1 (brassinazole-resistant 1) (Fig. 7), and BES1/BZR1 homologues 1–4 (BEH1–4)

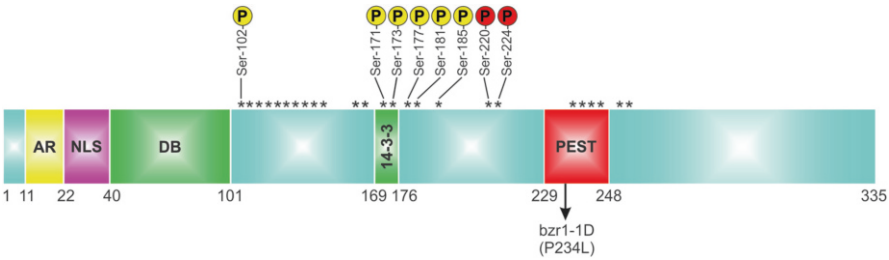

Fig. 7 The structure of the transcription factor BZR1. *AR* an alanine-rich domain, *NLS* nuclear localization signal, *DB* DNA binding domain, *PEST* proline, glutamic acid, serine, threonine rich domain, 14-3-3, binding motif. Putative BIN2 phosphorylation sites (as *blue box*) have been indicated by *asterisks*. *Yellow circles* containing the *letter P* indicate sites phosphorylated by BIN2 in vitro, and *red circles* indicate in vivo phosphorylation sites (adopted from Kim and Wang (2010))

that bind to the promoters of BR-regulated genes, and they are dephosphorylated in response to BR (Gruszka 2013).

BES1 and BZR1 are two major transcription factors that are regulated by BIN2 and mediate BR-regulated gene expression (Fig. 6) (Wang et al. 2002). BES1 and BZR1 are 88 % identical and are composed of DNA-binding domain (DBD), BIN2 phosphorylation domain with more than 20 putative BIN2 phosphorylation sites (Ser/ThrxxxSer/Thr, where x is any amino acid), and a *C*-terminal domain (CTD). The CTD is required for BES1 function as deletion of this domain leads to accumulation of inactive BES1 that acts as a dominant-negative form (Yin et al. 2005). The *C*-terminal domain of BES1 most likely acts as a transcription activation domain as it activates reporter gene expression in yeast. In addition, the *C*-terminal domain also contains a 12-amino-acid docking motif (DM) that binds BIN2, allowing BIN2 to phosphorylate BZR1. Since the same domain is conserved in BES1, it is likely that BIN2 interacts with DM to phosphorylate BES1 as well (Peng et al. 2010).

BIN2 phosphosphorylates BES1 and BZR1 at their central phosphorylation domain and inhibits their function likely through several different but non-exclusive mechanisms, including targeted protein degradation, nuclear export, and cytoplasmic retention by the phosphoprotein-interacting 14-3-3 proteins (Fig. 6). Polypeptides belonging to the group 14-3-3 function as another components of the BR signalling with dual role in regulation of this process. Recently, it has been reported that the 14-3-3 proteins may play a positive role in BR signalling by promoting BKI1 dissociation from the plasma membrane, what in consequence results in repressing of the BKI1 inhibitory effect on the BRI1 receptor (Lillo et al. 2006; Wang et al. 2011; Hao et al. 2013).

BZR1 can bind to a CGTG(T/C)G element, called BR-response element (BRRE) with its *N*-terminal domain to negatively feedback regulating the expression of genes involved in BR biosynthesis, such as *CPD*, *DWF4*, *ROT3*, and *BR6ox* (He et al. 2005). Apparently, BES1 may have a similar function in the feedback regulation of genes encoding BR biosynthetic enzymes (Yin et al. 2005; Vert and Chory 2006). Using transcript profiling and chromatin-immunoprecipitation

microarray experiments, Sun et al. (2010) reported 953 BR-regulated BZR1 target genes, which function in BR promotion of cell elongation and crosstalk between BR and other hormonal and light-signalling pathways at multiple levels.

Nuclear accumulation of dephosphorylated BES1/BZR1 plays important roles in directly regulating the expression of BR-responsive genes. Studies on the subcellular localisation of BES1 and BZR1 using green fluorescent protein (GFP) in *Arabidopsis* showed that, without BRs, BES1 and BZR1 are distributed in both the nucleus and cytoplasm, while BR treatment can rapidly promote the accumulation of BES1/BZR1 in nucleus in *Arabidopsis* hypocotyl cells (Wang et al. 2002; Yin et al. 2002). Later, another study showed that proteins BES1 and BZR1 labelled with GFP (BES1-GFP, BZR1-GFP) can be localised in both the cytoplasm and nucleus, and BR treatment can significantly induce the accumulation of dephosphorylated BES1-GFP and BZR1-GFP in the nucleus (Gampala et al. 2007; Ryu et al. 2010).

When BR levels are low, the GSK3-like kinase BIN2 phosphorylates and inactivates the BZR1 transcription factor to inhibit growth in plants. Brassinosteroid promotes growth by inducing dephosphorylation of BZR1 by protein phosphatase 2A (PP2A). PP2A is a heterotrimeric Ser/Thr phosphatase, which contains as scaffolding subunit A, catalytic subunit C, and a regulatory B subunit that interacts with substrates. Members of the B′ regulatory subunits of PP2A directly interact with BZR1's putative PEST domain containing the site of the *bzr1-1D* mutation. Interaction with and dephosphorylation by PP2A are enhanced by the *bzr1-1D* mutation, reduced by two intragenic *bzr1-1D* suppressor mutations, and abolished by deletion of the PEST domain. Therefore, PP2A plays a crucial function in dephosphorylating and activating BZR1 and completes the set of core components of the brassinosteroid-signalling cascade from cell surface receptor kinase to gene regulation in the nucleus (Tang et al. 2011).

In addition, BZR1 modulates the expression levels of many light-signalling components. Genome-wide protein-DNA interaction analysis revealed BZR1 binding to the promoters of a significant portion of light-regulated genes, suggesting that BR and light signals converge at the promoters of common target genes through direct interaction between BZR1 and some light-signalling transcription factors. BZR1 may also directly interact with phytochrome-interacting factors 4 (PIF4), which is accumulated in the dark to promote morphogenesis. BZR1 and PIF4 interact with each other in vitro and in vivo, bind to nearly 2,000 common target genes, and synergistically regulate many of these target genes, including the PRE family helix-loop-helix factors required for promoting cell elongation. Genetic analysis indicates that BZR1 and PIFs are interdependent in promoting cell elongation in response to BR, darkness, or heat. These results show that the BZR1-PIF4 interaction controls a core transcription network, enabling plant growth co-regulation by the steroid and environmental signals (Lillo et al. 2006; Oh et al. 2012).

Brassinazole (Brz), a specific inhibitor of BR biosynthesis, was used in experiments performed by Bekh-Ochir et al. (2013) to identify Brz-insensitive-long hypocotyls 2-1D (*bil2-1D*) mutant of *Arabidopsis*. The *BIL2* gene encodes a mitochondrial-localised DnaJ/heat-shock protein 40 (DnaJ/Hsp40) family, which is

involved in protein folding. *BIL2*-overexpression plants (*BIL2-OX*) showed cell elongation under Brz treatment, increasing the growth of plant inflorescence and roots, the regulation of BR-responsive gene expression, and the suppression against the dwarfed *BRI1*-deficient mutant. *BIL2-OX* also showed resistance against the mitochondrial ATPase inhibitor oligomycin and higher levels of exogenous ATP compared with wild-type plants. BIL2 participates in resistance against salinity stress and strong light stress. The results indicate that *BIL2* induces cell elongation during BR signalling through the promotion of ATP synthesis in mitochondria (Bekh-Ochir et al. 2013).

In addition, AtMYB30, another transcription factor, is also positively involved in BR signalling by promoting a subset of BR-responsive gene expression (Li et al. 2010). BES1 can interact with AtMYB30 both in vitro and in vivo to promote the expression of downstream target genes. It was discovered that BES1 can also physically interact with interacts-with-Spt6 (IWS1), which participates in RNA polymerase II (RNAPII) post-recruitment and transcriptional elongation processes (Li et al. 2010).

Apart from these transcription factors, BIN2 phosphorylates CESTA transcription factor belonging to the basic helix-loop-helix (bHLH) family. CESTA positively regulates expression of the BR-biosynthesis *CPD* gene by heterodimerisation with the close homologue of CESTA, BRI1-enhanced expression 1 (BEE1). BIN2-mediated phosphorylation of CESTA is assumed to regulate the nuclear localisation of this transcription factor. Based on the results derived from several different approaches, it has been suggested that BIN2 operates both in the nucleus and cytoplasm, and the exact mechanism may depend on developmental stage, tissue type, and BIN2 gene expression level (Clouse 2011; Poppenberger et al. 2011; Hao et al. 2013).

Brassinosteroid Signalling and Stress Tolerance

The molecular mechanisms of BR-induced plant stress tolerance remain poorly understood. Cui et al. (2012) reported that an endoplasmic reticulum (ER)-localised *Arabidopsis* ubiquitin-conjugating enzyme UBC32 is an essential factor involved in both BR-mediated growth promotion and salt stress tolerance. In vivo data in *Arabidopsis* showed that UBC32 is a functional component of the ER-associated protein degradation (ERAD) pathway, which is an important ubiquitin-proteasome system regulating plant growth and development, known to contribute to plant salt tolerance (Liu et al. 2011). UBC32 affects the accumulation of BRI1 and connects the ERAD pathway to BR-mediated growth promotion and salt stress tolerance. A recent study in tomato revealed one possible mechanism of BR-induced abiotic stress tolerance, especially for oxidative and heat stress (Nie et al. 2012). BRs trigger apoplastic H_2O_2 accumulation generated by NADPH oxidase, which is encoded by the RESPIRATORY BURST OXIDASE HOMOLOG 1 (*RBOH1*) gene. The *RBOH*s are involved in plant ROS production and plant response to various

abiotic stresses (Marino et al. 2012). NADPH oxidase in turn activates MAPKs, which play critical roles in plant signal transduction during stress responses (Mittler et al. 2004), giving rise to increased stress tolerance (Hao et al. 2013).

Conclusion Remarks

Brassinosteroids (BRs) are plant hormones implicated in a wide array of fundamental processes in plants ranging from triggering the cell cycle, genome expression, signalling, and plant growth and development to plant adaptation toward abiotic stresses. However, molecular mechanisms underlying BR participation in plant adaptation to stress are not completely understood. Understanding the signal transduction of BRs during abiotic stress is vital in developing plants for stress tolerance. There is an urgent need to identify the signalling components related to the biosynthesis and degradation and their coordination in gene expression events under stress conditions. The characterisation of the molecular mechanisms regulating hormone synthesis, signalling, and action is facilitating the modification of BR biosynthetic pathways for the generation of transgenic crop plants with enhanced abiotic stress tolerance.

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Salicylic Acid and Defense Responses in Plants

Chuanfu An and Zhonglin Mou

Abstract Salicylic acid (SA) is a simple phenolic compound distributed in a wide range of plant taxa. Depending on the plant species, developmental stage, and growth conditions, it can be synthesized from cinnamic acid produced by phenylalanine ammonia-lyase in the cytosol or from isochorismic acid generated by isochorismate synthase in chloroplasts. However, a fully defined SA biosynthetic pathway is still unavailable in plants. Besides its role in regulating various aspects of plant growth and development, SA is a plant immune signal essential for both local defense response and systemic acquired resistance. Significant progress has been made recently in understanding SA-mediated defense signaling networks including identification of SA receptors and elucidation of the crucial role of NPR1 (nonexpressor of pathogenesis-related genes 1) in SA signal execution. Understanding of SA-mediated plant defense has facilitated the development of disease-resistant crops through genetic manipulation of the SA signaling pathway. Although the use of *NPR1* and its orthologs in developing broad-spectrum transgenic disease resistance has been successfully extended to a variety of crop species, commercial application of these transgenic crops has been hampered by ethical concerns. In this regard, cisgenesis may hold the potential for application of bioengineered disease-resistant crops in agriculture.

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Introduction

Salicylic acid (SA, 2-hydroxy benzoic acid) is a small phenolic compound synthesized by a wide range of prokaryotic and eukaryotic organisms. It has a broad distribution in the plant kingdom as free phenolic acid and/or conjugated forms generated by glucosylation, methylation, amino acid conjugation, sulfonation, or hydroxylation (Pridham 1965; Pierpoint 1994; Vlot et al. 2009; Dempsey et al. 2011). Among these natural SA derivatives, salicin (β-glucoside salicylic alcohol) is the best known one. It accumulates to high levels in several willow species including *Salix alba*, *S. purpurea*, *S. daphnoides*, and *S. fragilis* whereby the name of salicylic acid was derived from (Raskin 1992; Foster and Tyler 1999). However, the highest levels of total SA were found in inflorescence of thermogenic plants and in spice herbs (Raskin et al. 1990). Under optimal conditions, rice, crabgrass, green foxtail, barley, and soybean have SA levels in excess of 1 μg g^{-1} fresh weight (FW) (Raskin et al. 1990). In the model plant *Arabidopsis thaliana*, basal levels of total SA range from 0.25 μg to 1 μg g^{-1} FW (Nawrath and Métraux 1999; Wildermuth et al. 2001; Brodersen et al. 2005). However, basal SA levels differ widely among species (up to 100-fold differences), even among members of the same family (Yalpani et al. 1991; Malamy et al. 1992; Navarre and Mayo 2004). As ubiquitous distributed secondary metabolites, salicylates (the general name of SA and its derivatives) have been known to possess medicinal properties since the fifth century BC when Hippocrates prescribed salicylate-rich willow leaf and bark for pain relief during childbirth (Weissman 1991). It eventually led to the development of aspirin, one of the world's most widely used drugs, in the 1890s (Raskin 1992). Recently, SA has been established as a distinct class of plant hormone because of its important regulatory roles in seed germination (Rajou et al. 2006), seedling establishment (Alonso-Ramírez et al. 2009), cell growth (Rate et al. 1999; Vanacker et al. 2001), trichome development (Traw and Bergelson 2003), flowering (Cleland 1974; Cleland and Ajami 1974; Martínez et al. 2004), thermogenesis (Raskin et al. 1987), nodulation (Stacey et al. 2006), respiration (Norman et al. 2004), stomatal responses (Manthe et al. 1992; Lee 1998), senescence (Morris et al. 2000; Rao and Davis 2001; Rao et al. 2002), and responses to biotic and abiotic stresses (Janda et al. 2007; Vlot et al. 2009).

The best-established role for SA is as a signal molecule functioning in plant immune responses (Enyedi et al. 1992; Alvarez 2000; Nishimura and Dangl 2010). Due to sessile nature and lacking specialized immune cells, plants have developed the capability to sense pathogen and mount immune response through individual cells. Recognition of pathogen-associated molecular patterns (PAMPs) leads to PAMP-triggered immunity (PTI) that prevents pathogen colonization. While PTI is sufficient to prevent further colonization by many microbes, some pathogens have evolved effectors to dampen PAMP-triggered signals. In turn, host plants have

evolved resistance (R) proteins to detect the presence of pathogen effectors and induce effector-triggered immunity (ETI) including hypersensitive response (HR) (Jones and Dangl 2006). Activation of defense signaling pathways (PTI or ETI) results in the generation of a mobile signal(s) that moves from local infected tissue to distal tissues to induce systemic acquired resistance (SAR), which is a long-lasting immunity against a broad spectrum of pathogens (Fu and Dong 2013). SA-mediated immune responses are important parts of PTI and ETI and also essential for the activation of SAR (Durrant and Dong 2004). Efforts to elucidate the crucial role of SA in immune responses have uncovered that pathogen infection leads to SA accumulation not only in the local infected tissue but also in systemic tissues that develop SAR (Malamy et al. 1990; Métraux et al. 1990) and that SA accumulation usually parallels or precedes the increase in expression of *pathogenesis-related* (*PR*) genes and development of SAR. Consistently, exogenous application of SA and its functional analogs induces *PR* gene expression and resistance against viral, bacterial, oomycete, and fungal pathogens in both dicotyledonous and monocotyledonous plants (Malamy and Klessig 1992; Wasternack et al. 1994; Gorlach et al. 1996; Ryals et al. 1996; Morris et al. 1998; Shah and Klessig 1999; Pasquer et al. 2005; Makandar et al. 2006). Conversely, blocking SA accumulation through expression of a bacterial *naphthalene* (*nah*)-catabolic gene *nahG*, which encodes a salicylate hydroxylase that converts SA to catechol, in transgenic tobacco and *Arabidopsis* plants compromises both HR and SAR (Gaffney et al. 1993; Delaney et al. 1994). Similarly, mutations of genes involved in SA biosynthesis and inhibition of SA biosynthesis have been shown to enhance susceptibility to pathogens, yet the resistance can be restored through exogenous SA application (Mauch-Mani and Slusarenko 1996; Nawrath and Métraux 1999; Wildermuth et al. 2001; Nawrath et al. 2002). Therefore, SA is an important endogenous marker and determinant of plant disease resistance.

In the past two decades, intensive studies have revealed a complex network of SA biosynthesis and signaling in plant immunity. Increasing knowledge of SA-mediated immunity in model systems has led to translational research on developing disease-resistant crop cultivars through transgenic approaches. Genetic screens, transcriptomics, proteomics, and protein interaction studies predominantly in *Arabidopsis* have provided a large number of candidate genes for biotechnological manipulation in crops. At the same time, outcomes of genetic engineering have enhanced our understanding of the SA-mediated immune responses in different plant species. Here, we describe the recent progresses in our understanding of SA biosynthesis, signal perception and execution, and their biotechnological applications in improvement of crop disease resistance.

Salicylic Acid Biosynthesis

Studies of SA biosynthesis in plants have discovered two distinct and differentially compartmentalized pathways: the phenylalanine ammonia-lyase (PAL) pathway starting in the cytosol and the isochorismate synthase (ICS) pathway operative in

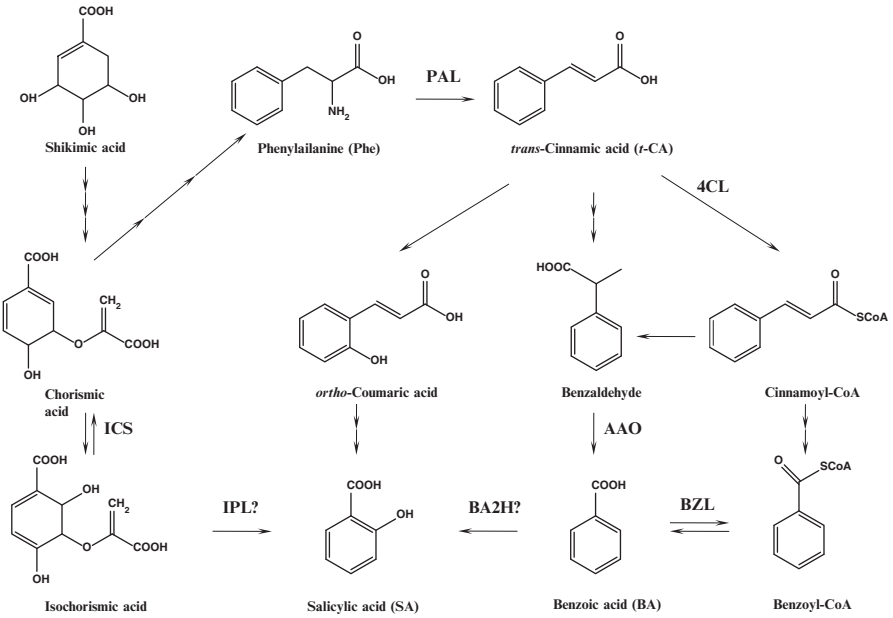

Fig. 1 Salicylic acid biosynthetic pathways in *Arabidopsis thaliana. AAO Arabidopsis* aldehyde oxidase, *BZL* benzoyl-CoA ligase, *BA2H* benzoic acid-2-hydroxylase, *4CL* 4-coumaroyl:CoA ligase, *ICS* isochorismate synthase, *IPL* isochorismate pyruvate lyase, *PAL* phenylalanine ammonia-lyase. Enzymes that have not been identified so far are marked with a question marker

chloroplasts (Fig. 1). Both pathways require the primary metabolite chorismate. However, to date neither biosynthetic route has been fully resolved.

The PAL Pathway

PAL (EC 4.3.1.5) is the first enzyme in the phenylpropanoid pathway, which catalyzes phenylalanine (Phe) to *trans*-cinnamic acid (*t*-CA) and NH_3 via a non-oxidative deamination reaction (Raes et al. 2003; Rohde et al. 2004). Early radiolabeling studies with Phe, *t*-CA, or benzoic acid (BA) suggested that SA is synthesized from Phe via *t*-CA, which is then converted to SA through two possible routes depending on the plant species and growing conditions (Klämbt 1962; El-Basyouni et al. 1964; Chadha and Brown 1974).

1. Hydroxylation of *t*-CA to *ortho*-coumaric acid followed by its decarboxylation to SA (Fig. 1). Feeding of ^{14}C-labled Phe and *t*-CA to young *Primula acaulis* and *Gaultheria procumbens* leaf segments leads to accumulation of *ortho*-coumaric acid and SA, indicating the function of *ortho*-coumaric acid pathway in SA biosynthesis (Griesebach and Vollmer 1963; El-Basyouni et al. 1964). Similarly, upon *Agrobacterium tumefaciens* infection, young tomato seedlings synthesize

SA through hydroxylation of *t*-CA to *ortho*-coumaric acid (Chadha and Brown 1974). Although the conversion of *t*-CA to *ortho*-coumaric acid is believed to be catalyzed by *trans*-cinnamate-4-hydroxylase in multiple species (Russel and Conn 1967; Alibert and Ranjeva 1971, 1972; Gabriace et al. 1991), the activity of 2-hydroxylation of *t*-CA to form *ortho*-coumaric acid was only detected in the suspension of chloroplasts instead of the cytosol of the sweet clover (*Melilotus alba* Desr.) (Gestetner and Conn 1974). Nevertheless, the enzyme(s) that catalyzes the conversion of *ortho*-coumaric acid to SA has not yet been identified.

2. Decarboxylation of the side chains of *t*-CA to generate BA followed by hydroxylation at C_2 position (Fig. 1). A growing body of evidence indicates that plants can potentially develop three biosynthetic subroutes to BA, including an β-oxidative route from cinnamoyl Co-A, a non-oxidative route from cinnamoyl Co-A, and a non-oxidative route from *t*-CA to BA (Wildermuth 2006). Radiolabeling studies using Phe or putative pathway intermediates performed in tobacco mosaic virus (TMV)-infected tobacco, smoke-treated coyote tobacco, or cucumber detected incorporation of radiolabeled carbon into BA and SA but not benzaldehyde, suggesting that SA is synthesized through the cinnamoyl-Co-A β-oxidative subroute (Ribnicky et al. 1998; Jarvis et al. 2000). Similar studies have not been performed in *Arabidopsis* to probe downstream components of SA biosynthesis via PAL pathway. However, a study of BA production in developing seeds identified an *Arabidopsis* aldehyde oxidase4 (AAO4) that catalyzes the conversion of benzaldehyde to BA, which is then incorporated into benzoyl glucosinolates (Ibdah et al. 2009). Additionally, the formation of [^{14}C]BA from [^{14}C]Phe through [^{14}C]*t*-CA was observed in *Tsuga canadensis*, young *Gaultheria procumbens* tissue, and uninfected tomato seedlings (Zenk and Muller 1964; Ellis and Amrhein 1971; Chadha and Brown 1974). Furthermore, ^{14}C-tracer studies with tobacco cell suspensions or TMV-inoculated leaves indicated that the label moves from *t*-CA to SA via BA (Yalpani et al. 1993). Similarly, rice shoots can convert both [^{14}C]*t*-CA and [^{14}C]BA to SA (Silverman et al. 1995).

The direct conversion of [^{14}C]BA to [^{14}C]SA discovered in etiolated *Helianthus annuus* hypocotyls, *Solanum tuberosum* tubers, *Pisum sativum* internodes, and infected cucumber plants was proposed to be catalyzed by an inducible BA 2-hydroxylase (BA2H) (Klämbt 1962; Meuwly et al. 1995). BA2H activity was further detected in ozone-exposed tobacco leaves, heat-treated pea plants, and salt-stressed rice seedlings (León et al. 1995; Ogawa et al. 2005; Sawada et al. 2006; Pan et al. 2006). Biochemical characterization indicated that tobacco BA2H is a soluble P450 oxygenase that specifically hydroxylates the *ortho* position of BA (León et al. 1995). Although there has been no subsequent report describing a BA2H-encoding gene in plants, similar activity has been observed in *Arabidopsis*, which converts neonicotinoid metabolite 6-chloropyridinyl-3-carboxylic acid to the SA mimic 6-chloro-2-hydroxypyridinyl-3-carboxylic acid *in planta* (Ford et al. 2010). Studies conducted in poplar and tobacco indicated that it might also be possible that the glucose-conjugated ester of BA acts as an intermediate for the synthesis of the SA glucose ester and SA (Chong et al. 2001; Ruuhola and Julkunen-Tiitto 2003).

The preference of SA biosynthetic route in the PAL pathway depends on plant species and growth conditions. Isotope-feeding experiments revealed that SA is mainly synthesized from BA in some plant species such as tobacco, rice, potato, cucumber, sunflower, and pea (Klämbt 1962; Yalpani et al. 1993; León et al. 1995; Silverman et al. 1995; Sticher et al. 1997), while other plant species can form SA through the route of *ortho*-coumaric acid (Yalpani et al. 1993; León et al. 1995; Silverman et al. 1995). However, feeding of ^{14}C-labeled Phe, *ortho*-coumaric acid, and BA to young *Primula acaulis* and *G. procumbens* leaf segments all leads to SA, suggesting that both routes are probably utilized in SA biosynthesis (El-Basyouni et al. 1964). Similarly, SA is formed mostly via BA in young tomato seedlings, but after infection with *A. tumefaciens*, SA biosynthesis is shifted to the route of hydroxylation of cinnamate to *ortho*-coumaric acid (Chadha and Brown 1974).

Elucidation of the above PAL pathway largely relied on isotope feeding of the perspective SA biosynthetic precursors to suspension cells or plant segments. Since isotope feeding is not an accurate reflection of *in planta* metabolism, the results might be misleading. Further supports to the PAL pathway in SA biosynthesis came from the evidence that pathogen-resistant tobacco and *Arabidopsis* show increased *PAL* expression and SA levels (Pellegrini et al. 1994; Mauch-Mani and Slusarenko 1996; Dempsey et al. 1999). Additionally, loss of PAL activity, due to sense suppression or treatment with the PAL inhibitor 2-aminoindan-2-phosphonic acid (AIP), reduces pathogen-induced SA accumulation in tobacco, cucumber, and *Arabidopsis*, and the defense phenotypes of PAL-inhibited plants can be complemented by exogenous SA application (Meuwly et al. 1995; Mauch-Mani and Slusarenko 1996; Pallas et al. 1996). Moreover, increases in BA2H activity parallel or precede SA accumulation induced by TMV infection, UV exposure, or treatment with BA or hydrogen peroxide in tobacco (Léon et al. 1993; Yalpani et al. 1993; León et al. 1995). Similarly, salinity induces BA2H activity and SA biosynthesis in rice seedlings, and the induced SA accumulation can be inhibited by uniconazole, a BA2H inhibitor, suggesting that inhibition of BA2H can prevent salinity-induced SA accumulation (Sawada et al. 2006). Importantly, genetic analysis of the *pal* quadruple mutant (*pal1 pal2 pal3 pal4*) revealed a ~75 % reduction in the basal level of total SA as compared with wild-type plants and a ~50 % reduction in total SA levels following avirulent bacterial pathogen infection (Huang et al. 2010). Therefore, it is generally believed that SA can be synthesized through the PAL pathway (Raskin 1992; Lee et al. 1995; Coquoz et al. 1998; Dempsey et al. 2011).

The ICS Pathway

Although early studies suggested that plants might synthesize SA through the PAL pathway, there have been accumulating data questioning its role in the overall SA biosynthesis. In some of the radiolabeling studies described above, the incorporation rate of labeled precursor into SA is lower than expected, particular under infection/induction conditions (Chadha and Brown 1974; Yalpani et al. 1993;

Coquoz et al. 1998). Inhibiting PAL activity by AIP can only reduce chemical- or pathogen-induced SA accumulation by several folds in potato or *Arabidopsis*, respectively (Mauch-Mani and Slusarenko 1996; Coquoz et al. 1998). These pieces of evidence indicated that there might be another pathway in plants leading to SA biosynthesis (Fig. 1).

Bacteria in several genera have been shown to synthesize SA in the production of iron-chelating siderophores (Garcion and Métraux 2006). In the bacterial pathway, chorismate is converted to SA through an isochorismate (IC) intermediate (Verberne et al. 1999). In some bacterial species, like *Pseudomonas aeruginosa* and *P. fluorescens*, chorismate is first converted to IC by isochorismate synthase (ICS, EC 5.4.4.2) and followed by conversion to SA and pyruvate by another unifunctional enzyme, isochorismate pyruvate lyase (IPL, EC 4.2.99.21) (Serino et al. 1995; Mercado-Blanco et al. 2001). In contrast, SA synthesis in *Yersinia enterocolitica* and *Mycobacterium tuberculosis* is achieved through a sole, bifunctional enzyme named SA synthase (SAS) that directly converts chorismate to SA via an isochorismate intermediate (Pelludat et al. 2003; Kerbarh et al. 2005; Harrison et al. 2006). Structurally, ICS and SAS are similar and contain conserved active sites (Harrison et al. 2006; Kerbarh et al. 2005; Kolappan et al. 2007; Parsons et al. 2008). Functionally, both enzymes begin with nucleophilic attack at C_2 of chorismate, with water as the nucleophile, concomitant with displacement of the C_4 hydroxyl group in an S_N2 reaction (He et al. 2004); however, reactions on SAS is followed by elimination of pyruvate and release of SA.

In plants, chorismate is synthesized in the plastid (Poulsen and Verpoorte 1991; Schmid and Amrhein 1995). Considering the fact that many plastid-localized pathways are derived from prokaryotic endosymbionts, it is possible that plants may also utilize a similar ICS pathway for SA biosynthesis (Verberne et al. 1999; Wildermuth et al. 2001). To assess whether plants contain an endogenous pathway to synthesize SA through IC, Wildermuth et al. (2001) identified two putative *ICS* genes in the *Arabidopsis* genome. ICS1 (At1g74710) and ICS2 (At1g18870) share 78 % identity at the amino acid level and ICS1 is 57 % identical to a *Catharanthus roseus* ICS, whose activity has been confirmed biochemically (van Tegelen et al. 1999; Garcion et al. 2008). However, only *ICS1* transcript is accumulated in leaves infected with fungal (*Golovinomyces orontii*) and bacterial (*P. syringae* pv. *maculicola*) pathogens (Wildermuth et al. 2001). *ICS1* expression correlates with SA accumulation and expression of the SA-inducible *PR1* gene. Subsequent analyses indicated that *ICS1* transcripts also accumulate in response to a variety of biotic or abiotic stresses, including UV light, ozone, PAMPs, (hemi)biotrophic pathogens, and exogenous SA treatment (Ogawa et al. 2005; Killian et al. 2007; Nobuta et al. 2007; Postel et al. 2010; Dempsey et al. 2011; Harrower and Wildermuth 2011). Two *Arabidopsis* mutants, *sid2-1* (*salicylic acid induction-deficient2-1*) and *eds16-1* (*enhanced disease susceptibility16-1*) (Nawrath and Métraux 1999; Dewdney et al. 2000), which can accumulate only 5–10 % of the wild-type level of SA following infection of virulent or avirulent pathogens, were found to contain lesions in the *ICS1* gene (Wildermuth et al. 2001). Exogenous SA application can complement their enhanced disease susceptibility phenotype (Wildermuth et al. 2001).

Biochemical and molecular analyses provided further evidence supporting the role of ICS1 in SA biosynthesis. As expected, ICS1 contains a putative plastid transit sequence and a cleavage site (Wildermuth et al. 2001). The high affinity of ICS1 for chorismate allows ICS1 to compete successfully with other pathogen-induced enzymes that use chorismate as their substrate, such as anthranilate synthase (Strawn et al. 2007; Ziebart and Toney 2010). Unlike the bifunctional SAS, the recombinant ICS1 only converts chorismate to IC, since no SA was detected in the products of this reaction (Strawn et al. 2007). Additional analyses revealed that proper function of ICS1 requires Mg^{2+}. However, ICS1 displays maximal activity over a broad range of pH and temperature, which is suitable for the light-mediated changes in the stromal environment.

Similarly to *ICS1*, *ICS2* encodes a functional ICS enzyme that can be imported into the chloroplast stroma (Strawn et al. 2007; Garcion et al. 2008). The fact that null *ics1* mutant still accumulates some SA suggests a likely role for ICS2 in SA biosynthesis. Comparison of SA accumulation in *ics1* and the double mutant *ics1 ics2* demonstrated that ICS2 indeed participates in the biosynthesis of SA. Upon UV exposure, *ics1* and *ics1 ics2* accumulate roughly 10 and 4 % of total SA compared to wild type, respectively. Therefore, the majority of SA (about 95 %) is synthesized from the ICS pathway in UV-treated *Arabidopsis* plants with the remaining through an alternative pathway (Garcion et al. 2008).

ICS homologs have also been identified in a wide variety of plant species (van Tegelen et al. 1999; Ogawa et al. 2005; Uppalapati et al. 2007; Yuan et al. 2007; Catinot et al. 2008). Given their role in phylloquinone synthesis, it is very likely that *ICS* homologs are present in all plant species. However, identification of an *ICS* gene in a given plant species is not sufficient to confirm its role in SA biosynthesis. Nevertheless, isotope-feeding experiment, with the intension to reflect *in planta* metabolism, revealed that most SA is synthesized via the ICS pathway in *Pythium aphanidermatum*-elicitated *C. roseus* cells. In addition, virus-induced gene silencing of *ICS* expression in *N. benthamiana* or tomato suppresses UV- and/or pathogen-induced SA accumulation (Uppalapati et al. 2007; Catinot et al. 2008).

Although it is becoming clear that SA is synthesized via the ICS pathway in various plant species, how isochorismate, the product of ICS, is converted to SA is still unclear. This conversion should be accomplished by an enzymatic reaction since nonenzymatic synthesis of SA from IC is negligible when the reactants are incubated under conditions consistent with chloroplast stroma (Strawn et al. 2007). In addition, it is expected that the enzyme(s) involved in SA synthesis from IC is plastid localized, as transgenic *Arabidopsis* expressing *nahG* fused to a chloroplast localization sequence fails to accumulate SA upon pathogen infection or UV treatment (Fragnière et al. 2011). However, no plant genes encoding IPL activity have been reported (Chen et al. 2009). Thus, whether plants contain IPLs that are structurally unrelated to or highly divergent from the bacterial counterparts or use a metabolic pathway distinct from that in bacteria and, consequently, catalyzed by enzymes unrelated to IPL merits further investigation.

Signal Perception and Execution of Salicylic Acid-Induced Responses

Over the past more than two decades, many genetic screens have been conducted to identify genes that are involved in SA biosynthesis/metabolism, perception, and signal transduction in *Arabidopsis*. These screens have yielded numerous mutants with genetic lesions either upstream or downstream of SA biosynthesis. Furthermore, recent studies have revealed the involvement of epigenetic factors in SA-mediated plant defense signaling. All these have sketched an integrated model for regulation of SA accumulation and a finely tuned SA-mediated defense signaling network. Here, we focus on SA perception and downstream signal execution. For regulation of SA accumulation, readers are referred to the recent review in The *Arabidopsis* Book (Dempsey et al. 2011).

SA Receptors

Although SA plays a pivotal role in galvanizing immune responses, until very recently it was unclear how plant cells perceived SA. There have been serious efforts to identify SA receptors using biochemical purification of SA-binding proteins (SABPs). To date, four types of SABPs have been identified including a catalase, a methyl salicylate esterase, a cytoplasmic ascorbate peroxidase, and a chloroplastic carbonic anhydrase (Du and Klessig 1997; Slaymaker et al. 2002; Kumar and Klessig 2003; Park et al. 2007; Vlot et al. 2008, 2009). Although these SABPs are involved in mediating some aspects of SA metabolism or action, genetic analyses suggested that none of them fulfill the criteria for a bonafide SA receptor, because these molecules do not have functional roles in plant immune signaling. Using different ligand-receptor binding methods, two research groups recently reported that NPR1 (nonexpressor of pathogenesis-related genes1) and NPR1-related proteins, NPR3 and NPR4, are the long-sought-after SA receptors in *Arabidopsis* (Fu et al. 2012; Wu et al. 2012). NPR1, NPR3, and NPR4 are all characterized by a conserved N-terminal BTB/POZ (broad complex, tramtrack, and bric-à-brac/poxvirus, zinc finger) domain and an ankyrin repeat in the middle of the proteins (Cao et al. 1997; Kinkema et al. 2000; Liu et al. 2005).

Using a special equilibrium dialysis ligand binding method, Wu et al. (2012) demonstrated that NPR1 binds to SA when NPR1 and SA are in equilibrium. SA binds strongly to a C-terminal transactivation (TA) domain of NPR1 through Cys^{521} and Cys^{529} via the transition metal copper (Rochon et al. 2006; Wu et al. 2012). Mutations of cysteines to serines or metal chelation abolish the binding of SA by NPR1. In the absence of SA, the NPR1 TA domain is inhibited by the BTB domain and thus fails to activate the expression of SA response genes. However, increased SA concentration upon pathogen infection facilitates binding of SA to Cys^{521}

and Cys^{529} through coordinated copper. Thus, the direct binding of NPR1 to SA and the functional importance of this interaction in plant immunity indicate NPR1 may be an SA receptor in *Arabidopsis*.

The presence of a BTB domain in NPR1 suggests that, like other BTB domain-containing proteins, NPR1 may interact with Cullin 3 (CUL3) E3 ligase and mediate substrate degradation. Even though the substrate for NPR1 has yet to be identified, NPR1 protein itself can be degraded by the proteasome both before and after SAR induction (Spoel et al. 2009). NPR1 paralogs NPR3 and NPR4 are adaptor proteins for the CUL3 E3 ligase that specifically targets NPR1 for degradation in an SA concentration-dependent manner (Fu et al. 2012). NPR1 and NPR4 interact with one another in the absence of SA; SA disrupts this interaction and promotes interaction between NPR1 and NPR3 instead. Using conventional ligand-receptor binding assays, Fu and colleagues (2012) found that the NPR1 protein does not have considerable SA-binding activity under different conditions but two NPR1-related proteins, NPR3 and NPR4, bind to SA with different affinity. Since NPR4 has high affinity for SA (nanomolar range) while NPR3 has low affinity for SA (micromolar range), low SA levels should reduce NPR1 degradation, whereas high SA levels should enhance it. According to the proposed model, in the absence of pathogen infection, NPR4 constantly removes most of the NPR1 protein through CUL3-NPR4-mediated degradation, and basal SA disrupts some of the NPR1–NPR4 interactions, allowing some NPR1 to escape degradation, which is required for keeping basal immunity (PTI). Following pathogen infection, recognition of pathogen effectors by plant resistance proteins induces a high level of SA in local infected tissue, which promotes interaction between NPR1 and NPR3, triggering CUL3-NPR3-mediated NPR1 degradation. As NPR1 is likely a negative regulator of programmed cell death (PCD) during ETI, degradation of NPR1 allows PCD to occur at the site of infection. In systemic tissues, on the other hand, an intermediate level of SA is insufficient to bring about NPR1–NPR3 interaction but high enough to disrupt NPR1–NPR4 interaction and, consequently, enables NPR1 to accumulation, leading to SAR activation. Thus, as SA receptors, NPR3 and NPR4 appear to regulate the homeostasis of NPR1, thus modulating the function of NPR1 in basal immunity, ETI, and SAR.

The seemingly conflicting results on the identification of SA receptors can be attributed to the different experimental approaches used to test the direct binding of SA to NPR1. Crystal structure analysis of NPR1, NPR3, and NPR4 will be the next crucial step to further unravel the binding sites and the exact SA-sensing mechanisms of these receptors. NPR3 and NPR4 may not be the merely SA-binding proteins that facilitate SA-mediated degradation of NPR1 and additional proteins are yet to be discovered (Kaltdorf and Naseem 2013). Alternatively, SA could be perceived by both NPR1 and NPR3/NPR4, resembling the multireceptor sensing of other phytohormones like abscisic acid (Spartz and Gray 2008). Given the fact of the existence of SA-dependent but NPR1-independent defense signaling pathway, in which NPR3/NPR4 may not participate, additional SA perception mechanisms may be present. Furthermore, it has now been well established that SA is also a prominent regulator of plant growth, development, and response to abiotic stresses

(Vicente and Plasencia 2011), suggesting the possible existence of additional SA receptors in plants. Regardless, identification of NPR1, NPR3, and NPR4 as SA receptors represents a great step forward in elucidation of SA immune signaling and is expected to have a long-lasting impact on future research in plant immunity.

NPR1-Dependent SA Signaling

As a central transcription coactivator, NPR1 is responsible for controlling approximately 95 % of SA-dependent genes, thus represents a key node in signaling downstream from SA (Dong 2004; Durrant and Dong 2004; Pieterse and van Loon 2004). The *NPR1* gene promoter contains W-box sequences, which are binding sites of WRKY transcription factors. Mutations in the W-box region of the *NPR1* gene affect its expression, suggesting that WRKY transcription factor(s) is crucial in mediating SA-induced *NPR1* expression (Yu et al. 2001). SA treatment or pathogen inoculation enhances *NPR1* expression. SA also promotes the translocation of NPR1 from cytoplasm to the nucleus. SA-induced changes in cellular redox state lead to reduction of disulfide bonds formed among conserved cysteine residues such as Cys^{82} and Cys^{216} likely though the function of TRX-H5 (thioredoxin-H5) and/or TRX-H3 (Mou et al. 2003; Tada et al. 2008). SA binding to the NPR1 protein appears to also play a role in this oligomer-to-monomer transition (Wu et al. 2012). Nevertheless, mutation of either Cys82 or Cys216 elevates the level of monomeric, nuclear localized NPR1, and consequently upregulates *PR1* gene expression (Mou et al. 2003). Since the NPR1 protein does not have DNA-binding capability, relaying NPR1-mediated signaling requires other transcription factors. Indeed, genome-wide expression profiling analysis indicated that several members of the WRKY transcription factor family act downstream of NPR1 (Wang et al. 2006), and protein–protein interaction assays revealed that NPR1 interacts with at least seven TGA (TGACG motif-binding factor) transcription factors (Zhang et al. 1999; Després et al. 2000; Zhou et al. 2000; Subramaniam et al. 2001; Song et al. 2011) and three structurally related NIMIN (noninducible immunity1 (NIM1)-interacting) proteins (Weigel et al. 2001, 2005).

The TGA transcription factors can directly interact with *PR1* gene promoter through binding to the activator sequence-1 (as-1) element in the promoter (Lebel et al. 1998). *In planta* analyses showed that the interaction between NPR1 and TGA1 and/or TGA4 needs the presence of SA (Després et al. 2000) and that the ability of TGA2 and TGA3 to activate transcription of downstream genes requires both SA and NPR1 (Johnson et al. 2003). In another study, however, interaction between NPR1 and TGA2 was detected in the absence of SA, but the interaction is weaker than in the presence of SA (Fan and Dong 2002). More recent studies suggested that the repressor activity of TGA2 is transformed into an activator activity by its incorporation into a transactivation complex with NPR1 (Rochon et al. 2006; Boyle et al. 2009). All these results indicate that SA and NPR1 likely enhance the DNA-binding activity of certain TGA factors and thus affect the transcription of *PR*

genes (Durrant and Dong 2004). Indeed, mutant characterization confirmed that TGA2, TGA5, and TGA6 function redundantly in SA signaling and SAR and that TGA3 and TGA7 are required for SA-mediated basal immunity (Zhang et al. 2003; Kesarwani et al. 2007; Song et al. 2011).

The NIMIN proteins appear to regulate SA/NPR1 signaling in a negative manner. While *NIMIN3* is expressed constitutively at a low level, both *NIMIN1* and *NIMIN2* are responsive to SA treatment (Weigel et al. 2001; Hermann et al. 2013). Overexpression of *NIMIN1* compromises ETI and SAR, whereas reducing its expression enhances SA-induced *PR1* gene expression (Weigel et al. 2005). NIMIN3 appears to also suppress SA-induced *PR1* gene expression, though to a lesser extent than NIMIN1 (Hermann et al. 2013). It was proposed that the NIMIN proteins act in a strictly consecutive and SA-regulated manner on NPR1 to repress the *PR1* gene at the onset of SAR (Hermann et al. 2013).

In a genetic screen for suppressors of *npr1*, a mutant named *sni1* (*suppressor of npr1-1*, *inducible1*) was identified (Li et al. 1999). The *sni1* mutation restores SA inducibility of *PR* genes and resistance to *npr1-1* and renders plants with a wild-type copy of the *NPR1* gene more sensitive to SAR signals. SNI1 is a nuclear protein with limited similarity to the mouse retinoblastoma protein, a negative transcription regulator, suggesting that SNI1 is likely a negative regulator of SAR (Mosher et al. 2006). Further genetic screens for suppressors of the *sni1* mutation identified a group of proteins including RAD51D (RAS associated with diabetes51d), BRCA2A (breast cancer2a), and SSN2 (suppressor of SNI1,2) that are required for SA-mediated defense gene transcription (Durrant et al. 2007; Wang et al. 2010; Song et al. 2011). Since RAD51D, BRCA2A, and SSN2 are all involved in homologous recombination or DNA repair, these results demonstrated that proteins from homologous recombination or DNA repair pathways play important roles in SA- and NPR1-mediated defense signaling (Moore et al. 2011).

Recent progresses have defined the function of a number of plant Mediator (MED) subunits in SA-mediated plant immune responses. As a conserved multiprotein cofactor of RNA polymerase II (RNAPII), the Mediator complex is recognized as an important player to fine-tune gene-specific and pathway-specific transcriptional reprogramming by acting as an adaptor/coregulator between sequence-specific transcription factor and RNAPII. Mutations in genes encoding the Mediator subunits MED14, MED15, and MED16 all affect SA-induced *PR* gene expression, compromise basal resistance against biotrophic bacterial pathogens, and block biological induction of SAR (Canet et al. 2012; Wathugala et al. 2012; Zhang et al. 2012b, 2013a). However, only *med15* causes SA hyperaccumulation and reduced SA tolerance like *npr1* (Canet et al. 2012). MED16 and NPR1 function largely independently of each other in basal immunity, whereas MED14 and NPR1 have significant overlapping functions in regulating basal immunity. Unlike the *med16* mutation, which differentially affects expression of several SAR positive and negative regulators, *med14* inhibits induction of a large group of defense genes including both SAR positive and negative regulators (Zhang et al. 2012b, 2013a). Both MED14 and MED15 appear to function downstream of NPR1 and do not affect NPR1 nuclear localization and/or stability (Canet et al. 2012; Zhang et al. 2013a),

whereas MED16 positively contributes to NPR1 protein accumulation (Zhang et al. 2012b). Interestingly, although the *med8* mutant displays enhanced susceptibility to bacterial pathogens, it has no significant defects in biological induction of SAR (Kidd et al. 2009; Zhang et al. 2012b). Furthermore, mutations in *MED25* attenuate the induction of SA-responsive genes but have no significant effects on resistance to biotrophic bacterial pathogens and biological induction of SAR (Kidd et al. 2009; Zhang et al. 2012b). Thus, these Mediator subunits employ distinct mechanisms to regulate SA-mediated defense gene expression and pathogen resistance.

NPR1-Independent SA Signaling

In *Arabidopsis*, ETI is suppressed by expression of the *nahG* gene, but not by the *npr1* mutation, suggesting the presence of NPR1-independent SA signaling in plant immunity (Raridan and Delaney 2002; Kachroo et al. 2001; Takahashi et al. 2002). The existence of NPR1-independent SA signaling is further supported by the results from characterization of a group of *Arabidopsis* mutants that either display SA inducibility of *PR* genes or constitutively accumulate SA and *PR* gene transcripts in the absence of a functional *NPR1* gene. The *sni1* mutation confers SA inducibility of *PR* genes to the *npr1-1* mutant, suggesting an NPR1-independent mechanism (Li et al. 1999). More components in the NPR1-independent SA signaling pathway were identified through screening for suppressors of the *npr1-5* mutant. The *ssi* (*suppressor of SA insensitivity*) *npr1* double mutants *ssi1 npr1*, *ssi2 npr1*, and *ssi4 npr1* constitutively accumulate SA and exhibit heightened resistance to a variety of pathogens (Shah et al. 1999, 2001; Shirano et al. 2002). The *ssi1* and *ssi2* single mutants accumulate higher levels of *PR1* gene transcripts than the *ssi1 npr1* and *ssi2 npr1* double mutants, respectively, indicating an NPR1-independent pathway functioning additively with the NPR1-dependent pathway (Shah et al. 1999, 2001). Another *npr1* suppressor, *snc1* (*suppressor of npr1-1 constitutive1*), displays constitutive SA-dependent, NPR1-independent resistance owning to a mutation in a Toll-interleukin-1 receptor-nucleotide binding site-leucine-rich repeat type *R* gene. The gain-of-function *snc1* mutation leads to constitutive activation of the R protein and downstream immune responses without the presence of pathogens. The *snc1* mutant also accumulates high levels of SA, constitutively expresses *PR* genes, and displays enhanced resistance to pathogens (Li et al. 2001). Further genetic screens for suppressors of *snc1* identified a series of *mos* (*modifier of snc1*) mutations affecting signal transduction downstream of *snc1* (Zhang and Li 2005). New members of the *snc* mutants such as *snc2-1D* (*suppressor of npr1-1*, *constitutive 2-1D*) and *snc4-1D* have been identified and characterized (Bi et al. 2010; Zhang et al. 2010b). Moreover, a set of genes that may be involved in SA-regulated, NPR1-independent signaling pathway encode WHIRLY (WHY) and MYB transcription factors. The single-stranded DNA-binding activity of WHY1 is stimulated by SA treatment in both wild-type and *npr1* mutant plants (Desveaux et al. 2002, 2004), indicating its important role in NPR1-independent *PR1* expression and resistance against

pathogens. The *Arabidopsis MYB30* (*myeloblastosis30*) gene positively regulates the HR in an SA-dependent, NPR1-independent manner (Raffaele et al. 2006). Additionally, the *cpr5* (*constitutive expressor of PR genes5*), *cpr6*, and *hrl1* (*hypersensitive response-like lesions1*) mutants exhibit NPR1-independent and SA-dependent immune phenotypes (Clarke et al. 2000; Devadas et al. 2002). Interestingly, the *cpr5*, *cpr6*, and *hrl1* mutations also activate jasmonic acid (JA)- and ethylene (ET)-mediated immune responses, indicating that the SA-dependent, NPR1-independent signaling may function synergistically with the JA/ET-mediated defense pathways (Clarke et al. 2000; Devadas et al. 2002).

In a genetic screen for suppressors of the *npr1* mutant based on its intolerance to SA, an *elp2* (*Elongator subunit2*) mutant allele was isolated (DeFraia et al. 2010). ELP2 is one of the six subunits of the Elongator complex, which interacts with elongating RNAPII to facilitate transcription (Winkler et al. 2002; Close et al. 2006). Despite the structural diversity of the Elongator subunits, loss of any Elongator subunit generally compromises its integrity and renders the complex inactive (Versées et al. 2010; Glatt et al. 2012). The Elongator catalytic subunit ELP3/ELO3 (ELONGATA3) harbors a C-terminal histone acetyltransferase (HAT) domain and an N-terminal cysteine-rich motif that resembles an iron-sulfur radical *S*-adenosylmethionine (SAM) domain (Chinenov 2002; Winkler et al. 2002; Nelissen et al. 2005). Both the HAT and SAM domains are required for Elongator's function in plant immunity (DeFraia et al. 2013). Mutations in *ELP2* and *ELP3* restore SA tolerance to *npr1*, suppress *npr1*-mediated hyperaccumulation of SA, and delay the induction of SA accumulation and defense gene expression (DeFraia et al. 2010, 2013). Although Elongator regulates the NPR1 transcriptional cascade, Elongator and NPR1 appear to function largely independently of each other in ETI, and mutations in *ELP2* and *ELP3* do not affect SAR (DeFraia et al. 2010, 2013). Further mutant characterization revealed that ELP2 is an epigenetic regulator required for *P. syringae*-induced rapid transcriptome reprogramming likely through maintaining histone acetylation levels in defense genes, modulating genomic DNA methylation landscape, and influencing pathogen-induced dynamic DNA methylation changes (Wang et al. 2013). Such chromatin modification has recently been described as an additional layer of regulation on plant immunity. Several reports have shown that the state of histone acetylation or DNA methylation is associated with SA-mediated defense responses (Mosher et al. 2006; Butterbrodt et al. 2006; Koornneef et al. 2008; van den Burg and Takken 2009; Choi et al. 2012; Luna et al. 2012). Compared with other epigenetic regulators, Elongator is unique in that it regulates both histone acetylation and DNA methylation status of defense-related genes (Winkler et al. 2002; Nugent et al. 2010; Xu et al. 2012). The NPR1 transcriptional cascade exemplifies a signal cascade where Elongator modulates the chromatin structure of both the key transcription regulator and its target genes, forming a transcriptional feed-forward loop and determining the kinetics of the transcription. However, the mechanism of the cooperative interaction between the specific transcription regulator NPR1 and the chromatin modulator Elongator in regulating gene transcription during immune responses is still unclear.

Biotechnological Manipulation of Salicylic Acid Signaling and Biosynthesis in Agriculture

Disease is a major threat to the yield and quality of crop plants worldwide. One major goal in plant science is the production of crops with increased and durable resistance to a spectrum of pathogens. Compared with other approaches employed to develop disease-resistant crops, genetic engineering is faster and allows transference of individual traits into crops in a calculated manner. Strategies for developing transgenic disease resistance have been evolved from overexpression of a single or combination of a small number of genes, which suffer from either incomplete efficacy or durability, to modification of existing innate signaling pathways, which can activate a battery of defense responses (Collinge et al. 2010). The accumulating knowledge of SA-mediated defense signaling pathways provides new opportunities for manipulating plant disease resistance. Several genes have received attention with respect to possible exploitation for developing transgenic disease-resistant crops. Among them *NPR1* is the most promising gene for generating broad-spectrum disease-resistant crop plants.

The *NPR1* gene was originally discovered in several independent genetic screens performed in *Arabidopsis*. The *npr1* (also known as *nim1* and *sai1* (*salicylic acid-insensitive1*)) mutants are unable to either mount a SAR response or accumulate *PR* transcripts and are hypersusceptible to biotrophic pathogens (Cao et al. 1994; Delaney et al. 1994; Shah et al. 1997). The original study in *Arabidopsis* using *NPR1* showed that overexpression of this gene increases resistance to two diverse biotrophic pathogens, the bacterium *P. syringae* pv. *maculicola* and the oomycete *Hyaloperonospora arabidopsidis* (Cao et al. 1998; Table 1). Since then transgenic studies using *NPR1* or its orthologs from other species have been extended to a large group of crop plants for resistance against pathogens with either biotrophic or necrotrophic lifestyle (Tables 1 and 2). In addition, overexpression of *NPR1* seems to enhance resistance to insect and root-knot nematode in tobacco plants (Meur et al. 2008; Priya et al. 2011). Interestingly, the majority of the transgenic plants display little or no constitutive expression of *PR* genes; rather, the transgenic plants exhibit a "primed" phenotype where induction of *PR* genes is faster, at higher intensity, and for a longer duration, resulting in a heightened capacity to undergo SAR when challenged with pathogens or treated with SA analogs. However, transgenic rice expressing either *NPR1* or the rice ortholog *OsNH1* (*Oryza sativa NPR1 HOMOLOGUES1*) is different, which exhibits constitutive expression of *PR* genes (Fitzgerald et al. 2004; Quilis et al. 2008).

Another avenue for boosting SA-mediated plant immunity is to manipulate SA biosynthesis. Tobacco plants overexpressing heterologous *PAL* transgenes display enhanced resistance to the fungal pathogen *Cercospora nicotianae* and the oomycete *Phytophthora parasittica* pv. *nicotianae* (Felton et al. 1999; Way et al. 2002). However, based on comparison of *PAL*-overexpressing plants and *PAL-overexpressing* plants harboring a *nahG* gene, which compromises SA accumulation, it has been suggested that the accumulation of phenylpropanoid intermediates

Table 1 Use of the *Arabidopsis NPR1* gene in transgenic disease resistance

Recipient plant	Pathogen resistance	Other resistance	Note	Reference
Arabidopsis thaliana	*Pseudomonas syringae* pv. *maculicola* and pv. *tomato*, *Erysiphe cichoracearum*, *Hyaloperonospora arabidopsidis*, and *Fusarium graminins*	N/A	Pathogens resistance is proportional to the NPR1 protein level; without notable yield penalty; observed fitness disadvantage in some conditions	Friedrich et al. (2001), Cao et al. (1998), Makandar et al. (2006), Heidel and Dong (2006)
Grapefruit/sweet orange	*Xanthomonas citri* subsp. *citri*	N/A	Grapefruit has fewer lesions and lower bacterial populations; no significant difference for sweet oranges	Zhang et al. (2010a)
Cotton	*Verticillium dahliae* isolate TS2, *F. oxysporum* f.sp. *Vasinfectum*, *Rhizoctonia solani*, and *Alternaria alternata*	Reniform nematode	Overexpression plants phenotypically normal; not resistance to all *V. dahliae* isolates	Parkhi et al. (2010a, b), Kumar et al. (2013)
Carrot	*Botrytis cinerea*, *Alternaria radicina*, *Sclerotinia sclerotiorum*, *E. heraclei*, *X. hortorum*, and *Thielaviopsis basicola*	N/A	Overexpression plants phenotypically normal	Wally et al. (2009)
Tomato	*F. oxysporum*, *Stemphylium solani*, *Ralstonia solanacearum*, and *X. campestris*	N/A	No adverse effects on growth or yield; enhanced susceptibility to *B. cinerea*	Lin et al. (2004), El Oirdi et al. (2011)
Rice	*X. oryzae*, *Erwinia chrysanthemi*, *Magnaporthe oryzae*, and *F. verticillioides*	N/A	Deleterious effect on rice growth; BTH- and environment-induced lesion-mimic/cell death phenotype; increased sensitivity to salt and virus	Fitzgerald et al. (2004), Quilis et al. (2008)
Tobacco	N/A	Nematode and insect	Up to 50 % improved resistance to both; proportional to *NPR1* expression levels; enhanced oxidative stress tolerance	Meur et al. (2008), Srinivasan et al. (2009), Priya et al. (2011)
Wheat	*F. graminearum*	N/A	Rapid defense response; 25 % infection level comparing to wild type; no yield penalty in lab	Makandar et al. (2006)
Canola	*P. syringae* pv. *tomato*	N/A	Effectively enhances basal resistance against *P. syringae* pv. *tomato*	Potlakayala et al. (2007)

Table 2 Use of *NPR1* orthologs in transgenic disease resistance

Source gene	Recipient plant	Pathogen resistance	Notes	Reference
Mustard *NPR1*	Mung bean	*Rhizoctonia solani*	No dry rot symptoms on transgenic shoots	Vijayan and Kirti (2012)
Soybean *NPR1*	*Arabidopsis*	*Pseudomonas syringae* pv. *tomato*	Soybean *NPR1-1* and *NPR1-2* complement the *Arabidopsis npr1-1* mutation; comparable levels of protection from both soybean orthologs as from *AtNPR1*	Sandhu et al. (2009)
Malus hupehensis NPR1	Tobacco	*Botrytis cinerea*	Increased resistance to fungus *B. cinerea*	Zhang et al. (2012a)
Malus hupehensis NPR1	Fuji apple	*Podosphaera leucotricha*	Induces *PR* gene expression and promotes SAR	Chen et al. (2012)
Malus pumila NPR1	Galaxy and M26 apple varieties	*Erwinia amylovora*, *Venturia inaequalis*, and *Gymnosporangium juniperi-virginianae*	Both varieties show significantly increased disease resistance	Malnoy et al. (2007)
Rice *NPR1*	*Arabidopsis*	*P. syringae* pv. *tomato*	Partially complements the *Arabidopsis npr1* mutation	Yuan et al. (2007)
Rice *NPR1*	Rice	*Magnaporthe oryzae* and *X. oryzae* pv. *oryzae*	Overexpressors are more resistant; RNAi lines are more susceptible; spontaneous lesions observed	Chern et al. (2005); Yuan et al. (2007); Feng et al. (2011)
Cacao *NPR1*	*Arabidopsis*	*P. syringae* pv. *tomato*	Partially complements the *Arabidopsis npr1* mutation	Shi et al. (2010)
Canola *NPR1*	*Arabidopsis*	*P. syringae* pv. *tomato*	Restores *PR1* gene expression; enhanced basal defense and SAR against *P. syringae* pv. *tomato*	Potlakayala et al. (2007)
Canola *NPR1*	Canola	*P. syringae* pv. *tomato*	Effectively enhances basal resistance against *P. syringae* pv. *tomato*	Potlakayala et al. (2007)
Grape *NPR1*	*Arabidopsis*	*P. syringae* pv. *maculicola*	Complements the *Arabidopsis npr1* mutation; increases tolerance to salinity but has not effect on the drought tolerance	Le Henanff et al. (2009, 2011), Bergeault et al. (2010), Zhang et al. (2013b)
Pepper *NPR1*	Tobacco	*Ralstonia solanacearum*	Resistance to *R. solanacearum* is coupled with enhanced transcript levels of defense-related maker genes	Dang et al. (2012)

such as chlorogenic acid is primarily responsible for the enhanced resistance to *C. nicotianae* in *PAL*-overexpressing plants, whereas SA accumulation has limited contributions (Shadle et al. 2003). Nevertheless, targeting the bacterial SA biosynthesis enzymes ICS and IPL to chloroplasts in transgenic tobacco plants increases SA and SA glucoside accumulation, leading to constitutive expression of defense genes and resistance to viral and fungal infection (Verberne et al. 2000). Importantly, overaccumulation of SA in transgenic tobacco plants does not affect plant growth, which is crucial for engineering disease-resistant crops. However, targeting a functional fusion enzyme of the bacterial ICS and IPL to chloroplasts in *Arabidopsis* strongly inhibits plant growth and significantly reduces seed production (Mauch et al. 2001).

As an increasing number of important SA signaling components are discovered, the list of candidate genes for genetic manipulation grows. Interestingly, many of the SA signaling components also plays important roles in nonhost resistance, which is the most common form of resistance exhibited by plants against a wide variety of microbial pathogens (An and Mou 2011). Therefore, manipulating these genes in crop species hold the potential to boost both host and nonhost resistance. However, limited investigations have been conducted on utilizing nonhost resistance to develop disease-resistant crops. Furthermore, manipulating SA-mediated immune responses through suppression of negative regulators or activation of positive regulators represents an attractive strategy for engineering disease resistance (Gurr and Rushton 2005b; Salomon and Sessa 2012). Thus far, the function of many defense regulators in manipulating disease resistance has been tested in *Arabidopsis*, but the efforts of translating these technologies to crops still lag behind.

It should be noted that because of the involvement of SA in diverse physiological processes other than plant immunity, increasing SA biosynthesis or signaling might lead to fitness penalties. Although little evidence for fitness penalties has been found for overexpression of *NPR1* in the laboratory, one study using controlled environments suggested that there seem to be fitness penalties for overexpression of *NPR1* under high nutrient conditions (Heidel and Dong 2006). To minimize the cost of defense activation on plant growth, pathogen- or chemical-inducible and tissue-specific promoters may be useful as they limit the cost of resistance by controlling temporal and spatial expression of the defense genes (Gurr and Rushton 2005a).

Although our understanding of the role of SA in plant defense against pathogens has increased considerably over the last two decades, much still remains to be elucidated. Among them, SA biosynthesis in plants is still not fully understood and the central signaling components, such as NPR1, still require more in-depth studies. Additionally, SA-mediated defense signaling pathways and other defense pathways are not isolated but rather interconnected to form a well-regulated network. Elucidating genetic components, especially those connecting multiple defense pathways, will continue to be a major task of the research community. On the other hand, understanding of SA-mediated plant defense has facilitated development of more effective ways for controlling important crop diseases. While gene efficacy in transgenic plants has often been good, field trials of transgenic disease-resistant crops have been hampered by ethical concerns. In this regard, the recently

developed cisgenic approach (Schouten et al. 2006), which utilizes target crop-derived genes and regulatory elements (promoters) together with improved transformation methods that do not rely on or subsequently eliminate selective marker genes, has the potential to develop resistant cultivars more acceptable to consumers.

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Jasmonates in Plant Growth and Stress Responses

Claus Wasternack

Abstract Jasmonates are lipid-derived compounds which are signals in plant stress responses and development. They are synthesized in chloroplasts and peroxisomes. An endogenous rise occurs upon environmental stimuli or in distinct stages of development such as that of anthers and trichomes or in root growth. Hydroxylation, carboxylation, glucosylation, sulfation, methylation, or conjugation of jasmonic acid (JA) leads to numerous metabolites. Many of them are at least partially biologically inactive. The most bioactive JA is the (+)-*7-iso*-JA–isoleucine conjugate. Its perception takes place by the SCF^{COI1}-JAZ-co-receptor complex. At elevated levels of JAs, negative regulators such as JAZ, or JAV are subjected to proteasomal degradation, thereby allowing positively acting transcription factors of the MYC or MYB family to switch on JA-induced gene expression. In case of JAM negative regulation takes place by anatagonism to MYC2. JA and COI1 are dominant signals in gene expression after wounding or in response to necrotrophic pathogens. Cross-talk to salicylic acid, ethylene, auxin, and other hormones occurs. Growth is inhibited by JA, thereby counteracting the growth stimulation by gibberellic acid. Senescence, trichome formation, arbuscular mycorrhiza, and formation of many secondary metabolites are induced by jasmonates. Effects in cold acclimation; in intercropping; during response to herbivores, nematodes, or necrotrophic pathogens; in pre- and post-harvest; in crop quality control; and in biosynthesis of secondary compounds led to biotechnological and agricultural applications.

Keywords Jasmonates • Oxylipins • Jasmonate biosynthesis • Jasmonate metabolites • Jasmonate perception • Jasmonate signaling • Cross-talk • Biotic stress • Abiotic stress • Root development • Flower development • Applied aspects

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Abbreviations

ABA	Abscisic acid
AM	Arbuscular mycorrhiza
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
BR	Brassinosteroids
COI1	CORONATINE INSENSITIVE1
ET	Ethylene
GA	Gibberellic acid
DAD1	DEFECTIVE IN ANTHER DEHISCECE1
13-HPOT	13-hydroperoxy octadecatrienoic acid
ISR	Induced systemic resistance
JA	Jasmonic acid
JA–Ile	JA–isoleucine conjugate
JAMe	JA methyl ester
JMT	JA methyltransferase
JAR1	JA resistant1
JAZ	JASMONATE ZIM DOMAIN
α-LeA	α-Linolenic acid (18:3)
LOX	Lipoxygenase
MYC	bHLHzip transcription factor
OPDA	12-Oxophytodienoic acid
OPR	OPDA reductase
PLA1	Phospholipase A1
RNS	Root nodule symbiosis
SA	Salicylic acid
ST	Sulfotransferase
TF	Transcription factor
SCF	Skp1/Cullin/F-box

Introduction

Jasmonic acid (JA) and its derivatives, commonly named jasmonates (JAs), are involved in developmental processes such as growth, lateral and adventitious root formation, seed germination, leaf senescence, glandular trichome formation as well as development of embryos and pollen (Fig. 1). Plants with their sessile lifestyle need constant adaptation to altering environmental cues, such as light, water deficit, salt, cold, and nutrient deficiency, in which JA-mediated responses play a crucial role. Furthermore, JAs are involved in biotic interactions such as responses to herbivores, pathogens, nematodes, or mutualistic symbiotic microorganisms, such as mycorrhizal fungi (Fig. 1). In these numerous interactions during plant stress

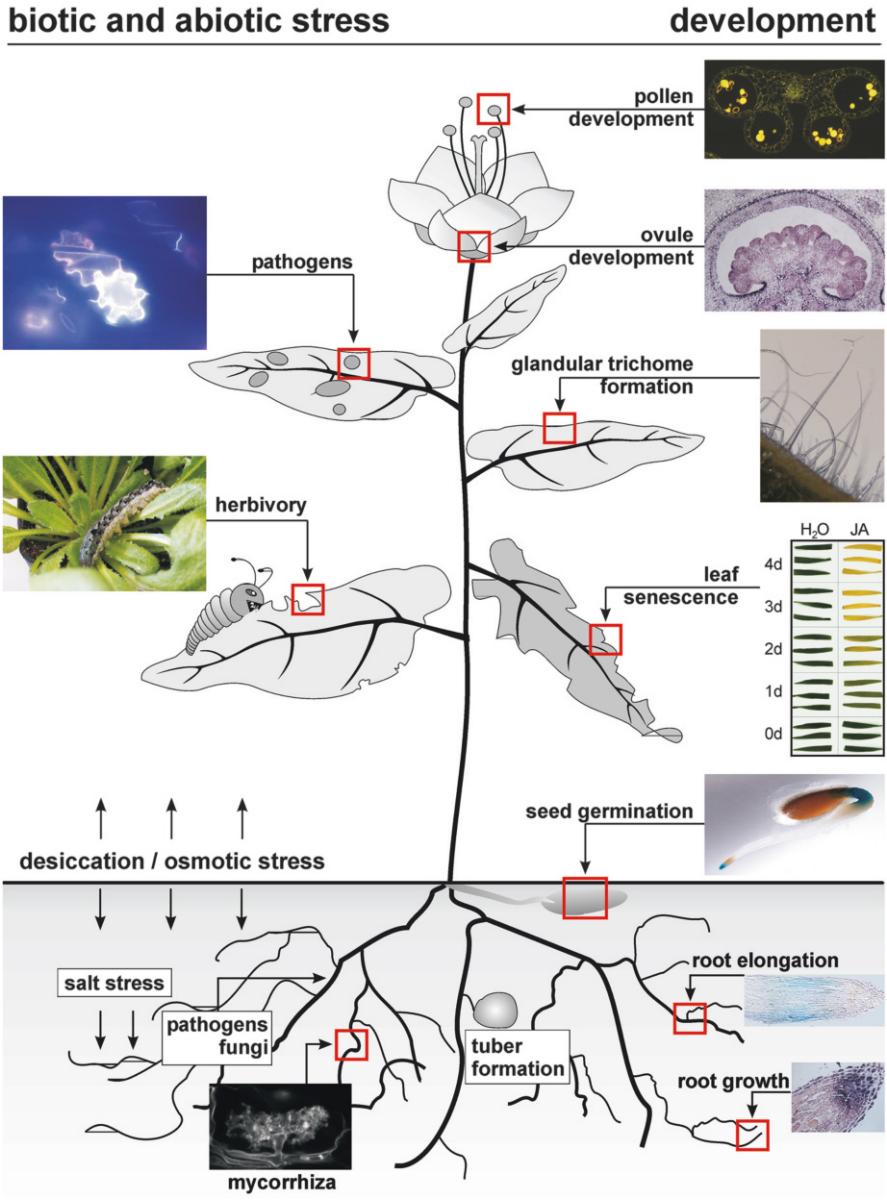

Fig. 1 Jasmonates in plant development (*right*) and plant responses to biotic and abiotic stress (*left*). Pictures for stress responses are given by a hypersensitive response upon pathogen attack, by herbivory on *Arabidopsis*, and by arbuscular mycorrhiza. The role of jasmonates in development is illustrated by a cross section of anthers of *Arabidopsis* showing pollen release, by immunocytochemical detection of allene oxide cyclase in cross section of tomato ovules, by trichomes, by senescing barley leaf segments upon treatment with jasmonate, by seedling growth and root elongation of a tomato seedling showing allene oxide cyclase promoter activity via GUS staining, and by root growth showing immunocytochemical detection of the allene oxide cyclase protein in the root tip. Jasmonates are also involved in growth inhibition, lateral root formation, adventitious root formation, attack by nematodes, light signaling, and freezing tolerance (with permission)

responses and development via JAs, various signal transduction pathways are involved. These pathways exhibit cross-talk to other plant hormones such as ethylene (ET), auxin, gibberellic acid (GA), salicylic acid (SA), brassinosteroids (BR), or abscisic acid (ABA).

The key components of JA biosynthesis, JA perception, and JA signaling have been identified. Several of these proteins were crystallized which allowed first mechanistic explanations. Since JA is perceived as its isoleucine conjugate (JA–Ile, cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones"), I will use here the term JA/JA–Ile. The present chapter will give an overview on JA/JA–Ile biosynthesis, JA/JA–Ile metabolism, JA/JA–Ile perception, JA/JA–Ile signal transduction and cross-talk to other plant hormones, and JA/JA–Ile functions in biotic and abiotic interactions as well as in plant growth and development and will discuss some biotechnological and horticultural applications of JA/JA–Ile. All these aspects have been continuously discussed in excellent reviews (Ballaré 2011; Browse 2009a, b; Kazan and Manners 2008, 2011, 2012; Kombrink 2012; Pauwels and Goossens 2011; Pieterse et al. 2012; Wasternack and Hause 2013; Wasternack and Kombrink 2010). Therefore, emphasis will be given on recently published data. The great amount of published data on JAs can be cited here only partially due to space limitation.

JA Biosynthesis

The JA and its derivatives are members of the class of oxylipins. Whereas JAs are generated by *13-lipoxygenases* (13-LOXs), other oxylipins are products of 9-lipoxygenases (9-LOXs, e.g., LOX1 and LOX5 of *Arabidopsis thaliana*) and α-dioxygenases (α-DOX) which form chemically unstable 2(*R*)-hydroperoxides. α-DOX is involved in defense against aphids (Avila et al. 2013), whereas AtLOX1 together with Atα-DOX1 is involved in the local and systemic response to *Pseudomonas syringae* pv. *tomato* (Vicente et al. 2012). AtLOX1 is also involved in an ABA-independent stomata closure and an immune defense response including SA and the MAP kinases MPK3 and MPK6 (Montillet et al. 2013).

The substrate of JA biosynthesis (Fig. 2) is derived from galactolipids of chloroplast membranes. α-Linolenic acid (18:3) (α-LeA) is released from the *sn-1* position of galactolipids by a phospholipase1 (PLA1). Initially, the PLA1 DEFECTIVE IN ANTHER DEHISCENCE1 (DAD1) was shown to be involved in JA formation (Ishiguro et al. 2001). A DAD1-activating factor (DAF) was identified upstream of DAD1 as putative RING-finger E3 ligase which positively regulates *DAD1* expression (Peng et al. 2013). DAD1 occurs preferentially in flowers and is controlled by the homeobox protein AGAMOUS. Involvement of DAD1 and DONGLE, another PLA1, in JA biosynthesis of leaves was excluded by wild-type-like phenotypes of *DAD1*- and *DONGLE*-RNAi lines in respect to leaf wounding and localization of the DONGLE protein in lipid bodies (Ellinger et al. 2010). Among the 16 lipase mutants of *Arabidopsis*, only that of PLA1γ1 (At1g066800) showed reduced JA

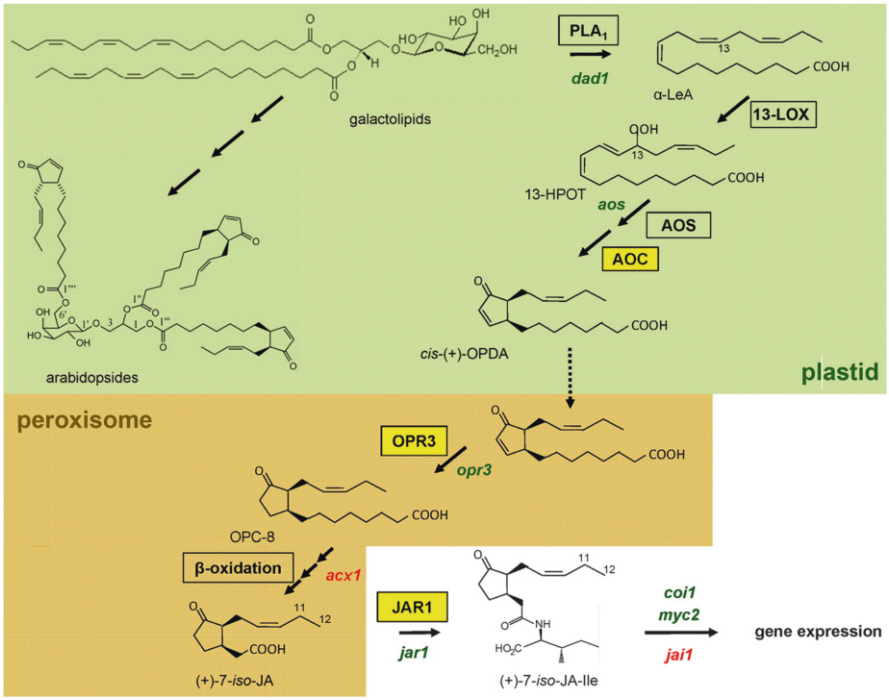

Fig. 2 Biosynthesis of jasmonic acid (JA) and its conjugate JA–isoleucine (JA–Ile) is initiated by the release of α-linolenic acid (α-LeA) from galactolipids of chloroplast membranes. A 13-lipoxygenase (13-LOX), an allene oxide synthase (AOS), and an allene oxide cyclase (AOC) catalyze formation of the cyclopentenone *cis*-(+)-12-oxophytodienoic acid (*cis*-(+)-OPDA). OPDA is released from the chloroplast and transported into peroxisomes, where reduction to the cyclopentanone ring by an OPDA reductase3 (OPR3) and shortening of the carboxylic acid side chain by the fatty acid ß-oxidation machinery take place. (+)-*7-iso*-JA is released into the cytosol, where conversion to JA–Ile and other metabolites takes place. Mutants of *Arabidopsis* are indicated in red, that of tomato in green. *acx1* acyl-CoA oxidase1, *coi1* coronatine insensitive1, *dad1* delayed anther dehiscence1, *13-HPOT* (13*S*)-hydroperoxy octadecatrienoic acid, *jai1* jasmonic acid insensitive1, *JAR1* JA amino acid synthetase1, *myc2* bHLHzip transcription factor MYC2, *OPC-8* 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid, *PLA$_1$* phospholipase A$_1$ (with permission)

levels upon wounding. The question, however, on activity of other PLA1s in other stress-induced JA formation is still open (Ellinger et al. 2010).

Free α-LeA is oxygenated in the C-13 position by 13-LOXs which occur among the six LOXs of *A. thaliana* as a family with four members (*LOX2, LOX3, LOX4, LOX6*) (Bannenberg et al. 2009). LOX2 is preferentially involved in early wound-induced JA formation (Glauser et al. 2009; Schommer et al. 2008) and JA formation during natural and dark-induced senescence (Seltmann et al. 2010). LOX2 is controlled by Ca^{2+} and a voltage-dependent vacuolar cation channel (Beyhl et al. 2009). This channel is under the control of members of the transcription factor (TF) family TEOSINTE BRANCHED/CYCLOIDEA/PROLIFERATING CELL FACTOR (TCP). Some of them such as TCP4 are targets of miR319 leading to control of JA

biosynthesis via LOX2 (Schommer et al. 2008). This and other examples indicate a developmental control of LOX2 (Danisman et al. 2012). Besides, LOX2 and also LOX3, LOX4, and LOX6 contribute to JA formation (Caldelari et al. 2011; Chauvin et al. 2013). The *LOX6* promoter is preferentially active in developing xylem cells of young tissues, whereas LOX3 and LOX4 are active in mature vascular tissues (Chauvin et al. 2013; Vellosillo et al. 2007), where other genes of JA biosynthesis such as allene oxide synthase (*AOS*) and allene oxide cyclase4 (*AOC4*) are expressed (Kubigsteltig et al. 1999; Stenzel et al. 2012). During fertility and anther development, JA formation including LOX3 and LOX4 activity is required, but LOX2 is not involved (Caldelari et al. 2011). LOX6 location attributes to the rapid increase in JA and JA–Ile after wounding in local and distal leaves (Chauvin et al. 2013). Only LOX6 is required for JA/JA–Ile formation in roots and is involved in responses to abiotic and biotic factors (Grebner et al. 2013). There are increasing examples that distinct isoforms catalyzing identical reactions in JA biosynthesis are involved in different JA/JA–Ile-mediated responses. Examples are the families of LOXs, AOCs, OPDA reductases (OPRs), and acyl-CoA oxidases (ACXs). In contrast to the four 13-LOXs of *A. thaliana*, LOX1 and LOX5 are 9-LOXs and are involved in defense reactions. Interestingly, in *Fusarium oxysporum* known to form many different jasmonates (Miersch et al. 1999), a nonheme iron 13*S*-LOX with multifunctional activity towards dihydroxy, keto, and epoxy alcohol derivatives has been identified (Brodhun et al. 2013). *F. oxysporum* infection activates expression of defense genes such as *THIONINS* (Vignutelli et al. 1998). The 13*S*-LOX detected in *F. oxysporum* suggests that fungal oxylipins including JA might modulate plant defense reactions upon *F. oxysporum* infection.

In JA biosynthesis the 13-LOX product 13-hydroperoxy octadecatrienoic acid (13-HPOT) is converted by the chloroplast-located *AOS,* the first specific step in the JA-specific branch of the LOX pathway. Other branches lead to leaf aldehydes and leaf alcohols as well as divinyl ether-, epoxyhydroxy-, keto-, and hydroxy-polyunsaturated fatty acids (Feussner and Wasternack 2002). AOS is a CYP450 enzyme (CYP74A) which does not require molecular oxygen nor NAD(P)H-dependent cytochrome P450 reductase as cofactor. Gene families of AOS, its substrate specificity and tissue-specific expression as well as the enzyme mechanism have been reviewed (Kombrink 2012; Schaller and Stintzi 2009; Wasternack and Kombrink 2010). Recently, a divinyl ether synthase could be converted into an AOS by a single point mutation indicating the close relationship of CYP74 enzymes (Toporkova et al. 2013). The AOSs of fungi seem to be evolved independently of CYP74, as suggested by the identification of a dioxygenase-cytochrome P450 fusion protein, a novel AOS with catalytic similarities to CYP74 and CYP8A1. This novel AOS has an analogous reaction mechanism to CYP74A enzymes (Hoffmann et al. 2013). A new type of CYP74 enzymes, CYP74C3 could be recently characterized with 9*S*-hydroperoxylinoleic acid as substrate (Brash et al. 2013). This enzyme forms besides the regularly generated *E*-isomer also a *Z*-isomer. Like the LOXs carrying positional specificity for carbon-9 or carbon-13, AOSs show at least preference for C-9 or C-13. An exception is the AOS1 of rice which shows dual specificity (Yoeun et al. 2013). The AOS of *A. thaliana* has been crystallized

(Lee et al. 2008). The highly unstable epoxide formed by AOS is converted by a chloroplast-located *AOC*. In the AOC-catalyzed step, *cis*-(+)-12-oxophytodienoic acid (OPDA) (9*S*,13*S*)-OPDA) is formed which contains the enantiomeric structure of the naturally occurring (+)-*7-iso*-JA. Even not proved experimentally so far, the exclusive occurrence of (9*S*,13*S*)-OPDA suggests that AOS and AOC act in a close vicinity avoiding the formation of a racemic mixture of *cis*-(+)-OPDA and *cis*-(−)-OPDA or spontaneous chemical decomposition leading to α-ketol and γ-ketol. The AOC2 of *A. thaliana* and both AOCs from *Physcomitrella patens* have been crystallized which allowed mechanistic explanation on the binding pocket (Hofmann et al. 2006; Neumann et al. 2012). The AOC of *A. thaliana* is encoded by a family of four members with different but overlapping expression pattern in organs and tissues (Stenzel et al. 2012). As suggested by the redundant expression in leaves and flower organs, interactions of all four AOCs occur by homo- and heteromerization which represents an additional regulatory level (Stenzel et al. 2012). The close association of LOX, AOS, and AOC within chloroplast membranes (Farmaki et al. 2007) may attribute to the formation of OPDA esterified within chloroplast membranes. This diverse group of abundantly accumulating compounds, called arabidopsides due to their exclusive occurrence in *Arabidopsis*, may be a storage form of OPDA (for review cf. Göbel and Feussner 2009; Ibrahim et al. 2011). In rice two photomorphogenic mutants (*hebiba, coleoptile photomorphogenesis 2* (*cpm2*) have been recently found to be defective in AOC genes. These genes encode functional AOCs which are active in defense against *Magnaporthe oryzae* (Riemann et al. 2013).

The second part of JA biosynthesis takes place in peroxisomes. *cis*-(+)-OPDA is assumed to be transported by the peroxisomal ATP-binding cassette (ABC) transporter protein COMATOSE (CTS1) and/or an ion trapping mechanism (cf. reviews of Hu et al. 2012; Wasternack and Kombrink 2010). In peroxisomes OPDA and/or its subsequently generated metabolites are activated by 4CL-like acyl-CoA synthetases (Hu et al. 2012; Kienow et al. 2008; Koo et al. 2006).The cyclopentenone ring of activated OPDA is reduced by an *OPR*. Among the six OPRs of *A. thaliana*, only OPR3 is involved in JA biosynthesis as shown by substrate specificity tests and crystallization of OPR1 and OPR3 (Breithaupt et al. 2001, 2006; Schaller and Stintzi 2009). In contrast, OPR1 seems to be involved in the synthesis of phytoprostanes, a group OPDA-like structures which are preferentially formed by nonenzymatic reactions (Mueller et al. 2008). Moreover, most of the OPRs except OPR3 are involved in detoxification by reduction of α,β-unsaturated aldehydes, ketones, maleimides, or acrolein. The OPRs of *A. thaliana*, rice, maize, and soybean occur in gene families of up to ten members. Their involvement in stress responses and development and even sex determination has been shown (Li et al. 2011).

The following reactions in JA biosynthesis include 4CL-like acyl-CoA synthetases, shortening of the carboxylic acid side chain by the fatty acid ß-oxidation machinery with acyl-CoA oxidase (ACX), the multifunctional protein (MFP), and 3-ketoacyl-CoA thiolase (KAT) (Kombrink 2012; Wasternack and Kombrink 2010). JA generated in peroxisomes is released into the cytosol, where it is metabolized.

The membrane-derived compounds JA and JA–Ile are involved in many responses to biotic and abiotic stress via distinct or overlapping signaling cascades

(cf. sections "Perception of JA-Ile and Cross-Talk to Other Hormones," "JA/JA-Ile in Biotic Interactions of Plants," "JA/JA-Ile in Abiotic Stress Response of Plants," and "JA/JA-Ile in Plant Growth and Development"). Another group of membrane-derived compounds are reactive electrophile species (RES), generated by lipid peroxidation. Whereas JA/JA–Ile- and CORONATINE INSENSITIVE1 (COI1)-mediated processes are involved in wounding, responses to necrotrophic pathogens, and developmentally regulated processes, RES are linked to the SA pathway that involves class II DNA-binding proteins (TGAs) (cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones"). There are numerous RES-mediated detoxification processes suggesting a "REScue" by cellular damage including photo-inhibition (reviewed in Farmer and Mueller 2013).

JA Metabolism

The most important reaction in metabolism of JA is its conjugation to amino acids catalyzed by JASMONATE RESISTANT1 (JAR1) (Fig. 3). *JAR1* is member of the *GRETCHEN HAGEN3* (*GH3*) gene family mainly involved in auxin conjugation (Staswick and Tiryaki 2004). The important role of JAR1 became obvious upon identification of (+)-*7-iso*-JA–Ile as the most bioactive compound among more than 40 JA compounds (Fonseca et al. 2009). JAR1 is a jasmonoyl amino acid conjugate synthase forming an acyl-adenylate/thioester intermediate by use of (+)-*7-iso*-JA as the substrate. JAR1/AtGH3.11 has been crystallized (Westfall et al. 2012). Most structure–activity relationships, recorded for numerous JA-dependent responses during the last two decades (for review cf. Wasternack 2007), can be explained now. In many plants JA and JA–Ile accumulate in a ratio of about 10:1. For a long time, the initial product of JA biosynthesis, (+)-*7-iso*-JA, was assumed to epimerize to the more stable (−)-JA. (−)-JA was taken as an indicator of endogenous rise of JAs upon any environmental stimuli. Now, an assay for quantification of (+)-*7-iso*-JA–Ile is available (Suza et al. 2010). Usually, however, levels of JA and JA–Ile are recorded without detection of the individual enantiomers. In *JAR1*-RNAi lines of tomato, up to 25–50 % residual JA–Ile was found upon wounding, suggesting the existence of other JA conjugating enzymes than JAR1 (Suza et al. 2010). Auxin homeostasis is sustained by amido-hydrolases such as IAA-LEUCINE RESISTANT (ILR)-LIKE GENE 6 (ILL6) and IAA-ALANINE RESISTANT 3 (IAR3) which cleave auxin amino acid conjugates. Recently, IAR3 and ILL6 were identified as JA–Ile and 12-OH-JA–Ile amido-hydrolases (Widemann et al. 2013). These enzymes attribute to homeostasis of the active signaling compound JA–Ile as well as formation of 12-OH-JA. Their activities represent a new and unexpected route of 12-OH-JA formation. A similar activity with JA–Ile occurs in *N. attenuata.* Here, a homologue of IAR3 has been cloned and shown to act as a JA–Ile amido-hydrolase (Woldemariam et al. 2012).

Besides amino acid conjugates of JA and their metabolites, twelve other JA derivatives have been identified in plant tissues, preferentially upon wounding

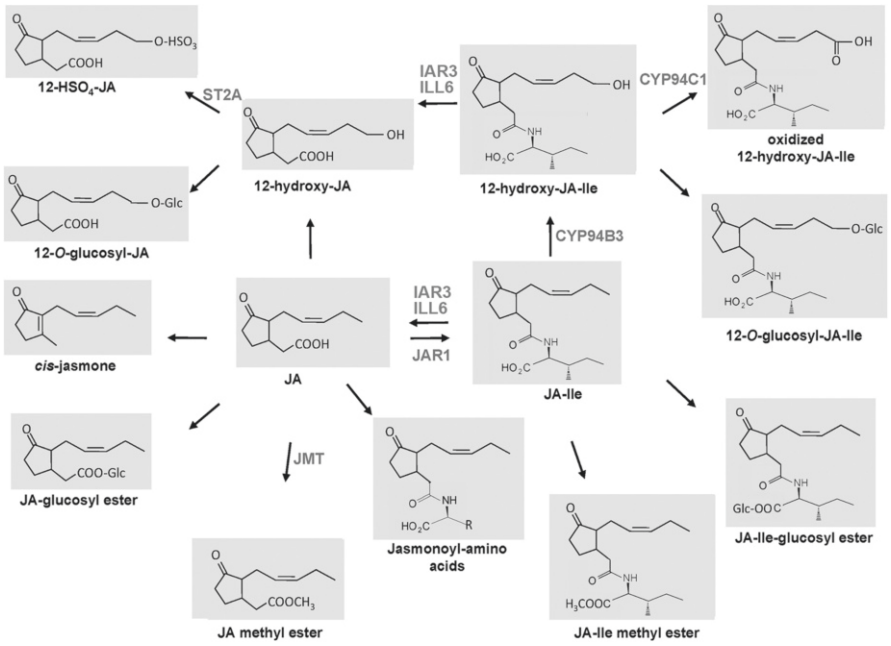

Fig. 3 Metabolism of jasmonic acid (JA) and JA–isoleucine conjugate (JA–Ile). Enzymes which have been cloned are indicated. *JAR1* JA amino acid synthetase, *JMT* JA methyltransferase, *ST2A* 12-OH-JA sulfotransferase 2A, *CYB94B3* JA–Ile hydroxylase, *CYP94C1* 12-OH-JA–Ile oxidase. Degradation of 12-hydroxy-JA–Ile and JA–Ile to 12-hydroxy-JA and JA, respectively, takes place by IAR3 and ILL6, two auxin amido-hydrolases (with permission and modified after Wasternack and Hause 2013)

(Wasternack and Hause 2013). Among them are JA methyl ester (JAMe), JA glucosyl ester, *cis*-jasmone, 12-*O*-glucosyl-JA, 12-HSO_4-JA, 12-hydroxy-JA, 12-hydroxy-JA–Ile, 12-COOH-JA–Ile, 12-*O*-glucosyl-JA–Ile, JA–Ile-glucosyl ester, and JA–Ile methyl ester. Similar derivatives can be assumed for OPDA, but such compounds were not identified so far.

Except JAR1, several enzymes active in JA metabolism have been cloned for *A. thaliana*, tomato, and tobacco. Among them are JA methyltransferases (JMT) (Seo et al. 2001): 12-OH-JA sulfotransferases (AtST2a) (Gidda et al. 2003), a JA–Ile hydroxylase (CYP94B3) (Heitz et al. 2012; Kitaoka et al. 2011; Koo et al. 2011), and a 12-OH-JA–Ile oxidase (CYP94C1) (Heitz et al. 2012). Some JAs accumulate abundantly and constitutively in distinct developmental stages and organs. Among them are 12-OH-JA, 12-HSO_4-JA, and 12-*O*-glucosyl-JA which can reach levels three orders of magnitude higher than that of OPDA, JA, or JA–Ile (Miersch et al. 2008). Many metabolites of JA and JA–Ile such as 12-HSO_4-JA, 12-*O*-glucosyl-JA, 12-hydroxy-JA, 12-hydroxy-JA–Ile, 12COOH-JA–Ile, JAMe, *cis*-jasmone, and 12-*O*-glucosyl-JA–Ile accumulate transiently upon wounding or other environmental stimuli (Glauser et al. 2008, 2009; Heitz et al. 2012; Koo et al. 2011; Miersch et al. 2008). Hydroxylation or other metabolic conversions can be an at least partial

deactivation of bioactivity of JA and JA–Ile (Heitz et al. 2012; Koo et al. 2011; Miersch et al. 2008). In case of the volatile *cis*-jasmone, the decarboxylated JA, bioactivity has been shown by expression data. A subset of genes is expressed by *cis*-jasmone which is different from that induced by JA or JA–Ile (Matthes et al. 2010). Pyrethrins such as cinerolone, jasmonolone, and pyrethrolone are thought to be synthesized from 7-OH-JA (Ramirez et al. 2013). Also 12-*O*-glucosyl-JA has been shown to be active. A distinct enantiomer of the jasmonoyl moiety of this compound was identified as leaf-closing factor of *Albizia* and *Samanea* (Nakamura et al. 2011).

Perception of JA–Ile and Cross-Talk to Other Hormones

One of the most exciting results of the last couple of years in plant biology was the genetic and biochemical proof on hormone perception via the ubiquitin—proteasome system. Similar modules were identified for perception of JA–Ile, auxin, GA, and ET (Chini et al. 2009; Kelley and Estelle 2012). In case of auxin and JA/JA–Ile, similarities are exceptional (Perez and Goossens 2013). A Skp1/Cullin/F-box (SCF) complex functioning as an E3 ubiquitin ligase binds the hormone to the complex. Subsequently, negative regulators of transcription can be recognized by the F-box protein of the complex and are ubiquitinated and thereby subjected to proteasomal degradation (Fig. 4). This allows positively acting TFs to become active. In case of JA–Ile the SCF complex contains the F-box protein *COI1* which was identified via the JA/JA–Ile insensitive mutant of *A. thaliana coi1* (Xie et al. 1998). Coronatine is a bacterial toxin of *Pseudomonas syringae* acting as a molecular mimic of JA–Ile (Zheng et al. 2012), but does not occur in plants. The structural similarity between coronatine and (+)-*7-iso*-JA–Ile led to identification of the latter compound as the most bioactive JA (Fonseca et al. 2009) and finally as the ligand of the JA–Ile receptor (Sheard et al. 2010; Yan et al. 2009). The *SCF^{COI1}-JAZ-co-receptor complex* has been crystallized and mechanism of binding of (+)-*7-iso*-JA–Ile together with inisitol-5-bisphosphate, a co-activator, was shown (Mosblech et al. 2011; Sheard et al. 2010). Targets of the SCF^{COI1} complex are JASMONATE ZIM (ZINC-FINGER PROTEIN EXPRESSED IN INFLORESCENCE MERISTEM) (JAZ) proteins, a new protein family with twelve members in *Arabidopsis* (Chini et al. 2007; Thines et al. 2007; Yan et al. 2007). At low JA–Ile levels, TFs such as MYC2 which binds to the G-box of a promoter of a JA-inducible gene are repressed by JAZ proteins (Fig. 4). At higher JA–Ile levels, however, the SCF^{COI1} complex binds a JAZ protein via JA–Ile binding resulting in ubiquitinylation and degradation of the JAZ protein and derepression of the transcriptional activators. This basic scenario of JA–Ile perception via the SCF^{COI1}-JAZ-co-receptor complex and the subsequent activation of JA/JA–Ile-induced gene expression became more complex upon identification of the corepressor TOPLESS (TPL) and the adaptor protein "Novel Interactor of JAZ" (NINJA) (Pauwels et al. 2010). NINJA interacts with JAZ and TPL. Repression of gene expression takes place by binding of JAZ to TFs such as the *basic*

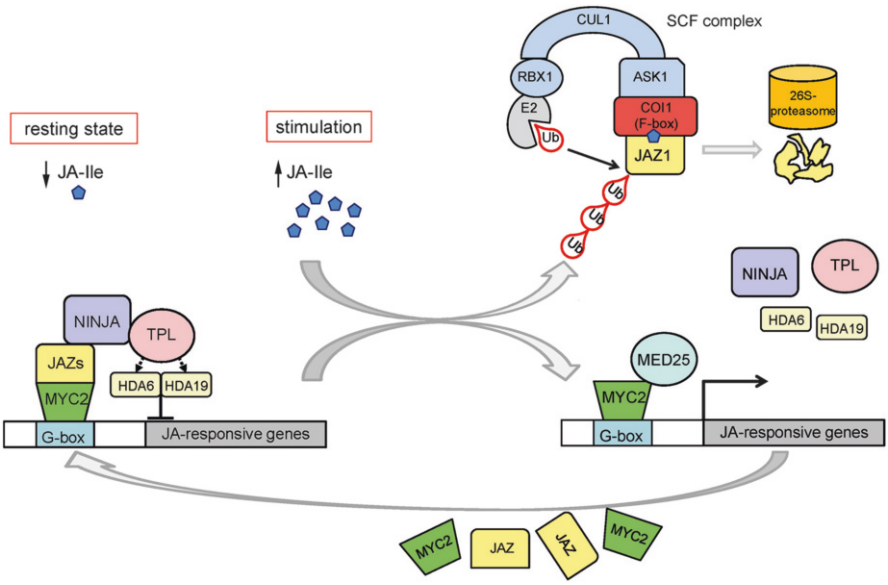

Fig. 4 JA/JA–Ile perception by the SCF^{COI1}-JAZ-co-receptor complex leads to JA/JA–Ile-induced gene expression. There is a low JA/JA–Ile level without environmental stimuli. MYC2 which bounds to a G-box of a JA/JA–Ile-responsive gene is repressed by negative regulators such as JAZs, mediated by corepressors NINJA and TOPLESS (TPL) which act via the HISTONDEACETYLASE6 (HDA6) and HDA19. In addition to JAZ proteins, JAMs (JASMONATE-ASSOCIATED MYC2-LIKE1, JAM2, JAM3) (Nakata et al. 2013) and JAV1 (JASMONATE-ASSOCIATED VQ MOTIF GENE 1) act as repressors. In case JAV1 the interacting ubiquitin E 3 ligase is unknown (Hu et al. 2013a), whereas JAMs compete with MYC2 in binding to the G-box. Dimerization is experimentally shown only for JAZ proteins so far. Upon increase of JA/JA–Ile levels by any stress, JAZs, and JAV1 proteins are subjected to ubiquitinylation and subsequent degradation by the 26S proteasome. Therefore, MYC2 can switch on transcription of JA/JA–Ile-responsive genes including early genes such as *JAZs* and *MYC2*. MED25, the subunit 25 of the Mediator complex, mediates transcription (cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones"). Ub, ubiquitin; E2, Rbx, Cullin, ASK1, and the F-box protein COI1 are components of the SCF complex (with permission)

helix-loop-helix (bHLH) TF MYC2 and corepressor activity of TPL mediated by histone deacetylases 6 and 19. In the derepressed state JA/JA–Ile-responsive gene expression is mediated by subunit 25 of the Mediator complex (MED 25) (Çevik et al. 2012; Chen et al. 2012). TFs such as MYC2 and the JAZ proteins are JA/JA–Ile inducible. Therefore, a futile cycle may occur which will attribute to a fine tuning of JA/JA–Ile-induced gene expression at different levels.

The interaction between MYC2 and JAZ takes place via the JAZ INTERACTING DOMAIN (JID) of MYC2 and the Jas domain of JAZ. Jas is absolutely required for repressor function of JAZ (Browse 2009a; Thines et al. 2007). The ZIM domain of JAZ mediates interaction to NINJA but is also responsible via its TIFY domain for homo- and heterodimerization of JAZs (Chung and Howe 2009). The NINJA–TPL interaction takes place via the ET-RESPONSIVE ELEMENT BINDING FACTOR-ASSOCIATED AMPHIPHILIC REPRESSION (EAR) motif of NINJA. Some JAZ

proteins contain such an EAR motif which allows direct binding of TPL without NINJA. These versatile interaction domains occur also in homologous components of ABA and auxin signaling (Pauwels et al. 2010). Consequently, NINJA and TPL are integrators of different signaling pathways. The SCF^{COI1}-JAZ-co-receptor complex and its interactors exhibit several exciting regulatory components:

1. The Jas domain of JAZ interacts with COI1 in the presence of JA–Ile and is strongly increased by IP_5 (Mosblech et al. 2011; Sheard et al. 2010). Stability of COI1 depends on its integration in the SCF complex (Yan et al. 2013).
2. Alternative splice variants of JAZ attribute to multiple JAZ functions and negative feedback control of JA/JA–Ile signaling (Moreno et al. 2013).
3. Enhanced stability of JAZ proteins such as that of JAZ8 being unable to strongly interact with COI1 may attribute to JAZ activity (Shyu et al. 2012).
4. Homo- and heterodimerization of JAZ proteins is another regulatory level (Chung and Howe 2009).
5. *JASMONATE-ASSOCIATED VQ MOTIF GENE 1* (*JAV1*) has been identified recently as another negative regulator of JA/JA–Ile-mediated plant defense with similarities to JAZ (Hu et al. 2013a; Zhu and Zhu 2013). The interacting ubiquitin E 3 ligase, however, is unknown for JAV1. In contrast to JAZ proteins, JAV1 is a repressor against necrotrophic pathogens and herbivorous insects, but not active in plant growth and development.
6. A JASMONATE-ASSOCIATED MYC2-LIKE1 TF, called JAM1, was identified as an ABA-inducible bHLH-type transcriptional repressor of JA responses against herbivores and in JA-dependent growth and development (Nakata et al. 2013). JAM1 competes with MYC2 to target sequences of MYC2 thereby attributing to a fine tuning in JA/JA–Ile-induced gene expression. Together with JAM2 and JAM3, many JA/JA–Ile responses are negatively regulated by JAM1 (Sasaki-Sekimoto et al. 2013). This includes also expression of genes involved in JA biosynthesis and metabolism. The degree of repression by JAZs or/and JAMs is unknown so far.
7. MYC2 activity is sustained by a phosphorylation-coupled proteolysis leading to a distinct amount of "fresh" MYC2 which is able to activate transcription in a positive manner (Zhai et al. 2013). This nuclear located regulatory loop has similarity to SA signaling via the NPR1 protein, the NONEXPRESSOR OF *PR* GENE1 active in SA-induced transcription as co-activator of defense gene expression (cf. Pieterse et al. 2012).
8. Among the bHLH TFs, the subgroup IIId has been identified as novel target of JAZ proteins and as transcriptional repressors in root growth inhibition and anthocyanin formation (Song et al. 2013a). These repressors act redundantly to JAZs indicating a fine tuning in JA/JA–Ile signaling by increased number of signaling components.
9. ILL6, a member of *GH3* gene family coding for amido-hydrolases, has been identified as a new negatively acting regulatory component in JA/JA–Ile responses by comparing expression profiles of individual wild-type plants (Bhosale et al. 2013). ILL6 is involved in cleavage of JA–Ile and 12-OH-JA–Ile,

thereby attributing to JA–Ile homeostasis as well as generation of 12-OH-JA without direct hydroxylation of JA (Widemann et al. 2013).

10. A screen with a JAZ10 reporter system revealed mutants of NINJA which showed constitutive activation of JA responses in roots and hypocotyls indicating organ-specific activation of JA signaling (Acosta et al. 2013).

This plethora of components and regulatory principles in JA signaling is used by downstream components as well as in the cross-talk to other hormones. Targets of JAZs in JA signaling are TFs of the bHLH-type MYC and the *R2R3-type MYB* family. MYC2 was the first TF for which an interaction with a JAZ protein was shown (Chini et al. 2007). MYC2 is a key player in JA/JA–Ile-induced gene expression and is involved in synthesis of auxin, tryptophan, glucosinolates (GS), ET, and JA as well as in responses to herbivores, oxidative stress, pathogens, and ABA-dependent drought stress (Dombrecht et al. 2007; Kazan and Manners 2008). The central role of MYC2 is documented by (1) the regulation of its cross-talk with SA, ABA, GA, and auxin signaling pathways; (2) the link between JA/JA–Ile and other signaling pathways such as light, phytochrome and circadian clock; (3) the regulation of lateral and adventitious root formation, flowering, and shade avoidance syndrome; (iv) the innate immunity in roots; (5) induced systemic resistance (ISR) by beneficial soil microbes; as well as (6) the antagonistic coordination of responses to herbivores and pathogens. Some of the MYC2-dependent JA-regulated processes have been verified by proteome analysis of wild-type and *myc2* mutant plants (Guo et al. 2012). All these aspects reflect the central role of MYC2 and have been reviewed recently (Kazan and Manners 2013). Besides the master regulator MYC2, other targets of JAZs are MYC3, MYC4, MYB21, and MYB24. All MYC TFs have a JID domain and a conserved ACT-like domain at the C-terminus being involved in homo- and heterodimerization of MYCs (Cheng et al. 2011; Fernández-Calvo et al. 2011, Pauwels and Goossens 2011). MYC2, MYC3, and MYC4 are partially redundant (Fernández-Calvo et al. 2011). The *myc2,3,4* triple mutant plants are free of GS and show altered insect performance and feeding behavior (Schweizer et al. 2013). MYC2 binds directly to promoters of GS biosynthesis genes. All three MYCs interact with GS-related MYB TFs indicating the complex scenario in JA/JA–Ile-induced gene expression (Schweizer et al. 2013). The bHLH TFs involved in anthocyanin formation and trichome initiation contain also a JID domain and are targets of JAZ1 and JAZ8 (Qi et al. 2011). JAZ targets active in development were identified in a transcriptome analysis of developing stamen of JA-treated *opr3* plants (Mandaokar et al. 2006). Among them are *MYB21* and *MYB24* which interact with JAZ1 and JAZ8 via the N-terminal R2R3 domain (Song et al. 2011). Both TFs are specifically involved in fertility but less in other JA/JA–Ile-dependent processes such as root growth or anthocyanin formation.

The *cross-talk* between *JA/JA–Ile and auxin* was shown in several processes. Prominent examples are (1) the MYC2-mediated suppression of PLETHORA, a central regulator in auxin-mediated root meristem and root stem cell niche development (Chen et al. 2011); (2) the regulatory activity of JA/JA–Ile in expression of *ANTHRANILATE SYNTHASE1* (*ASA1*), which encodes the initial enzyme in auxin

biosynthesis (Sun et al. 2009); and (3) COI1- and JA/JA–Ile-dependent regulation of *YUCCA8* and *YUCCA9*, two important genes in auxin biosynthesis (Hentrich et al. 2013).

The *cross-talk* between *JA/JA–Ile and ET* is synergistic and takes place by MYC2 activated upon herbivore attack and by ETHYLENE RESPONSE FACTOR1 (ERF1). ERF1 is activated upon infection by necrotrophic pathogens and JA/JA–Ile-dependent degradation of JAZs, the repressors of MYC2 and TFs in ET signaling such as ETHYLENE INSENSITIVE3/EIN-LIKE1 (EIN3/EIL1) and OCTADECANOID-RESPONSIVE *ARABIDOPSIS* AP2/ERF domain protein (ORA59) (Pieterse et al. 2012). The final output of JA/JA–Ile-ET cross-talk is an antagonistic activity between the MYC2 branch and the ERF1 branch and is of benefit for plants due to the naturally occurring simultaneous attack by herbivores and necrotrophic pathogens (Pieterse et al. 2012; Verhage et al. 2011).

Cross-talk between *JA/JA–Ile and GA* signaling takes place synergistically during stamen development and antagonistically in the balance between growth and defense (Kazan and Manners 2012; Wasternack and Hause 2013). During stamen development, the repressors in GA signaling, the DELLA proteins, repress *DAD1* and *LOX* expression in the absence of GA leading to JA/JA–Ile deficiency, to downregulation of *MYB21* and *MYB24* by JAZ, and finally to male sterility (Cheng et al. 2009; Song et al. 2011). The opposite scenario takes place by GA-induced SCF^{GID}-mediated DELLA degradation. JA/JA–Ile and GA act antagonistic in growth and defense which is of benefit for the plant, since plant defense is costly and occurs at the expense of plant growth (Hou et al. 2013; Kazan and Manners 2012). Plant growth can occur at sufficient GA level which represses DELLAs and attenuates DELLA binding to JAZ followed by JAZ binding to MYC2. Consequently, JA-dependent defense response is suppressed during growth (Kazan and Manners 2012; Wager and Browse 2012; Wasternack and Hause 2013). There is a balance of the modules of the SCF complexes for JA and GA. It has to be kept in mind, however, that these complexes are part of the COP9 signalosome (CSN) multiprotein complex which regulates both SCF activities (Stratmann and Gusmaroli 2012). In addition to the GA—JA/JA–Ile cross-talk, the balance between disease resistance and growth is regulated by ABA, SA, and auxin (Denancé et al. 2013). Here, pathogens evade hormone-mediated defense responses with a negative effect on fitness leading to less growth and development.

Cross-talk between *BR and JA/-JA–Ile* is antagonistic in respect to growth as shown by mutants (Huang et al. 2010) and is synergistic in case of anthocyanin biosynthesis, where BR acts upstream of JA/JA–Ile (Peng et al. 2011; Song et al. 2011). Another cross-talk of BR and JA/JA–Ile occurs in defense to herbivores (Yang et al. 2013). Surprisingly, BR receptor impairment downregulates herbivore-induced accumulation of JA–Ile and diterpene glycosides without effects on JA levels and trypsin proteinase inhibitor levels (Yang et al. 2013). An important gene in BR biosynthesis is *DWF4* (*DWARF4)* which encodes a steroid C22 α-hydroxylase (CYP90B1). Its expression is auxin inducible and is repressed by JA/JA–Ile. Consequently, the balance between growth and defense is sustained by JA/JA–Ile via BR (Kim et al. 2013).

The *cross-talk* between *ABA and JA/JA–Ile* was clearly detected for the wound response. Here, the rise of ABA and JA/JA–Ile and JA/JA–Ile-induced formation of PYL4 and PYL5, which are ABA receptors, have been shown (Kazan and Manners 2008; Lackman et al. 2011). Many components of the *cross-talk* between *JA/JA–Ile and SA* have been identified, and synergistic and antagonistic interactions were shown (Boatwright and Pajerowska-Mukhtar 2013; Gimenez-Ibanez and Solano 2013; Pieterse et al. 2012). JA/JA–Ile is the key player in responses to necrotrophic pathogens and herbivores, whereas SA is the central signaling compound in responses to biotrophic pathogens (Pieterse et al. 2012). Key components of both pathways such as glutaredoxins, thioredoxins, TFs such as WRKY70 for the SA pathway, and MYC2 as well as COI1 for the JA pathway are involved in the cross-talk. Final steps in this cross-talk are nuclear modulation of both signaling pathways (Gimenez-Ibanez and Solano 2013; Pieterse et al. 2012). The well-known suppression of JA-responsive gene expression takes place downstream of JA formation (Leon-Reyes et al. 2010) and of the SCF^{COI1}-JAZ-co-receptor function. The suppression includes the TF ORA59 (Van der Does et al. 2013). Another interesting cross-talk was shown by coronatine-mediated increase in *P. syringae* virulence (Zheng et al. 2012). Here, ARABIDOPSIS NAM, ATAF1,2, CUC2 (NAC) TFs (ANACs) are involved. Coronatine activates the three homologous TFs, ANAC019, ANAC055, and ANAC072, in an MYC2-dependent manner, leading to inhibition of initial steps in SA synthesis. A similar scenario for these ANAC TFs was found during senescence (cf. section "JA/JA-Ile in Plant Growth and Development"). In parallel, coronatine allowed bacterial propagation locally and systemically upon induction of stomata reopening (Xin and He 2013) or inhibition of stomatal closure (Lee et al. 2013). These data reflect the multiple virulence activities of coronatine (Zheng et al. 2012). The properties of coronatine as a multifunctional suppressor of defense include also COI1- and SA-independent signaling (Geng et al. 2012). The JA/JA–Ile - SA cross-talk is a conserved mechanism and is transmitted to the next generation (Luna et al. 2012). Obviously, these pathways allow in nature the flexibility of plants to adapt to simultaneously and/or subsequently occurring changes in the environment (Thaler et al. 2012). It is interesting to note that nuclear targeted effectors of pathogenic fungi, nematodes, and beneficial microbes are similar in their action and reprogramming of hormonal pathways such that of SA and JA/JA–Ile (Gimenez-Ibanez and Solano 2013).

JA/JA–Ile signaling versus OPDA signaling is an intriguing question rose by the fact that the SCF^{COI1}-JAZ-co-receptor complex accept exclusively (+)-*7-iso*-JA–Ile (Fonseca et al. 2009) but not OPDA (Thines et al. 2007). The mechanistic proof was given upon crystallization of the complex (Sheard et al. 2010). There are, however, OPDA-specific reactions such as tendril coiling (Blechert et al. 1999), gene expression (Mueller et al. 2008; Taki et al. 2005), embryo development in tomato (Goetz et al. 2012), inhibition of seed germination (Dave et al. 2011), activation of *PHO1* genes which are involved in phosphate accumulation (Ribot et al. 2008), PHYTOCHROME A signaling (Robson et al. 2010), hypocotyl growth inhibition (Brüx et al. 2008), or insect-induced closure of the Venus flytrap (Escalante-Pérez et al. 2011) (reviewed in Wasternack and Hause 2013, Wasternack et al. 2012).

In *P. patens* which does not contain JA (Stumpe et al. 2010), OPDA is involved in responses to *B. cinerea* infection by reinforcement of the cell wall and programmed cell death (Ponce de Leon et al. 2012). Even JA is absent in *P. patens*, the moss can respond to applied JA suggesting perception via the SCF^{COI1}-JAZ-co-receptor complex or a perception mechanism not yet identified.

Some of the OPDA-specific effects might be mediated by RES since OPDA contains an α,β-unsaturated carbonyl group (Farmer and Mueller 2013). An interesting new example of OPDA-specific signaling was given recently by data on OPDA-binding to cyclophilin 20-3 which is involved in stress responses (Park et al. 2013). As a consequence of OPDA-binding to this cyclophilin, a hetero-oligomeric cysteine synthase complex is formed in the chloroplast leading to activation of sulfur assimilation and cellular redox homeostasis (Park et al. 2013).

JA/JA–Ile-Regulated Metabolism of Secondary Compounds

Besides JA-induced proteins of barley (Weidhase et al. 1987) and wound-induced PROTEINASE INHIBITOR (PIN) formation in tomato (Farmer and Ryan 1990), the elicitor-induced alkaloid synthesis of plant cell cultures was among the first JA-induced gene expression programs which were analyzed (Gundlach et al. 1992). Meanwhile, JA/JA–Ile-induced synthesis of secondary compounds has been shown for many plant species and diverse secondary compounds. This led to biotechnological and agricultural applications (reviewed in Wasternack 2013). OCTADECANOID DERIVATIVE RESPONSIVE CATHARANTHUS AP2 DOMAIN2 and 3 (ORCA2 and ORCA3) were the first TFs involved in synthesis of secondary metabolites, here terpenoid indole alkaloids (TIA) in *Catharanthus roseus* (van der Fits and Memelink 2000). Transcriptional control of secondary metabolite biosynthesis has been shown in detail and includes the SCF^{COI1}-complex, JAZ proteins, MYC2, ORCAs and/or ERFs, MYBs, and WRKYs which are active in distinct pathways. For *nicotine* biosynthesis requirement of functional SCF^{COI1}-JAZ-co-receptor complex, MYC2, and AP2/ERFs has been shown (De Boer et al. 2011; Shoji and Hashimoto 2011). AP2/ERFs are encoded by the *NIC* locus in tobacco, comprise 239 members (Rushton et al. 2008), and are close homologues of ORCA3 of *C. roseus*. Obviously, these TFs evolved as a regulatory module in two species and two pathways in parallel due to evolutionary advantage.

The abovementioned "machinery" of SCF^{COI1}, JAZ, MYC2, ORCA2, and ORCA3 is also active in *vinblastine* biosynthesis of *C. roseus* (Zhang et al. 2011), whereas *artemisinin* biosynthesis is controlled by ERF1, ERF2, MYC2, and WRKY1 (Ma et al. 2009). The trichome-specific TF of *Artemisia annua* ORA, a member of the AP2/ERF TF family, is a key player in artemisinin biosynthesis (Lu et al. 2013). Interestingly, artemisinin biosynthesis genes are coordinately activated with genes involved in the formation of trichomes, the storage organ of artemisinin (Maes et al. 2011).

Many genes encoding enzymes of *glucosinolate/camalexin* biosynthesis are JA/JA–Ile regulated via SCF^{COI1}, JAZ, MYC2, MYC3, MYC4, and an MAP

kinase—WRKY cascade (De Geyter et al. 2012; Schweizer et al. 2013). Members of the NAC TF family such as ANAC42 are also involved. In summary, the TFs active in alkaloid biosynthesis belong to the families of bHLH, MYC, ERF, and WRKY TFs, and most of them are JA/JA–Ile inducible. These aspects have been reviewed recently (Yamada and Sato 2013).

Anthocyanin is the most prominent secondary compound formed upon JA/JA–Ile treatment or any environmental stimuli leading to endogenous rise of JA/JA–Ile. Any stress of plant tissues is frequently visible by red cell layers indicating anthocyanin formation. Involvement of JA/JA–Ile biosynthesis and signaling has been repeatedly shown by lack of anthocyanin formation in mutants of *A. thaliana* or tomato affected in JA biosynthesis or signaling. Prominent examples are *coi1* and *opr3* for *A. thaliana* and *jai1*, *spr2,* and *acx1* for tomato (Browse 2009b) (Table 1). Important TFs active in anthocyanin synthesis are PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1), ENHANCER OF GLABRA3 (EGL3), GLABRA3 (GL3), MYB75, and TRANSPARENT TESTA8 (TT8). All of them are targets of JAZ proteins (Qi et al. 2011). Like artemisinin, anthocyanin formation and trichome formation are coordinately regulated as shown by identification of the tomato homologue of COI1, JAI1 (Li et al. 2004). In *jai1* mutant plants no anthocyanin formation and trichome formation takes place.

JA/JA–Ile in Biotic Interactions of Plants

Due to their sessile lifestyle, plants have to respond to any attack by herbivores, leaf or root pathogens, nematodes, and sucking insects. Biotic interactions can be, however, also beneficial for plants as in case of mutualistic interactions, such as arbuscular mycorrhiza (AM), growth-promoting rhizobacteria leading to ISR, or root nodule symbiosis (RNS). Even plant–plant interactions occurring by near growth of different plant species can be beneficial for both partners. Leaf volatiles or root exudates can attribute to such interaction. The benefit for the plants is obvious by the so-called intercropping, the mixed growth of two or more plant species (cf. section "Applied Aspects on Jasmonates"). In all these interactions JA is a signal.

Response to *herbivory* and *mechanical wounding* is one of the most prominent and early observed JA responses. There was the observation by C. A. Ryan (Pullman, USA) that a sagebrush plant led to less attack by herbivores of a neighboring tomato plant (Farmer and Ryan 1990). Volatile JAMe was identified as the compound emitted by sagebrush leaves which induced in the neighboring tomato leaves formation of PIN2, a deterrent protein for the gut of herbivores. Worldwide is a dramatic loss in agriculture by herbivores, mechanical wounding, or sucking/piercing insects. This led to intensive research. Plant responses to herbivores are induced by oral secretion of the herbivore which contain inducers of wound-induced gene expression such as volicitin (cf. rev. of Wasternack and Hause 2002). There are two defense mechanisms: (1) *direct defense* by formation of toxic compounds such as nicotine in tobacco or other deterrent secondary metabolites, by synthesis of many defense proteins such as PINs or polyphenol oxidase (PPO) which have deterrent role in the

Table 1 Mutants of JA biosynthesis and JA signaling in *Arabidopsis* and tomato (modified after Wasternack 2006)

Mutants	Phenotype	Gene product	References
Deficient JA biosynthesis			
dad1	Reduced filament elongation, *d*elayed *a*nther *d*ehiscence, JA deficient in flowers	Phospholipase A1	Ishiguro et al. (2001)
fad3-2fad7-2fad8[a]	Male sterile, delayed anther development, altered α-LeA level	ω-7-*f*atty *a*cid *d*esaturase	McConn and Browse (1996)
spr2[a,b]	Deficient in α-LeA and JA levels, no wound response, *s*uppressed *pr*osystemin expression	ω-3-fatty acid desaturase	Li et al. (2003)
aos	JA deficient, decreased resistance to pathogens	*AOS*	Park et al. (2002)
dde2-2	Male sterile, delayed anther development	*AOS*	von Malek et al. (2002)
opr3	JA deficient, decreased resistance to pathogens, reduced filament elongation	*OPR3*	Stintzi and Browse (2000)
acx1[a]	JA-deficient, reduced wound response	Acyl-CoA oxidase1	Li et al. (2005)
aim1[c]	Abnormal inflorescence meristem	Multifunctional protein1	Richmond and Bleecker (1999)
comatose	Reduced JA content	*COMATOSE/PXA1*	Theodoulou et al. (2005)
Constitutive JA response			
cev1	*C*onstitutive *e*xpression of *v*egetative storage proteins	Cellulose synthase CeS3A	Ellis et al. (2002)
cet1-9	*C*onstitutive *e*xpression of *t*hionins, increased JA levels	?	Hilpert et al. (2001)
cas1	Constitutive expression of *AOS*	?	Kubigsteltig and Weiler (2003)
ore9/max2[d]	Delayed leaf senescence, strigolactone-dependent shoot branching	F-box protein	Woo et al. (2001) Domagalska and Leyser (2011)
Reduced sensitivity to JA			
coi1	Reduced root growth inhibition, male sterile, reduced filament elongation, enhanced sensitivity to necrotrophic pathogens	F-box leucine reach repeat (LRR) COI1	Xie et al. (1998)
jai1[a]	Female sterile, altered trichome development, increased sensitivity to pathogens, decreased wound response	Tomato homologue of COI1	Li et al. (2004)
jar1/jin4/jai2	Reduced root growth inhibition by JA, increased sensitivity to necrotrophic pathogens	JA amino acid conjugate synthase	Lorenzo et al. (2004), Staswick et al. (1992), Staswick and Tiryaki (2004)
cyp94b3	Reduced JA responses	JA–Ile-hydroxylase	Koo et al. (2011), Heitz et al. (2012)

cyp94c1	Reduced JA responses	JA–Ile-carboxylase	Heitz et al. (2012)
jin1/jai1/myc2	Reduced root growth inhibition	AtMYC2 (bHLHzip TF)	Lorenzo et al. (2004)
mpk4	Dwarf phenotype, altered expression of JA- and SA-response genes	*AtMPK4*	Petersen et al. (2000)
axr1	Reduced root growth inhibition by JA	RUB-activating enzyme	Xu et al. (2002)
jai4/sgt1b[e]	Reduced root growth inhibition in the *ein3* background	*AtSGT1b*	Lorenzo et al. (2004)
Increased JA response			
jam1[f]	Increased anthocyanin formation and resistance to herbivores	JAM1 TF	Nakata et al. (2013)
jam2[f], *jam3*[f]	Increased defense responses, anthocyanin formation, and root growth inhibition	JAM2 TF JAM3 TF	Sasaki-Sekimoto et al. (2013)
jav1[g]	Increased resistance to pathogens and herbivores, but unaltered development	JAV1 TF	Hu et al. (2013a)

[a]Tomato mutants
[b]*SUPPRESSOR OF PROSYSTEMIN EXPRESSION*
[c]*ABNORMAL INFLORESCENCE MERISTEM1*
[d]*ORESA9/MORE AXILLARY GROWTH2*
[e]*JASMONATE INSENSITIVE4/SUPPRESSOR OF THE G2 ALLELE OF SKP 1*
[f]*JASMONATE-ASSOCIATED MYC2-LIKE1,2,3*
[g]*JASMONATE-ASSOCIATED VQ MOTIF GENE 1*

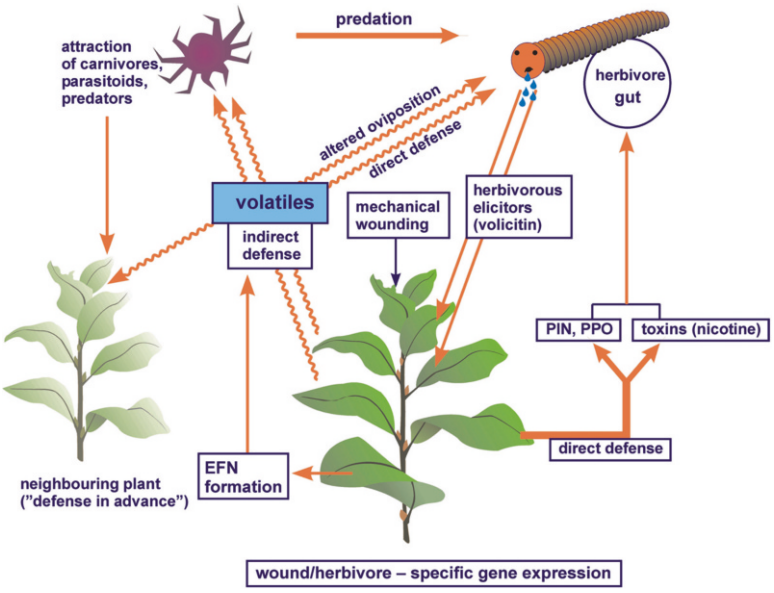

Fig. 5 Mechanical wounding and herbivory leads to direct and indirect defense. Upon elicitation by oral secretions of herbivores or mechanical damage of leaves, defense proteins such as proteinase inhibitors (PINs) or polyphenol oxidase (PPO) as well as toxic compounds such as nicotine in case of tobacco are formed. All of them affect digestion of the leaf tissues in the herbivorous gut due to deterrent properties of these proteins or compounds. Indirect defense upon herbivory is initiated by emission of leaf volatiles which attract parasitoids and carnivores or alter oviposition of herbivores. Additionally, volatiles can induce defense reactions in neighboring plants. Extra floral nectar (EFN) formation can also attribute to defense (with permission)

digestion of the herbivorous gut, and (2) *indirect defense* by emission of volatiles such as leaf alcohols or aldehydes or terpenoids (Fig. 5). These volatiles attract carnivores, parasitoids, or predators and alter the oviposition of herbivores. There is a specific volatile blend which differs among various insect communities. Under field conditions, the volatile emission can reduces the number of herbivores up to 90 % (Kessler et al. 2004). The scenario, however, is more complex than previously recognized, e.g., oral secretions of herbivores contain bacteria which downregulate plant defense reactions (Chung et al. 2013). Another issue is the reallocation of resources within a plant by herbivore attack. JA/JA–Ile-mediated defense is costly, e.g., herbivore attack on leaves reduces sugar and starch levels in roots and reduces regrowth from the rootstock (Machado et al. 2013). Besides wounding by mechanical damage or herbivores, touch of aboveground plant parts increases endogenous JA/JA–Ile levels and leads to growth inhibition (Tretner et al. 2008). This is even different to soft mechanical stress which generates ROS (*reactive oxygen species*) in a JA-independent manner leading to resistance to *B. cinerea* (Benikhlef et al. 2013).

Due to the overwhelming literature on wound responses and herbivory available already in reviews, we refer here to some of them to avoid overlap (Ballaré 2011; Bonaventure et al. 2011; Dicke and Baldwin 2010; Erb et al. 2012; Fürstenberg-Hägg et al. 2013; Meldau et al. 2012; Reymond 2013; Santino et al. 2013).

Arbuscular mycorrhiza (*AM*) is a mutualistic interaction of about 80 % of land plants with fungi of the phylum *Glomeromycota* (Schüssler et al. 2001). AM leads to supply of mineral nutrients and water as well as improved tolerance to some abiotic and biotic stressors (Cameron et al. 2013; Hause and Schaarschmidt 2009). Some of participating proteins have been identified mainly by RNAi approaches. Among them are components of membrane biosynthesis, transport, sucrose cleavage, and carotenoid biosynthesis (Recorbet et al. 2013). Several data accord with a role of JA/JA–Ile in the establishment and maintenance of AM: (1) AM roots of *M. truncatula* have increased JA levels and increased expression of JA biosynthesis genes (Hause et al. 2002; Isayenkov et al. 2005), (2) transgenic tomato lines with enhanced JA levels exhibit increased mycorrhization (Tejeda-Sartorius et al. 2008), (3) AOC-RNAi lines of *M. truncatula* carrying reduced JA biosynthesis have significantly less mycorrhization (Isayenkov et al. 2005), and (4) repeated wounding of *M. truncatula* leaves elevates JA levels and increases AM (Landgraf et al. 2012) (cf. also review of Wasternack and Hause 2013). The establishment of AM leads to systemic protection against many attackers similar to systemic acquired resistance (SAR) following pathogen attack and ISR after colonization by nonpathogenic rhizobacteria (Cameron et al. 2013). Therefore, the term "mycorrhiza-induced resistance" (MIR) was proposed. Four phases have been proposed, where in the last phase a systemic priming of JA- and ET-dependent defense reactions occur (Cameron et al. 2013).

ISR is induced by nonpathogenic microbes and, as mentioned above, by mycorrhizal fungi. JA/JA–Ile is the central regulator in generation of ISR (Van der Ent et al. 2009). There is a close interconnection between ISR and MIR due to putative priming of JA-dependent defenses caused by ISR-related rhizobacteria in the mycorrhizosphere (Cameron et al. 2013).

RNS has been controversially discussed in respect to putative role of JA/JA–Ile (cf. rev. of Wasternack and Hause 2013). Whereas in limited light supply JA/JA–Ile seems to be a positive regulator (Shigeyama et al. 2012; Suzuki et al. 2011), no increased JA level during nodulation under normal growth conditions was found (Landgraf et al. 2012). Autoregulation, a systemic effect in RNS, is a complex scenario, for which involvement of shoot-derived JA/JA–Ile has been proposed (Hause and Schaarschmidt 2009; Kinkema and Gresshoff 2008). RNS and AM have some common signaling components. Ca^{2+} and calmodulin-dependent protein kinases are the central signaling hubs, whereas specificity for AM and RNS is given by transcriptional regulators (Singh and Parniske 2012). These common sequences in AM and RNS seems to be inhibited by shoot-derived JA/JA–Ile during autoregulation (Hause and Schaarschmidt 2009).

JA/JA–Ile in Abiotic Stress Response of Plants

Involvement of JA/JA–Ile has been shown for plant responses to salt, drought, and osmotic and chilling stresses and has been reviewed recently (Santino et al. 2013). For several of these signaling pathways, JA/JA–Ile-specific signaling modules such as

SCF^{COI1}, JAZ, and MYC2 or expression of JA/JA–Ile biosynthesis genes has been identified. An example is the response to cold stress being positively regulated by JA/JA–Ile (Hu et al. 2013b). Key players in cold stress response are JA/JA–Ile inducible, and the INDUCER OF CBF EXPRESSION1 (ICE) is a target of JAZ1 and JAZ4.

JA/JA–Ile in Plant Growth and Development

The involvement of jasmonates in plant growth and development has been unequivocally shown by *mutants* affected in JA/JA–Ile biosynthesis and JA/JA–Ile signaling. These mutants preferentially identified for *A. thaliana* and tomato showed an altered phenotype in root growth inhibition and flower development. These aspects have been reviewed (Browse 2009a, b). For comparison, a brief summary of several mutants is shown in Table 1. These mutants can be subdivided into mutants of JA biosynthesis, mutants with reduced sensitivity to JA/JA–Ile, mutants with constitutive JA response, and mutants with increased JA response. Among JA biosynthesis mutants, *fad3-2fad7-2fad8*, *spr2*, *aos*, and *dde2-2* are prominent examples for JA/JA–Ile and OPDA deficiency. In contrast, *opr3* and *acx1* plants are JA deficient but still able to accumulate OPDA upon wounding. Constitutive JA/JA–Ile responses occur in *cev1* plants, where the subunit 3 of the cellulose synthase complex of *A. thaliana* is altered (Ellis et al. 2002). Recently, a set of mutants with increased JA responses was identified. Here, *JAM1, JAM2, and JAM3* were identified as bHLH TF/JA-associated MYC2-like negative regulators of MYC2 signaling (Nakata et al. 2013) (cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones") . Another negative regulator is encoded by the *JAV1* gene. In *jav1* mutant plants defense responses to necrotrophic pathogens and herbivores are increased without influencing growth and development (Hu et al. 2013a). This indicates repressor function of JAV1 at least partially like the JAZ proteins (cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones"). Male sterility is among the most prominent phenotypes described for JA-insensitive (*coi1, jai1*) or JA-deficient plants (*opr3, dde2-2, fad3-2 fad7-2 fad8*).

Flower Development: The altered phenotype of mutants affected in JA/JA–Ile biosynthesis and signaling led to detailed analyses of flower development (Browse 2009a; Song et al. 2013b; Wilson et al. 2011). Among the male sterile *A. thaliana* plants, insufficient filament elongation (*opr3*), nonviable pollen, and delayed anther dehiscence (*dad1*) have been described. Stamen transcriptome analysis in JA-treated *opr3* plants led to the identification of several MYB-type TFs (Mandaokar et al. 2006) (cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones"). Among them, MYB21, MYB24, and MYB57 were identified as JAZ targets being essential for stamen development (Song et al. 2011). Cross-talk to auxin in anther development was clearly shown by control of JA biosynthesis genes such as *DAD1*, *LOX2, AOS,* or *OPR3* by AUXIN RESPONSE FACTOR6 (ARF6) and ARF8 (Nagpal et al. 2005; Reeves et al. 2012) and accumulation of JA in auxin receptor quadruple mutant (*tir1, afb1-3*) (Cecchetti et al. 2013) (cf. review of Song et al. 2013b). There is also a cross-talk between JA/JA–Ile and GA as briefly described in section "Perception of JA-Ile

and Cross-Talk to Other Hormones". Here, DELLAs suppress expression of JA biosynthesis genes, thereby reducing JA/JA–Ile levels which are required for *MYB21/MYB24/MYB57* expression, the essential TFs in stamen development (Song et al. 2011, 2013b). Another indication for the role of JA/JA–Ile in flower development is given by binding of the TF AGAMOUS to the promoter of *DAD1*, encoding the PLA1 involved in JA formation in flowers (Ishiguro et al. 2001) (cf. section "JA Biosynthesis"), and by controlling of the bHLH TF BIGPETALp by JA/JA–Ile. This TF is involved in petal growth (Brioudes et al. 2009).

Seed Germination: Although GA, ABA, and ET are key players in seed germination, also JA/JA–Ile is active in an inhibitory manner (cf. review of Linkies and Leubner-Metzger 2012). Seed germination data for many mutants affected in JA biosynthesis and JA signaling revealed involvement of COI1. The mechanism of the suggested involvement of the SCF^{COI1}-JAZ-co-receptor complex is, however, not clear. The compound which inhibits seed germination is OPDA and not JA/JA–Ile, as checked with mutants of enzymatic steps downstream of OPDA formation (Dave et al. 2011; Dave and Graham 2012; Goetz et al. 2012). OPDA cannot be perceived via the SCF^{COI1}-JAZ co-receptor complex (Thines et al. 2007) (cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones").

Growth and Light: Plant growth is influenced by light in developmental programs such as photomorphogenesis, skotomorphogenesis, and shade avoidance syndrome (SAS) which have been studied intensively (Chory 2010; Lau and Deng 2010). Involvement of JA/JA–Ile, however, was analyzed only recently. Requirement for MYC2 activity, decreased defense against herbivores or necrotrophic pathogens upon silencing of JA/JA–Ile signaling components, and involvement of the JA/JA–Ile-linked MED25 (cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones") in phytochrome B-mediated SAS are few examples. The different aspects of JA/JA–Ile in light signaling have been reviewed (Lau and Deng 2010; Ballaré 2011; Ballaré et al. 2012; Kazan and Manners 2011; Wasternack and Hause 2013) and are not repeated here to avoid overlap.

Growth inhibition is an early observed physiological effect of JAs (Dathe et al. 1981). An explanation could be given by wound-induced inhibition of mitosis (Zhang and Turner 2008). The endogenous rise in JA after wounding of leaves occurs in all dicotyledonous plants tested so far. Even repeated touching of leaves leads to increase in JA which is sufficient to inhibit growth (Chehab et al. 2012; Tretner et al. 2008). Recently performed analysis of effects of JA showed COI1-dependent arrest in endo-reduplication cycle, in mitotic cycle during the G1 phase, and in downregulation of key determinants of DNA replication (Noir et al. 2013). The final output of these JA/JA–Ile effects is reduced expansion, growth, size, and number of cells which leads to reduced leaf size.

Root growth inhibition is a regularly performed assay for action of jasmonates and was used for screening of mutants in JA biosynthesis and JA/JA–Ile signaling, e.g., *jar1*, a JA-insensitive mutant (cf. Table 1), has been identified via root growth inhibition (Staswick et al. 1992). Root growth inhibition is COI1 dependent. Involvement of JA/JA–Ile is also indicated by the stunted root growth phenotype of *cev1* plants which have constitutively elevated JA/OPDA levels (Ellis et al. 2002).

NINJA, the corepressor of JA/JA–Ile signaling acting together with JAZ proteins (cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones"), is indispensible in repressing JA/JA–Ile signaling in roots and keeps normal root growth (Acosta et al. 2013). The complex nature of root growth is now studied by system biology approaches (Band et al. 2012a) which showed hierarchic interaction of GA, auxin, CK, and JA. Due to the abovementioned cross-talk among these hormones during JA/JA–Ile perception and signaling (cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones"), the outcome of root growth inhibition is given by altered cell division, membrane traffic, cell wall loosening and synthesis, as well as altered turgor and growth rate. All of them affect hormonal and mechanic signaling (Band et al. 2012b). Auxin, the key player in root growth, is influenced by (1) JA/JA–Ile-induced *ASA1* expression, required for auxin biosynthesis (Sun et al. 2009); (2) JA-induced redistribution of PIN-FORMED2, an auxin transporter (Sun et al. 2011); and (3) JA/JA–Ile-induced MYC2-dependent repression of PLETHORA, required for stem cell niche activity (Chen et al. 2011). Furthermore, in rice the outcome of root growth inhibition is determined by root cell elongation which is regulated by a ternary complex of JAZ proteins, bHLH TFs, and a nuclear factor active in rice salt stress (Toda et al. 2013).

Lateral root formation is influenced by JA/JA–Ile via the abovementioned cross-talk with auxin. Genes involved in JA/JA–Ile formation such as *AtAOC3* and *AtAOC4* have high promoter activity in emerging lateral roots (Stenzel et al. 2012), and the JA/JA–Ile-insensitive *coi1-16* plants have less lateral roots (Zhang and Turner 2008). But also a JA/JA–Ile-independent signaling seems to be involved, since 9-LOX products derived from LOX1 and LOX5 negatively regulate lateral root formation (Vellosillo et al. 2007).

Adventitious root formation is a multifactorial process with involvement of auxin, cytokinin, and JA/JA–Ile (Da Costa et al. 2013). Key player is auxin that acts as an inducer by regulating JA/JA–Ile homeostasis (Gutierrez et al. 2012). Auxin regulates ARF6 and ARF8 in a positive manner. Downstream of auxin, adventitious root formation is negatively regulated by JA/JA–Ile in a COI1- and MYC2-dependent manner. Consequently, *coi1-16, myc2, myc3, myc4*, and *jar1* mutant plants have more adventitious roots than the wild type (Gutierrez et al. 2012).

Gravitropism is a morphogenic response caused by auxin redistribution and intra- and intercellular communication. Besides the mechanistic framework of cross-talk in auxin and JA/JA–Ile signaling, gradients of auxin, JA/JA–Ile, and auxin responsiveness have been detected during gravitropic response. This supports the traditionally used Cholodny–Went hypothesis for explanation of asymmetric growth (Gutjahr et al. 2005).

Trichomes, preferentially glandular trichomes, are "factories" for production of secondary metabolites such as terpenoids, flavonoids, alkaloids, and defense proteins (Tian et al. 2012; Tissier 2012). Therefore, glandular trichomes are involved in resistance to insects as shown by the *odorless-2* tomato mutant (Kang et al. 2010). Identification of *jai1*, the tomato homologue of *AtCOI1,* clearly showed requirement for intact JA/JA–Ile-signaling in trichome formation (Li et al. 2004). Trichome density and JA/JA–Ile-inducible defense compounds such as monoterpenes,

sesquiterpenes, and PINs are involved in resistance to herbivores (Tian et al. 2012). Trichome initiation is dependent on TFs such as MYB75, GL3, and EGL3 which are targets of JAZ proteins (Qi et al. 2011) (cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones").<> Among trichome-specific enzymes involved in synthesis of secondary metabolites such as pyrethrins of *Pyrethrum* are two LOXs which convert α-LeA to 13-HPOT (Ramirez et al. 2013). The pyrethrins cinerolone, jasmolone, and pyrethrolone are assumed to be synthesized from the JA derivative 7-OH-JA (cf. section "Metabolism").

Tuber formation was assumed to be dependent on 12-OH-JA. In the late 1980s, 12-OH-JA was named tuberonic acid (TA) due to its tuber-inducing activity (reviewed by Wasternack and Hause 2002). Later on, involvement of StLOX1 in tuber formation (Kolomiets et al. 2001) and accumulation of JA and TA in stolons under low tuber-inducing temperature were shown (Nam et al. 2008). These data on TA, however, are only correlative. The effect could be indirect. Meanwhile, a conclusive scenario of tuber formation has been established. In this scenario, the potato orthologues of CONSTANS and FLOWERING LOCUS T are involved (Rodríguez-Falcón et al. 2006).The gene encoding the homeobox TF BEL5 is expressed in a phytochrome B-dependent manner, and its mRNA is transported under short-day conditions and at low temperature from leaves to the stolon tip via the phloem (Hannapel 2010; Lin et al. 2013). Finally, the *GA-20 oxidase1* promoter binds StBEL5 and another TF, POTH1, leading to increased GA levels (Banerjee et al. 2006; Lin et al. 2013). Interestingly, the phloem transport of *StBEL1* mRNA is accompanied with a phloem transport of mRNAs of Aux/IAA-encoding genes which leads to suppression of root growth (Hannapel 2013). Possibly, the role of TA is indirect by altering cell expansion.

Senescence: Senescence is a complex developmentally and environmentally regulated process. Nutrient availability, biotic and abiotic stress, and light/dark conditions influence senescence. Among senescence-related hormones, JA is known for a long time as a senescence-promoting factor (Ueda and Kato 1980). Aspects on senescence were reviewed recently (Guo and Gan 2012; Zhang and Zhou 2013). Transcript profiling in different stages of senescence led to a leaf senescence database (Buchanan-Wollaston et al. 2005; Liu et al. 2011) and identification of JA-linked TFs such as WRKY53 (Miao and Zentgraf 2007), WRKY54, and WRKY70 (Besseau et al. 2012) and TFs of the NAC family (Balazadeh et al. 2010). For the latter, e.g., ANAC019, ANAC055, and ANAC072, a regulatory network was shown recently indicating similarities and divergence among activities of TFs in stress responses (cf. section "Perception of JA-Ile and Cross-Talk to Other Hormones") and senescence downstream of MYC and MYB TFs (Hickman et al. 2013). The NAC TF ORE1 (ANAC092) is a positive and central regulator of senescence (Matallana-Ramirez et al. 2013). Other components of JA/JA–Ile-mediated senescence are (1) the COI1-dependent downregulation of RUBISCO activase (Shan et al. 2011), (2) the JA/JA–Ile-induced chlorophyll degradation (Tsuchiya et al. 1999), (3) the cross-talk to ET (Wang et al. 2013) or CK (van Doorn et al. 2013), and (4) the recruitment of JA/JA–Ile signaling in the absence of functional plastoglobule kinases accompanied with conditional de-greening (Lundquist et al. 2013).

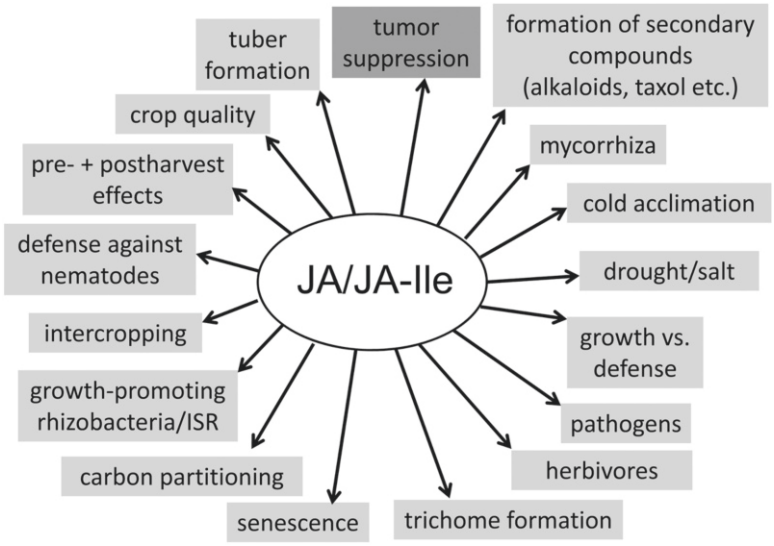

Fig. 6 Scheme on applied aspects of jasmonates in horticulture, pharmacy, and biotechnology. The accumulated knowledge on role of jasmonates in formation of secondary compounds; in defense reactions against pathogens, nematodes, or herbivores; in senescence, pre- and post-harvest, crop quality; or in arbuscular mycorrhiza led to their increased application (with permission)

Applied Aspects on Jasmonates

Upon two decades of JA research on JA-biosynthesis and JA-mediated signal transduction pathways in plant stress responses and development, an increasing interest is obvious to use this knowledge for horticultural applications. There are several examples summarized in Fig. 6, showing how JA/JA–Ile-mediated processes can be used in agriculture for improved plant growth, harvest, biotechnological production of secondary metabolites, or improvement of plant immunity. Applied aspects on jasmonates have been reviewed recently (Wasternack 2014). Therefore, only few examples will be briefly discussed here.

Freezing Tolerance: JA/JA–Ile is clearly a positive regulator of freezing tolerance (Hu et al. 2013b). Inhibition of JA/JA–Ile biosynthesis and signaling leads to hypersensitivity to freezing. The key players in cold stress, CBF1/DREB1, are JA/JA–Ile inducible, and ICE (INDUCER OF CBF EXPRESSION1) is a target of JAZ1 and JAZ4.

Defense Against Root Nematodes: Roots are attacked by root-knot and cyst nematodes which are endoparasites. These parasites use plant nutrients for their own lifestyle (Gheysen and Mitchum 2011). Worldwide there is about 5 % crop loss by root-knot nematodes of the genus Melogyne which attack about 200 mono- and

dicotyledonous species. Nematodes inject after invasion effector proteins into the host leading to a dramatic reprogramming of gene expression. Besides auxin, ET, and BR, JA is involved in systemically induced defense reactions against root nematodes (Nahar et al. 2013). Knowledge on participating signaling components will improve putative application. Here, simultaneously active shoot-feeding insects have to be taken into account. There is a compensatory plant growth response by herbivores which affects nematode invasion (Wondafrash et al. 2013).

Intercropping: Mixed growth of two or more crops, called intercropping, is of increasing interest due to obvious disadvantages of plant growth in monocultures. More than 28 million hectare in China is used already by intercropping. An interesting example is the maize/peanut intercropping which improves iron content of plants on calcareous soil (Xiong et al. 2013). In both plants, stress-related proteins are downregulated in a JA-dependent manner, initiated by interactions via the rhizosphere. A JA/JA–Ile-mediated advantage in intercropping systems is also given by volatile organic compounds (VOCs) which strongly interfere with insect interactions (Poveda and Kessler 2012).

A pesticide-free management of agroecosystems is envisaged by growing the right plants together. Maize plants growing together with legumes are much less attacked by the adult stem borer moth due to VOC emission, whereas grasses growing at the boarder of a maize field can attract gravid females away from maize plants (Hassanali et al. 2008). There are increasing examples, how plant–plant communications can be used for agricultural improvement. In the rhizosphere, root exudates attribute to communication, whereas in the atmosphere volatile compounds such as VOCs including JAMe are active.

Pre- and Post-harvest Effects and Crop Quality: Infection by *Botrytis* and green mold is the reason for the most frequently appearing loss in post-harvest (Rohwer and Erwin 2008). The role of JA/JA–Ile in infection by necrotrophic pathogens like *B. cinerea* is well understood. Consequently, application of JA and JA/JA–Ile-mediated volatile production are frequently used to establish resistance by pre- and post-harvest treatments. Crop quality can be improved by JAMe treatment. Here, (1) accumulation of "healthy" compounds such as resveratrol in case of *Vitis vinifera* leaves (Ahuja et al. 2012), (2) JA-induced accumulation of anthocyanins and antioxidant compounds in fruits and vegetables (Wang and Zheng 2005), or (3) JA/JA–Ile-induced GS formation in cruciferous vegetables (Grubb and Abel 2006) can be of interest. The latter aspect can be reached by JA treatment under field conditions without loss in post-harvest quality (Ku et al. 2013). Compounds of pharmaceutical interest such as alkaloids, taxol, or saponins are "produced" in plant cell cultures or via transgenic approaches due to their induction by JA/JA–Ile. During post-harvest of crops, herbivore resistance can be enhanced by using plant-circadian clock function for fitness (Goodspeed et al. 2013).

Jasmonates in Cancer Therapy: Jasmonates are unique for plants and do not occur in human tissues. There is, however, an anticancer activity of several JA compounds at least in several human cell lines (cf. review of Cohen and Flescher 2009).

JAs exert cytotoxic effects on cancer cells by direct cell death induction via interference with energy production, mitochondrial perturbation, and ROS production and/or via cell cycle arrest, redifferentiation, and anti-inflammatory properties (Raviv et al. 2013). Most strategies for use of JAs in anticancer therapy are based on improved chemical synthesis, increase in pharmacokinetic stability, and development of new JA compounds. There are, however, already natural sources of plants which are used for a long time for preparation of pharmaceutical drugs with anticancer activity. Among them are extracts of mistletoe (*Viscum album*). A putative explanation was found recently. Mistletoe plants have a JA content of about four orders of magnitude higher levels than most other plants, such as *A. thaliana*, tomato, or tobacco, even if these plants were wounded (Miersch and Wasternack, unpublished). Natural sources such as algae extracts or treatment with JAMe have been repeatedly described to exert anticancer activity in prostate cancer (Farooqi et al. 2012).

Soil Microbe Communities: There is a remarkable growth promotion of *Arabidopsis* by soil microbes which includes a facilitation of iron uptake, downregulation of genes involved in nitrogen uptake, redox signaling, and SA-mediated signaling, whereas genes involved in JA signaling, photosynthesis, and cell wall synthesis were upregulated (Carvalhais et al. 2013). There are about 10^{11} microbes with up to 30,000 prokaryotic species per gram roots in the rhizosphere near the roots (Berendsen et al. 2012). Among them are pathogenic, beneficial, and commensal microbes. Pathogen infection leads to damage by root growth inhibition caused by toxic compounds of bacterial origin. Colonization by beneficial microbes, however, can result in growth promotion or ISR. Soil-borne beneficial microbes such as *Pseudomonas* spp. *rhizobacteria* can establish protection against abiotic stress, may prime the plant immune system, and can change the root architecture (Zamioudis et al. 2013).

Simultaneously Applied Stresses: Most analyses of stress responses include single stress scenarios. In nature, however, several biotic and abiotic stresses occur simultaneously and/or subsequently. Consequently, for any application in agriculture, data collection has to be envisaged by simultaneously performed, multiple stresses. In an initial transcriptome-based comparison of single and double stresses, about 60 % of transcripts upon double stress could not be predicted by single stress data (Rasmussen et al. 2013). Another transcriptome data set on simultaneously performed biotic and abiotic stress showed regulation of specific genes, which are involved in several stress responses, but also an overriding property of abiotic stress on the response to biotic stress (Atkinson et al. 2013). Transcriptome and metabolome analyses of a multifactorial stress experiment including heat, drought, and virus infection revealed specific genes for single, double, and triple stress conditions including altered biotic stress responses by abiotic stress application (Prasch and Sonnewald 2013). This balance between abiotic and biotic stress responses was inversed in case of photoprotection versus defense. *Arabidopsis* mutants affected in key components of the chloroplast photoprotection system showed elevated oxylipin levels (JA/JA–Ile, OPDA) and increased defense against herbivores and pathogens (Demmig-Adams et al. 2013). Obviously, any balance between abiotic and biotic stresses is not optimal in plants and is of great impact on any agricultural application.

Conclusions

After two decades of JA research based on analytical, genetic, molecular, and cell biological approaches, principles in biosynthesis, perception, signaling, and action of JA/JA–Ile have been elucidated. Signaling modules and similarities to other hormones as well as the network of cross-talk among all of them are milestones in this new knowledge. Transcriptomic, proteomic, lipidomic, and metabolomic analyses led to a vast amount of data which will be extended on new conditions and will lead to system biology approaches. Complex analyses will be performed on:

1. JA/JA–Ile action in stress responses and development under natural conditions
2. Simultaneous and/or subsequent action of two or more stresses in relation to JA/JA–Ile signaling
3. JA/JA–Ile-dependent balance of growth and development
4. JA/JA–Ile-based communication of plants via the rhizosphere and the atmosphere
5. JA/JA–Ile-mediated plant productivity in terms of secondary and macromolecular compounds

These global questions will be underpinned by mechanistic studies in JA/JA–Ile-signaling leading to identification of:

1. New regulatory components around the well-established SCF^{COI1}-JAZ-co-receptor complex
2. Translational and posttranslational control mechanisms including protein phosphorylation and protein stability
3. Epigenetic regulation of biosynthesis and signaling of JA/JA–Ile
4. Stress-specific and developmentally specific regulators active in JA/JA–Ile signaling

It will be fascinating to see the concerted progress in plant hormone research including JA/JA–Ile.

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Strigolactones: Biosynthesis, Synthesis and Functions in Plant Growth and Stress Responses

Hinanit Koltai and Cristina Prandi

Abstract Strigolactones, terpenoid lactones derived from carotenoids, are plant hormones with various biological roles. They act in both shoots and roots to regulate several aspects of plant growth and architecture. They also affect plant communication in the rhizosphere. In this chapter, we will present the role of strigolactones as plant hormones and highlight the known modes of strigolactone signalling and transport and their crosstalk with other plant hormones. Also, we will review growing bodies of evidence that strigolactones contribute to plant response to nutrient and light conditions. Furthermore, the recent development in strigolactone synthetic chemistry and their future applications for the benefit of agriculture will be discussed.

Keywords Strigolactones • Shoot • Root • Lateral buds • Phosphate • Hormones • Ethylene • Cytokinin • Auxin • Root hairs • Primary root • Lateral root • Light

Introduction

Strigolactones (SLs) are now known to be plant hormones and to have diverse biological roles. As plant hormones, they were shown to regulate shoot development, acting to repress lateral bud outgrowth (Gomez-Roldan et al. 2008; Umehara et al. 2008). In the shoots, they promote shoot secondary growth (Agusti et al. 2011) and repress adventitious root formation (Rasmussen et al. 2012). In the roots, they

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regulate lateral root formation and induce root hair elongation (Kapulnik et al. 2011a; Ruyter-Spira et al. 2011). However, SLs are also involved with communication in the rhizosphere. They stimulate seed germination of parasitic plants including *Striga* and *Orobanche* (Cook et al. 1966; reviewed by Xie et al. 2010) but thought to play a very minor role, if any, in germination per se (Shen et al. 2012). SLs also regulate hyphal branching of the symbiotic arbuscular mycorrhizal fungi (AMF; reviewed by Koltai et al. 2012). SLs are produced mainly in roots as a family of substances by plants and are found in a wide variety of plant species, including dicots, monocots and primitive plants (e.g. Xie et al. 2010; Proust et al. 2011; Delaux et al. 2012; Liang et al. 2010; Koltai et al. 2010a, b).

Recent findings suggest that SLs are major players in optimising plant growth and development in response to environmental stimuli. This is evident from the early stages of plant evolution. SLs are produced in primitive plants, such as moss, liverworts and the charophyte green algae, stoneworts, but not other green algae (Proust et al. 2011; Delaux et al. 2012). In moss, they determine the patterns of growth and response between neighbours (Proust et al. 2011). In higher plants, SLs regulate both shoot and root architecture and may also affect fungal symbiosis to enhance nutrient uptake (discussed below). More and detailed information on the activities of SLs in parasitic plants and mycorrhizal fungi can be found in Koltai et al. (2012). This chapter will focus on the biosynthesis of SLs in plants and their roles in plant growth, development and environmental responses. We will highlight aspects of SL chemistry and introduce some future aspects as to the applications of SL-related biotechnological strategies for agriculture sustainability.

Strigolactone Biosynthesis

The elucidation of the biosynthetic pathway of SLs started from the identification of mutant plants in a range of species which displayed an undersized and bushy phenotype not due to any of other hormones that had been already known to influence shoot branching. Combinations of exogenous SL application and grafting experiments were instrumental to discriminate genetic determinants involved in biosynthesis rather than perception or downstream signalling of SLs. Studies with carotenoid biosynthesis inhibitors and mutants demonstrated that SLs are secondary metabolites derived from a carotenoid precursor (Matusova et al. 2005). These mutant sets include *more axillary growth* (*max*) in *Arabidopsis*, *ramosus* (*rms*) in pea (*Pisum sativum*), *dwarf* (*d*) or *high-tillering dwarf* (*htd*) in rice (*Oryza sativa*) and *decreased apical dominance* (*dad*) in petunia (*Petunia hybrida*) (Beveridge and Kyozuka 2010). Genetic, biochemical and molecular approaches clarified that SLs were the compounds whose biosynthesis or perception was defective in these mutants (Gomez-Roldan et al. 2008; Umehara et al. 2008). To date, more than 19 natural SLs have been characterised from various plant species, and all of them share a common four-cycle skeleton (A, B, C and D), with cycles A and B bearing various substituents and cycles C and D being lactone heterocycles connected by an

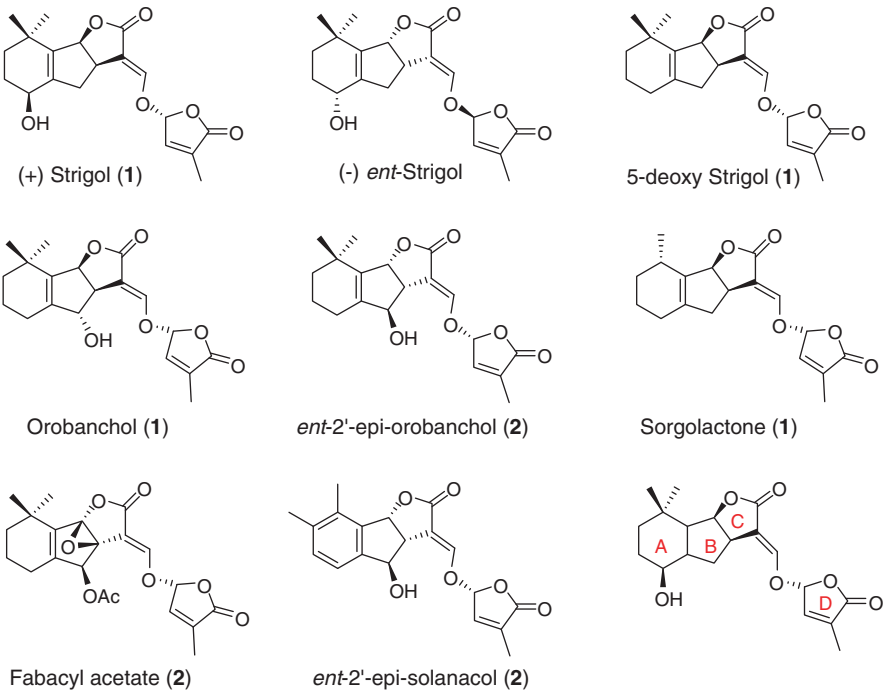

Fig. 1 Chemical structure of natural strigolactones. (**1**) Means same stereochemistry of strigol and (**2**) same stereochemistry as *ent*-2′-epi-orobanchol

enol-ether bond (Fig. 1). (+)-5-Deoxystrigol is thought to be the precursor of other identified SLs (Matusova et al. 2005). Plants produce a so small amount of SLs in the roots and lower part of shoots that they can only be analysed and quantified using the highly sensitive mass spectrometry approach (Xie et al. 2010); it is anticipated that many more SLs will be discovered with the development of better analytical protocols (Yoneyama et al. 2009; Zwanenburg et al. 2009). In spite of the advances of our knowledge, both biosynthesis and perception of SLs are still far from being completely elucidated; namely, only a few gene products crucial for biosynthesis have been identified (Fig. 2). Strigolactone production in higher plant species tested to date originates in the plastid from carotenoid molecules (Booker et al. 2004; Matusova et al. 2005). Three plastid-localised proteins are involved in the first stages of strigolactone synthesis (Fig. 2). One is a carotenoid isomerase, DWARF27 (D27), which has been characterised so far in rice and *Arabidopsis* and, as demonstrated by in vitro studies, is able to convert all-*trans*-β-carotene into 9′-*cis*-β-carotene (Liu et al. 2009; Waters et al. 2012a, b). The *cis*-configure substrate is then oxidatively tailored by two double bond-specific cleavage enzymes (carotenoid cleavage dioxygenases, CCDs) (Alder et al. 2012; Kohlen et al. 2012). Therefore, first, the 9′, 10′ bond of β-carotene is attacked by CCD7 yielding β-ionone (C_{13}) and 9′-*cis*-β-apo-carotenal, the 9′-*cis*-configured aldehyde (C_{27}).

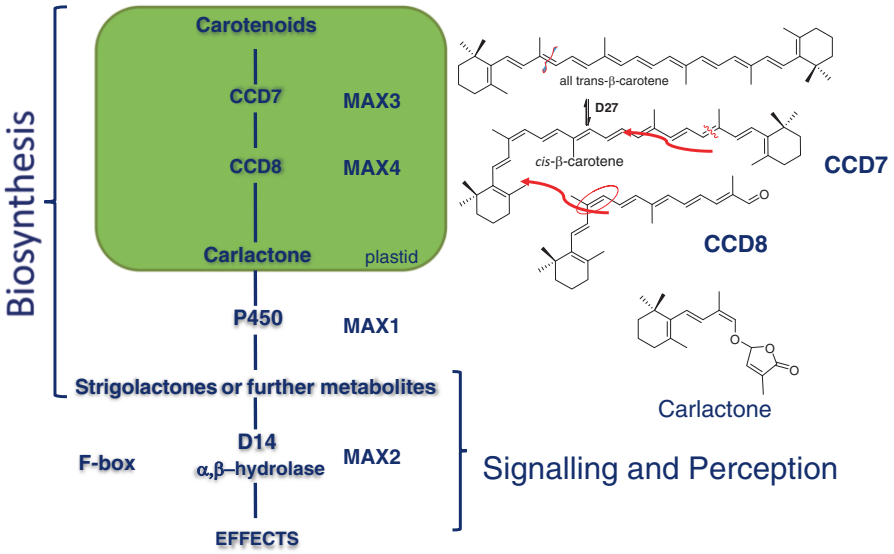

Fig. 2 *Left*: SLs biosynthetic pathway. *Right*: Chemical structures of some intermediates

The latter compound can be then further cleaved and cyclised by CCD8 into a bioactive SL precursor named carlactone (CL) (Booker et al. 2004; Schwartz et al. 2004; Alder et al. 2012). Orthologous CCD enzymes have been found in several and diverse higher plants (reviewed by Dun et al. 2009a, b; Beveridge and Kyozuka 2010): MAX3 and MAX4 in *A. thaliana* (Sorefan et al. 2003; Booker et al. 2004), RMS5 and RMS1 in *P. sativum* (Morris et al. 2001; Sorefan et al. 2003), DAD3 and DAD1 in *P. hybrida* (Snowden et al. 2005; Drummond et al. 2009) and D17/HTD1 and D10 in *O. sativa* (Arite et al. 2007; Zhang et al. 2010). Moss, which can produce SLs, contains homologues of these three genes and displays mutant phenotypes that connect SL biosynthesis to colony growth (Proust et al. 2011). Only certain of these genes occur in other basal plants (Drummond et al. 2009) and algae (Delaux et al. 2012). Carlactone possesses the D ring connected to a six-membered cycle through a dienyl enol ether; with respect to the SLs structure, the B and C ring are missing (Fig. 2) (Alder et al. 2012). Based on grafting studies with *d27*, *ccd7* and *ccd8* mutants, the precursors of CL presumably do not move out of the plastid (Booker et al. 2005; Morris et al. 2005; Smith and Waters 2012). Carlactone has not yet been detected in plants and it is not known whether it could move out of the plastid or cell. In the current view, however, further but so far uncharacterised enzymatic steps are required to yield SL molecules from CL. For example, MAX1, a class-III cytochrome P450 monooxygenase, is a candidate to such a role. In fact, it is required for SL biosynthesis and assumed to convert CL into 5-deoxystrigol, a general precursor for various SLs (Fig. 1), but its biochemical action still needs to be resolved experimentally (Booker et al. 2005; Alder et al. 2012). Very recently, it was reported that synthetic CL represses *Arabidopsis* shoot branching and influences leaf morphogenesis via a mechanism that is dependent on the

cytochrome P450 MAX1. While MORE AXILLARY GROWTH 2 (MAX2) is also necessary for normal seedling development, Dwarf14 (D14) (discussed below) and the known SL-biosynthesis genes are not (Fig. 2), raising the question of whether endogenous, canonical SLs derived from CL have a role in seedling morphogenesis. The authors demonstrated that while the commonly employed synthetic SL GR24 [(3aR*,8bS*,E)-3-(((R*)-4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-3,3a,4,8b-tetrahydro-2H-indeno[1,2-b]furan-2-one (Fig. 3)] acts non-specifically through both D14 and KARRIKIN INSENSITIVE 2 (KAI2; D14 paralogue), CL is a specific effector of SL signalling that acts through MAX1 and D14 (Scaffidi et al. 2013). Whereas GR24 appears to lack specificity, *Z*-carlactone (via MAX1-dependent conversion to SLs) provides specificity for D14 signalling.

Considering the substantial structural differences between carotenoids and the diverse 'mature' SLs, and the compartmentalisation issues specific for the hydrophobic precursor and the hydrophilic final products, it is hard to imagine that the four enzymes mentioned above are the only ones involved. Indeed, other genes were recently identified in SL biosynthesis, among which are two encoding photosynthesis-related enzymes [SlORT1 in tomato (*Solanum lycopersicum*) and AtPPD5 in *Arabidopsis*] (Koltai et al. 2010a, b; Roose et al. 2010).

Putative regulators of the SL-biosynthesis pathways in rice and *Medicago* were suggested to be the GRAS (GAI, RGA, SCARECROW)-type transcription factors NODULATION SIGNALING PATHWAY 1 (NSP1) and NSP2 (Liu et al. 2011). Once produced, SLs or their precursor(s) is transported upwardly to the shoot or exported into the rhizosphere by the ABC (ATP-binding cassette) transporter PLEIOTROPIC DRUG RESISTANCE PROTEIN (PDR1) identified in petunia (Kretzschmar et al. 2012).

Strigolactone Function in Plant Growth

Role of Strigolactones in Shoot Development

The first indication of SLs activity as plant hormones came from examination of hyperbranching mutants. This class of mutants had altered levels of a graft-transmissible signal that suppressed shoot branching, and since their phenotype could not be attributed to altered levels of one of the established plant hormones, a novel signal that was associated with this phenotype was suggested (Beveridge et al. 1997). Later on, this signal was identified to be SLs and to act as long-distance branching factors that suppress growth of preformed axillary buds dependent on the F-box protein MAX2 signalling (Gomez-Roldan et al. 2008; Umehara et al. 2008) by promoting axillary bud 'dormancy'. However, this dormancy is an active state of metabolically active buds and it is not clear whether in SL mutant plants axillary buds ever enter a phase of dormancy or whether they are always released to grow, although even in SL mutant plants usually some axillary buds do not grow out and hence other dormancy mechanisms exist (Koltai and Beveridge 2013).

SLs were shown to act on bud outgrowth both locally and at a distance. SLs were shown to inhibit growing pea buds to an extent and to act directly in the bud itself (Dun et al. 2012, 2013) and were suggested to be an auxin-promoted secondary messenger that moves up into the buds to repress their outgrowth (Brewer et al. 2009; Ferguson and Beveridge 2009; reviewed by Dun et al. 2009a). Alternatively, or additionally, SLs act to mediate reduction in the capacity of the main shoot for polar auxin transport from the apical meristem. One of the proposed mechanisms for shoot branching control is that the establishment of auxin export from the bud is crucial for the bud to be activated. Accordingly, this SL-mediated reduction in auxin transport is suggested to lead to inhibition of polar auxin transport from the buds, thereby restraining their outgrowth (e.g. Bennett et al. 2006; Mouchel and Leyser 2007; Ongaro and Leyser 2008; Crawford et al. 2010; Domagalska and Leyser 2011). Strong lines of evidence to support the involvement of polar auxin transport level in bud activation come from *Arabidopsis* SL mutants, which show both hyperbranching and increased polar auxin transport (Domagalska and Leyser 2011).

Role of Strigolactones in Shoot Secondary Growth

The secondary growth of the shoots consists of lateral growth of the shoot axes, leading to enhanced girth. It is caused by activity of the vascular cambium, a stem cell-like tissue, resulting with production of secondary vascular tissues and wood production. The secondary growth from the vascular cambium is regulated through auxin (and additional hormones) (Miyashima et al. 2012). Based on studies of cell-specific activation of SL signalling, SLs were found to promote secondary shoot growth by positively regulating cambial activity by a local induction of the cambium-specific stem cell niche and of vascular tissue formation. This was demonstrated in *Arabidopsis*, pea and Eucalyptus and requires the same MAX2-dependent signalling as for shoot branching inhibition (Agusti et al. 2011). This effect of SLs was suggested to be local and independent from their effect on shoot branching (Agusti et al. 2011). Moreover, expression in *max2-1* mutants of MAX2 under the control of the (pro)cambium-specific *WUSCHEL-RELATED HOMEOBOX 4* (*WOX4*) promoter background was sufficient to confer secondary growth at wild-type (WT)-like levels, suggesting a local, cambium-specific, MAX2-dependent activity of SLs (Agusti et al. 2011). Therefore, in this case SL signalling may regulate the process of secondary growth in the cambium in a cell-autonomous manner, as a secondary messenger of auxin (Agusti et al. 2011).

Role of Strigolactones in Root Development

SLs affect different aspects of root development. In *Arabidopsis* they regulate early lateral root formation following seed germination, negatively under conditions of sufficient phosphate nutrition and positively once phosphate in deficient conditions

(Kapulnik et al. 2011a; Ruyter-Spira et al. 2011). In accordance, mutants of SL response or biosynthesis had more lateral roots than the WT (Kapulnik et al. 2011a; Ruyter-Spira et al. 2011), whereas treatment of seedlings with GR24 repressed lateral root formation. The effect of SLs on lateral root development was in the WT and SL-synthesis mutants, but not in the SL-response mutant, suggesting that the negative effect of SLs on lateral roots formation is MAX2 dependent (Kapulnik et al. 2011a; Ruyter-Spira et al. 2011). Treatment of seedlings with GR24 led to a decrease in PIN-FORMED (PIN1)-GFP intensity in lateral root primordia, suggesting an involvement for PIN1 in the GR24-mediated reduction of lateral root development. Upon exogenous supplementation of both auxin and GR24, lateral root development was increased and no reduction in PIN1-GFP intensity was observed, suggesting that SLs modulate auxin flux in roots, and as a result alters the auxin optima necessary for lateral root formation (Ruyter-Spira et al. 2011). Similarly, cytokinins (CKs) act to negatively regulate lateral root formation by interference with auxin transport in lateral root primordia (Bishopp et al. 2011). Hence, strigolactones and CKs may be considered to act similarly in the case of lateral roots, since both influence auxin distribution.

Exogenous supplementation of SLs also led to root hair elongation in the WT and SL-deficient mutants, but not in the SL-response mutant, suggesting that the effect of SL on root hair elongation is mediated via MAX2 as well (Kapulnik et al. 2011a). The hormonal balance in the epidermal cell layer was suggested to determine root hair tip elongation. Analysis of the SL-response mutant suggested that auxin signalling was required, at least in part, for the positive effect of SLs on root hair elongation. However, SL signalling is not necessary for the root hair elongation induced by auxin (Kapulnik et al. 2011b). Moreover, ethylene was also shown to be involved in the root hair response to SLs. This is because the ethylene-signalling mutants *ethylene-response gene* (*etr*) and *ethylene insensitive* (*ein*) had significantly reduced response to GR24 (Kapulnik et al. 2011b). Hence, SLs exert their effect on root hair growth at least partially through the auxin and ethylene pathways (Koltai 2011).

Under conditions of carbohydrate limitation that usually leads to a reduction in primary root length (Jain et al. 2007), GR24 led to elongation of the primary root and to an increase in meristem cell number in a MAX2-dependent manner. Accordingly, under these conditions, the SL-deficient and SL-response mutants had a shorter primary root and less primary meristem cell number than those of the WT plants (Ruyter-Spira et al. 2011). GR24 supplementation also affected root directional growth in both tomato and *Arabidopsis* (Koltai et al. 2010a, b; Ruyter-Spira et al. 2011). Ruyter-Spira et al. (2011) suggested this effect to be a result of distorted expression of the PIN auxin efflux carriers. However, SLs seem not to be associated with the gravitropic response of roots (Shinohara et al. 2013).

Role of Strigolactones in Adventitious Root Formation

The process of adventitious root formation from stems was found to be negatively regulated by SLs in *Arabidopsis* and pea. In SL-deficient and SL-response mutants of both species, enhanced adventitious rooting was found. Consistently, SL treatments

reduced adventitious rooting in the SL-biosynthesis mutant and WT, but not in the SL-response mutant (Rasmussen et al. 2012). SLs and CKs were suggested to act independently, but a partial dependency between SLs and auxin activity was found in this process (Rasmussen et al. 2012). As in the case of lateral root formation, auxin plays a pivotal role in adventitious root development (Li et al. 2009). Accordingly, tomato transgenic plants with reduced *SlCCD8* expression, and thereby reduced SL levels, had excessive adventitious root development (Kohlen et al. 2012), further supporting a negative role for SLs in this process.

Strigolactone Signalling and Transport

Strigolactones, similar to other plant hormones, are sensed by the plants via a specific perception system. Two of the components of SL signalling are likely to be the α-/β-fold hydrolase, D14 (Arite et al. 2009; Hamiaux et al. 2012; Waters et al. 2012a, b) and the F-box protein, MAX2/D3/RMS4 (Stirnberg et al. 2002; Ishikawa et al. 2005; Johnson et al. 2006) (Fig. 2). Mutants in these genes are hyperbranching and show a reduced response to SLs (reviewed by, e.g. Smith and Waters 2012). Based on in vitro experiments, a physical interaction was suggested between these two components, since Hamiaux et al. (2012) showed that D14 from petunia (DAD2) interacts with petunia MAX2 in a yeast two-hybrid assay, but only in the presence of GR24, the synthetic and biologically active SL (e.g. Umehara et al. 2008). It was suggested that under these conditions, DAD2 is able to hydrolyze GR24 into non-bioactive products (Hamiaux et al. 2012). Based on putative protein functions and similarly to other hormonal perception systems (e.g. gibberellin signalling; Ueguchi-Tanaka and Matsuoka 2010), it was suggested that the MAX2-D14 duplex function as an SCF complex that tags transcriptional regulators for degradation (Hamiaux et al. 2012). However, their protein targets are yet to be identified (Smith and Waters 2012).

As elaborated below, SLs are involved in regulation of shoot branching. As such, they would need to integrate into the hormonal regulatory network that controls axillary bud outgrowth. Models for controlling bud outgrowth involve downward-moving auxin that come from the shoot tip (apex). This auxin flow provides the below shoot tissue with information about the growth status of the apex and allows for decision making about lateral growth (Leyser 2009). However, other hormone and non-hormone signals are clearly involved (Morris et al. 2005), including SLs (Gomez-Roldan et al. 2008; Umehara et al. 2008). CKs were also found to be regulators of shoot bud outgrowth (Sachs and Thimann 1967), however antagonistically from SLs (Brewer et al. 2009; Dun et al. 2012). Both hormones are regulated conversely by auxin (reviewed by Dun et al. 2009a). In garden pea, both SLs and CKs act to repress or induce the bud-specific target gene *BRANCHED1* (*BRC1*) that encodes a transcription factor repressing bud outgrowth (Aguilar-Martínez et al. 2007; Braun et al. 2012; Dun et al. 2012). In other species, related genes also repress

bud outgrowth, but respond to SLs or CKs in a species-specific manner (reviewed by Muller and Leyser 2011; Brewer et al. 2013).

Studies showed that SLs are produced in shoots, although to a lesser extent than in roots. Evidently, the pea *rms1* (*CCD8*) is expressed in many other plant tissues in addition to roots, including mainly epicotyl and internode tissues (Foo et al. 2005; Dun et al. 2009b). Also, the shoot is actually better than the root at inhibiting branching since branching inhibition is greater in WT shoots grafted to SL-deficient roots, rather than the reciprocal combination (Foo et al. 2001; Morris et al. 2005). Hence, SLs could act locally to directly repress bud outgrowth (Brewer et al. 2009; detailed below). This fact as well as the effect of SLs on roots, their main site of production (as described below), suggests that SLs might act in the same cells in which they are produced, or very nearby at least in some cases of SL activity.

A higher resolution as to the site of SL signalling in roots was obtained by expressing *MAX2* under root tissue-specific promoters in *max2* mutant background (Koren et al. 2013). *MAX2* expression under the *SCARECROW* (*SCR*) promoter, which is expressed mainly in the root endodermis and quiescence centre (Perilli et al. 2012 and references therein), was found to be sufficient to confer sensitivity to GR24 in roots (Koren et al. 2013). Accordingly, the root endodermis has been suggested to play an important regulatory role in lateral root initiation via regulation of PIN3 auxin transporter (Marhavy et al. 2012). Accordingly, several indications suggested that SLs affect auxin efflux in root tips. One came from the fact that only 2,4-D (2,4-dichlorophenoxyacetic acid, a synthetic auxin that is not secreted by efflux carriers) led to reversion of the GR24 effect on roots (Koltai et al. 2010a, b). The second indication was the decrease in PIN1-GFP intensity in lateral root primordia that was detected upon GR24 application, suggesting that PIN1 is involved in the SL-mediated reduction of lateral root development (Ruyter-Spira et al. 2011). Third indication was SLs' positive effect on meristem size (Ruyter-Spira et al. 2011; Koren et al. 2013). The interplay between auxin and CKs in the root tip carefully balances cell differentiation and cell division in the meristem to determine root meristem size (Perilli et al. 2012). The fact that endodermal SL signalling is sufficient in regulating the proliferation of adjacent meristematic cells (Koren et al. 2013) may also result from SL signalling's ability to regulate auxin flux in the root tip (Koltai and Kapulnik 2013).

Also, in the shoots considerable amount of data suggest that SLs regulate auxin flux. SLs act to dampen auxin transport (e.g. Domagalska and Leyser 2011). Consistent with this observation, in SL-deficient or SL-response mutants, PIN protein levels and the amount of polar auxin stream were increased compared with WT plants. In accordance, in both rice and *Arabidopsis* SL mutants, restoring polar auxin transport to WT level rescued the branching phenotype, suggesting that the branching phenotypes of SL mutants are linked to their auxin transport in the stem (Domagalska and Leyser 2011). Indeed, SL signalling was found to trigger PIN1 depletion from the plasma membrane of xylem parenchyma cells in the stem, further supporting the hypothesis that SLs regulate shoot branching by modulating the competition between shoot apices for a common auxin transport path to the roots (Shinohara et al. 2013).

However, since SLs are produced mainly in roots, for execution of their action on axillary shoot buds, they would need to be transported upwards to shoots. Indeed, grafting studies have indicated that SLs, their metabolites or other unknown secondary messengers move in the root-to-shoot direction to inhibit shoot bud outgrowth (reviewed by Dun et al. 2009a). Moreover, the presence of the SL orobanchol in the xylem sap of *Arabidopsis* was indicated (Kohlen et al. 2011). These findings suggest that (i) orobanchol is indeed produced in the root and move towards the shoot through vasculature and (ii) SLs themselves as the active compounds may be actively transported to their target organs (e.g. in or near shoot buds) for their activity, rather than their hydrolysis or downstream products.

According to the suggestion of active transport of SLs, an SL putative transporter was identified. This came from a study of an ABC transporter in petunia (Kretzschmar et al. 2012), the PDR1, which was suggested to function as a cellular SL exporter. The *pdr1* mutant had enhanced branching phenotype and reduced mycorrhizal symbiosis, whereas overexpression of the petunia *PDR1* in *Arabidopsis* resulted with increased tolerance to high concentrations of GR24. *PDR1* was shown to be expressed in root tissues, extensively in individual subepidermal cells of the lateral roots. It was also expressed in the stem, restricted mainly to the vasculature and nodal tissues adjacent to leaf axils, but absent from dormant buds, consistent with PDR1's function as an SL transporter. However, it seems that further work is required to verify whether PDR1 is involved in SL import into axillary buds. At the subcellular level, PDR1 was allocated to the plasma membrane, again consistent with its suggested role in secretion. It was suggested that PDR1 may confer cellular mobility between cells that might be required to deliver SLs to their site of action (Kretzschmar et al. 2012).

The level of SLs should be carefully regulated. This might take place as part of a careful balance between different plant hormones. Three groups of molecules are suggested to regulate SL levels by feedback regulation. One is auxin that positively regulates SL levels in roots and stems by inducing both *MAX3* (*CCD7*) and *MAX4* (*CCD8*) transcription in pea and *Arabidopsis*. Auxin depletion treatments reduced SL-biosynthesis gene expression in pea (for *RMS5* and *RMS1*; Foo et al. 2005; Johnson et al. 2006) and *Arabidopsis* (Hayward et al. 2009). Also, both transcripts are upregulated in SL-response and SL-synthesis mutants, consistent with the increased auxin flow found in these mutants (Bennett et al. 2006). Moreover, it was shown that this feedback regulation of auxin on SL biosynthesis involves auxin signalling (Hayward et al. 2009). Apically derived auxin was shown to induce SL synthesis in the root via the AUXIN RESISTANT/TRANSPORT INHIBITOR RESPONSE1 (AXR1/TIR1) signal-transduction pathway, involving stability of the AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) protein, IAA12 (reviewed by Beveridge and Kyozuka 2010).

A second group of molecules that regulate SLs levels are SLs themselves. SL mutants showed higher levels of SL-biosynthesis gene expression and/or SL content (e.g. Foo et al. 2005; Dun et al. 2009b; Hayward et al. 2009; Umehara et al. 2010). In rice roots, D10 (MAX4 homologue) expression was upregulated in SL pathway mutants, whereas SL application led to restoration of its expression to WT levels in

the biosynthesis mutant but not the response mutant (Umehara et al. 2008). It was shown in several other plant species that GR24 treatments reduced expression of SL-biosynthesis genes (Mashiguchi et al. 2009; Dun et al. 2012).

However, for a systemic feedback regulation of SL production, another signal that moves basipetally from shoots to roots was suggested. A yet unidentified component, RMS2, was suggested to be essential for long-distance feedback regulation of CK export from roots in pea (Foo et al. 2001, 2005, 2007). Unlike other SL-biosynthesis and SL-response mutants in *Arabidopsis* and pea, the hyperbranching mutant *rms2* does not have reduced levels of the major CKs in xylem sap in comparison to WT plants (Foo et al. 2005, 2007). It was suggested that RMS2 is essential for long-distance feedback regulation of CK export from roots in addition to regulating expression of SL-biosynthesis genes (Foo et al. 2001, 2005, 2007).

Strigolactone Function in Stress Responses

Nutrient

As stated above, SL pathways show high conservation across the plant kingdom. This conservation suggests that SLs play a pivotal role in plant development. Indeed, it was found that when the plant encounters certain suboptimal conditions, such as reduced nutrient availability, SL levels rise in order to optimise and adapt the plant's growth strategy to fit the conditions (Umehara et al. 2008; Kohlen et al. 2011).

A suboptimal plant growth condition that was the most extensively studied in relation to SLs is phosphate deprivation. One of the essential macronutrients required by plants for growth and development is phosphorus (P). Plants acquire P from the soil, mostly in its inorganic phosphate (Pi) form; Pi levels vary considerably in the soil and are limiting factors for development in many habitats (Bieleski 1973; Maathuis 2009). Under low phosphate conditions, SL levels increase in red clover. Phosphate deprivation may exceed SL production as much as 100,000-fold. Nitrate deficiency was also shown to have a similar effect and to increase SL exudation. However, nitrate deficiency may affect Pi levels in the shoot and thereby SL exudation, and a correlation was found between shoot Pi levels and SL exudation across plant species (Yoneyama et al. 2007a, b, 2012).

The increase in SL levels may lead to several outcomes. The first one is a positive effect on the hyphae branching of AMF (Akiyama et al. 2005). Increased SL production and exudation are likely to encourage mycorrhizal symbiosis (Yoneyama et al. 2007a, b) that promotes plants to acquire Pi from the soil (Smith and Read 2008). The second outcome relates to architectural changes of the plants, which help them adapt to the changing growth conditions. These include changes in the shoots, as shoot branching is inhibited (e.g. Umehara et al. 2008; Kohlen et al. 2011). As for the roots, under limited nutritional conditions lateral roots are promoted for

increased foraging of subsurface soil, but then inhibited after extended deprivation (Nacry et al. 2005). Root hair length and density are increased to expand root surface area and promote nutrient acquisition (Bates and Lynch 2000; Peret et al. 2011; Gilroy and Jones 2000), while the primary root elongation is inhibited (Sánchez-Calderón et al. 2005).

In both *Arabidopsis* and rice, the SL pathway was shown to be important for the shoot response to low Pi conditions. In *Arabidopsis*, in correlation with the changes in shoot architecture, SL (orobanchol) was detected in xylem sap and was enhanced under Pi deficiency (Kohlen et al. 2011). In rice, under these conditions, tiller bud outgrowth was inhibited and root SL (2′-epi-5-deoxystrigol) levels were increased.

As described above, SLs positively regulate root hair elongation and negatively lateral root formation (Kapulnik et al. 2011a), suggesting that they regulate at least some of the root architectural features which are associated with adaptation to Pi conditions. Also, under low Pi conditions elevated levels of SLs in plants repress shoot branching (Umehara et al. 2010; Kohlen et al. 2011), increase lateral root formation (Ruyter-Spira et al. 2011) and promote root hair density (Mayzlish Gati et al. 2012). However, results from low nutrient conditions may depend on the species and exact treatment. For example, the primary root growth is inhibited in some *Arabidopsis* ecotypes but not others (Chevalier et al. 2003) and is promoted in rice under Pi deprivation (Peret et al. 2011). Moreover, SLs are essential for the plant ability to sense or respond to low Pi conditions. Mutants defective in SL biosynthesis or response are less able to respond to low Pi in both roots and shoots (e.g. Umehara et al. 2008; Kohlen et al. 2011; Mayzlish Gati et al. 2012). Conceivably, this lack of response to stress in these mutants would greatly suppress their competition and survival in challenging environments and would suggest an important role for SLs in plant adaptation to stress, even in species that do not undergo AM fungi symbiosis, such as *Arabidopsis*.

Plants may respond to nutrients as a result of interplay between several plant hormones, including auxin, CKs and SLs. Auxin is required for the low Pi response in roots (reviewed by López-Bucio et al. 2003; Chiou and Lin 2011), and increase in auxin sensitivity was detected under reduced Pi availability, resulting from induction of the auxin receptor *TIR1* expression (Lopez-Bucio et al. 2002; Perez-Torres et al. 2008). In accordance, the SL-response mutant, under the conditions of Pi deprivation, displayed a reduction, rather than induction of *TIR1* (Mayzlish Gati et al. 2012). Also, exogenous supplementation of auxin to SL-insensitive and SL-biosynthesis mutant roots resulted in complementation of the mutants' phenotypes to that of the WT (Mayzlish Gati et al. 2012). Cytokinin levels are decreased upon nutrient deficiency (Ei-D et al. 1979) and CK addition can counteract the root response to low Pi (Martín et al. 2000). Also, under optimal Pi conditions, ethylene is one of the modulators of the root hair response to SLs (Kapulnik et al. 2011b). Therefore, SLs, by interacting with other plant hormones, may be an important link in the complex interplay among hormones that confer plant response to stress conditions, particularly nutrient availability (Koltai and Kapulnik 2013).

Light

Another important environmental factor that affects SL levels or signalling is light. SLs induce expression of light-harvesting components (Mayzlish-Gati et al. 2010) and mimic light-adapted seedling growth (Tsuchiya et al. 2010). Also, WT plants display elongated leaves and a tall and slender main stem, whereas some of the SL-response and SL-synthesis mutants in *Arabidopsis* display rounded leaves and short stature (Stirnberg et al. 2002). The SL-response *max2* mutant is insensitive to some light-related responses and displays smaller cotyledons, elongated hypocotyls and reduced expression of light responsive genes, such as *ELONGATED HYPOCOTYL 5* (Stirnberg et al. 2002; Shen et al. 2007; Tsuchiya et al. 2010; Nelson et al. 2011).

Light response is particularly relevant to shading responses. Daylight consists of roughly equal proportions of red (R) and far-red (FR) light, but within vegetation red light absorption is taking place by photosynthetic pigments, and as a result the ratio R:FR is lowered. This light quality change is perceived through phytochromes and deactivates PHYTOCHROME B (PHYB) and is associated with the shade avoidance response that includes rapid elongation of stems and leaves, apical dominance and an upward reorientation of leaves (leaf hyponasty) (Ruberti et al. 2012). Under condition of high R:FR ratio, the *Arabidopsis phyB* mutant grows as a tall slender plant with reduced branching (Finlayson et al. 2010). However, under high R:FR light double mutants of *phyB* and SL response or SL synthesis in *Arabidopsis* show high branch numbers similar to the SL mutants, repressing the *phyB* phenotype (Finlayson et al. 2010). Thus, the SL pathway may act downstream of the *PHYB*-dependent response and SLs are required for response to the changes in R:FR ratio. Potentially SL biosynthesis may be repressed by PHYB under high R:FR and released from PHYB repression under low R:FR light conditions. In accordance, since auxin production is increased in shaded plants (Tao et al. 2008), and auxin positively regulates SL biosynthesis, it might be expected that low R:FR ratio will increase SL production and thus promote shade avoidance phenotypes. Thus, SLs may act as regulators of optimisation of growth under conditions of changed light.

Strigolactone Chemistry

Natural SLs

The first weed germination stimulant was isolated in 1966 from root exudates of cotton; later on in 1973 the structure of strigol (Fig. 1) was elucidated and the absolute stereochemistry definitively established by X-ray analysis 20 years later (Zwanenburg and Pospisil 2013). Strigol is the major *Striga* germination stimulant produced by maize and proso millet. The collective name ‘strigolactones’ was then proposed for this class of molecules. Sorgolactone (Fig. 1) was isolated in 1992

from sorghum roots and orobanchol from red clover; the structure of these three SLs has been confirmed by total synthesis (Zwanenburg et al. 2009). The elucidation of the molecular structures is sometimes hampered by the minute amounts of sample; in most cases a definitive confirmation of the structure came from total synthesis. Up to now 19 naturally occurring SLs (a selection of which is reported in Fig. 1) have been isolated and identified, but it can be inferred that new ones will be detected as far as the methodological and technological methods of purification become more sensible to small amount of compounds. The structural core of SLs is a tricyclic lactone (ABC rings, Fig. 1), with different substituents on AB rings and connected to a fourth butenolide ring (D ring) through an enol-ether bridge. The bioactiphore involves the CD part of the molecule (Zwanenburg et al. 2009). A full understanding of the importance of stereochemistry in bioactivity has been possible with the total synthesis of all its eight stereoisomers of strigol and the control of their activity on parasitic seeds (Reizelman et al. 2000). SLs contain in fact several stereogenic centres and can in principle exist as mixture of stereoisomers; as it frequently happens in natural compounds, the bioactivity of the different isomers is dramatically different. According to the CIP (Cahn, Ingold and Prelog) rules in the IUPAC system (International Pure and Applied Chemistry), each stereocentre can be described as R or S indicating the sense of chirality. The random combinations of R or S stereochemistry for three stereocentres give rise to a maximum number of eight stereoisomers. Natural biosynthetic processes are very selective. There is no need to produce several different stereoisomers when only one is sufficient for bioactivity (natural cholesterol is one out of 256 possible stereoisomers). Frequently, in case of natural compounds a notation specifying the stereochemical relationship with a parent structure is preferred to the indication of the absolute configuration (R,S system). To this purpose, the prefix *ent-* stays for enantiomer and *epi-* for epimer, meaning the opposite configuration only at one stereocentre. As an example, natural (+)-strigol is notably more active than its enantiomer *ent*-strigol (Fig. 1). The absolute configuration of the BCD moiety in naturally occurring (+)-sorgolactone, (+)-deoxystrigol and (+)-sorgomol is the same as in the parent natural (+)-strigol. Recently, the structure of the SLs in red clover exudates has been reinvestigated, fully elucidated and identified as *ent*-2′-*epi*-orobanchol (Ueno et al. 2011).

Stereochemistry

As new natural SLs are isolated and identified, it is evident that they can be grouped into two families. In the first one, the absolute configuration of the BCD part is the same as parent (+)-strigol (Fig. 1); many naturally occurring SLs belong to this family, namely, (+)-sorgolactone, (+)-sorgomol and (+)-5-deoxystrigol. In the second family of natural SLs, the stereochemistry of the BCD part of the molecule is the same as in the natural (−)-orobanchol (*ent*-2′-*epi*-orobanchol, Fig. 1). This latter absolute stereochemistry of the BCD rings was also found in fabyl acetate, *ent*-2′-*epi*-orabanchyl acetate and *ent*-2′-*epi*-solanacol as well (Zwanenburg and Pospisil 2013). The difference between the two families lies in the stereochemistry of the BC

junction, whereas the stereochemistry at C-2′ remains the same as strigol. Only three natural SLs do not fit in the proposed classification: the 2′-epi-orobanchol, 7-oxo-orobanchol and 7-hydroxy-orobanchyl acetate. It is reasonable to presume that the absolute configuration of these three molecules should be reconsidered in light of the recent insights.

Analogues

SLs are produced in very small amounts (pg-scale/plant/day); consequently, their isolation from root exudates sometimes cannot secure the structures, which will have to be confirmed by total synthesis. Because of their scarcity, natural SLs cannot be used for bioactivity experiments either, in which higher quantities of product are required. In this sense, chemical synthesis of structural analogues, i.e. molecules with simpler structures but retaining most of the activity, is a valuable tool to deepen the structure–activity relationship (SAR) knowledge on one side and to develop new active compounds suitable for practical applications on the other. Extensive SAR studies led to a better understanding of the molecular mechanism at the base of the perception as well as of the minimum structural requirements for activity. Among the synthetic SL analogues, GR24 (Fig. 3) was initially developed as highly active germination stimulant with increased stability compared to natural SLs. In addition, GR24 can be synthesised on a multi-gram scale and is worldwide used as a standard compound in most biological assays. Due to its large use in different biological assays, stereochemistry of GR24 deserves to be discussed more in details. With its three stereocentres, GR24 could in principle exist as eight stereoisomers that are reduced to four as a consequence of the *cis* junction between rings B and C. The bioactivity of the four stereoisomers has been evaluated (Reizelman and Zwanenburg 2002; Zwanenburg and Pospisil 2013). The isomer with the ‘natural’ configuration (GR24 and ent-2′epi GR24 in Fig. 3) has the highest activity, *ent*-GR24 the lowest. Commercial GR24 is usually a mixture of two or four stereoisomers. In GR7 and GR5, a reduction of molecular complexity is achieved at the cost of a slightly lower activity on parasitic seeds. Due to the great impact of stereochemistry on biological activity, the design of new SL analogues should consider a minimal number of stereocentres to avoid mixtures of diastereomers. Some indolyl derivatives (EGO10) with interesting activity features both on parasitic seeds and AMF were also reported (Bhattacharya et al. 2009; Prandi et al. 2011).

Mimics

All the SL analogues show a common functional group, the enol-ether bridge, linking the C and D rings, which is the putative bioactiphore of the active SLs. An interesting recent development concerns the bioactivity of molecules in which

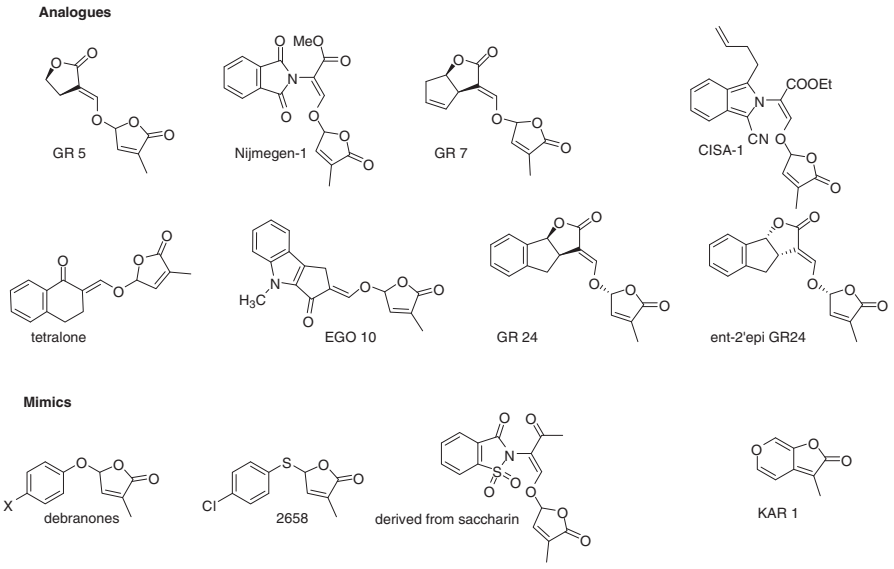

Fig. 3 Chemical structures of some SL synthetic analogues (retaining the enol-ether bridge conjugated to a carbonyl) and mimics (D ring is simply linked to a good leaving group)

the D ring is directly connected with an aromatic ring with different substitution patterns. These simpler molecules are grouped under the class of SLs' 'mimics' (Fig. 3), among which are debranones (Fukui et al. 2013), the thia-derivative 2658 (Boyer et al. 2012) and a saccharin derivative (Zwanenburg and Pospisil 2013), just to list some.

Mode of Action Mechanism

The mode of action of the three classes of compounds (SLs, SL analogues and mimics) will be completely understood only once the receptors in the different biological systems are characterised. The mechanism of SL perception occurring at the receptor site is still under discussion (Fig. 4). Structure-activation studies demonstrated that there are nuances between the plant and fungal system with respect to activity (Akiyama et al. 2010; Xie et al. 2010). Moreover, a number of stimulants other than SLs have been reported to have a strong bioactivity on seeds of parasitic plants, among which karrikins (Fig. 3) have been often associated to SLs and supposed to share part of the perception system with SLs (Joel et al. 2011; Nelson et al. 2012). The general mechanism so far accounted for activity on parasitic seeds and relaying on a Michael addition on the enol ether of SLs followed by the release of the D ring has been overshadowed by recent and new data. Interestingly, SL mimics lacking

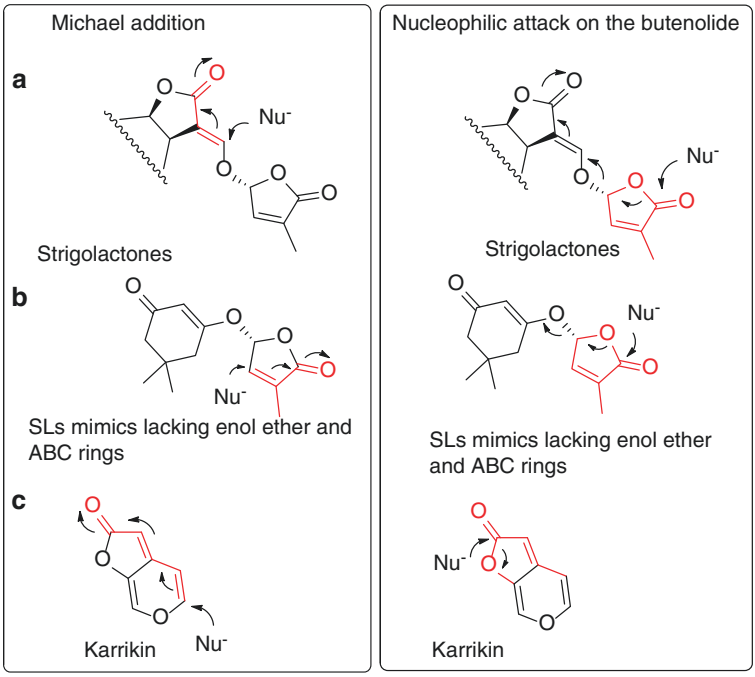

Fig. 4 Mechanism at the receptor. *Left*: Zwanenburg hypothesis of a Michael addition to the enol ether (**a**) or to the D ring (**b**). Conjugated Michael addition on karrikins (**c**). *Right*: Nucleophilic attack to the D ring in SLs (**a**), mimics (**b**) and karrikins (**c**)

both the enol ether and the ABC systems were proved to be active (Fukui et al. 2011; Boyer et al. 2012) as plant hormones. These compounds are simpler molecules with respect to natural SLs and to the most used synthetic analogues and are formed by a butenolide (D ring in SLs) with a good leaving group at C2′ (Fig. 3) (Zwanenburg and Mwakaboko 2011; Asami and Ito 2012). Based on this last data, Zwanenburg proposed an alternative mechanism relying on a Michael addition on the D ring only (Fig. 4b, Zwanenburg and Pospisil (2013)). A Michael addition mechanism has been also proposed to explain the activity as germination stimulants of karrikin (Fig. 4c). Besides, very recently Scaffidi et al. (2012) proposed a different mechanism to support structure-reactivity data on a series of karrikin analogues based on the attack of a nucleophile to the butenolide ring. This last mechanistic hypothesis is fully consistent with the α,β-hydrolasic function of D14, the so far most promising candidate as SLs receptor (Gaiji et al. 2012; Hamiaux et al. 2012). Very recently, the first extensive SAR for SLs and their role in the control of shoot branching in *Pisum sativum* has been reported (Boyer et al. 2012). According to these data, the presence of the Michael acceptor motif as well as the methyl butenolide (D ring) in the same molecule is mandatory to induce activity.

Concluding Remarks

Strigolactones are likely to be key regulators of plant development in adaptation to environmental conditions and may have been first developed, about 450 million years ago, as an adaptation of plants to terrestrialisation. Since then, their role may have expanded into diverse roles in plant growth and development and communication in the rhizosphere.

SLs with their multifaceted biological roles can undeniably become a potent and valuable tool to develop new agricultural methodologies and technologies according to emerging concepts of sustainable agriculture. New physiological effects of SLs on shoot and root architectures will be hopefully discovered in the near future, and their roles in the enhancement of plant resilience to environmental stresses, including climate changes, will be completely unveiled. One example is the use of SL inhibitors to enhance rooting (Rasmussen et al. 2012). Inhibition of the SL-related rooting may lead to overcoming the restrictions of woody plants to adventitious rooting, thereby substantially promoting propagation of woody plants for industry and for conservation of endangered species. Another example is changing root architecture. Since SLs modulate root branching (Kapulnik et al. 2011a), their use or their inhibition may lead to root system with desired architecture, for example, hyperbranched root system for increased tolerance to nutrient deficiency or deeper roots for increase water use efficiency.

Up to date, the differences observed in the response of parasitic weeds, fungi and plants for the hormonal activity suggest that each system uses distinct perception system. The design of new targeted SL analogues would be possible once the receptor proteins involved in the perception, as recently found in rice, *Arabidopsis* and petunia (Zhao et al. 2013; Hamiaux et al. 2012; Kagiyama et al. 2013), were confirmed and the mechanism occurring at the receptor site was fully elucidated. In addition, once the SL receptor in AM fungi and in parasitic plant seeds will be identified, the research in understanding the communication in the rhizosphere will be boosted. From the above-cited results, it seems evident that more structure-reaction data are needed for elucidation of the mechanisms involved in the perception/signalling of SLs and for the syntheses of molecules specifically targeted for each of the various roles of SLs.

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Phytohormonal Crosstalk Under Abiotic Stress

Aurelio Gómez-Cadenas, Carlos de Ollas, Matías Manzi, and Vicent Arbona

Abstract As sessile organisms, plants cannot escape from adverse conditions. Thus, responses to the changing environment are more complex than in animals that usually just try to flee. Plant responses to abiotic constrains involve changes in gene expression, protein activity, cellular metabolite, and ion levels and must be perfectly coordinated by phytohormones that are the compounds that transduce signals. Recent data indicate that the signaling pathways are not isolated but interconnected in complex networks. Moreover, supporting evidence points to specific transduction pathways in different types of tissues or organs. This chapter will revise molecular mechanisms conserved among different hormone signaling pathways, which accounts for their evolutive importance together with particular interactions. The work is organized in sections that contextualize crosstalks of the main phytohormones in particular physiological processes. Data revised in this chapter support the importance of finding divergent experimental systems in the future. Therefore, whereas simplified plant systems will allow finding new phytohormone crosstalks, considering the plant as a whole will provide further information among interactions that can be hidden at this point due to the massive use of model plants in early stages of growth or cultivated in artificial conditions. Specific hormone interactions could represent targets for breeding/managing for yield resilience under multiple stress situations.

Keywords Signal transduction • Hormone interactions • Physiological responses • Gene expression

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Introduction

Plants have evolved to integrate diverse environmental cues into their developmental programs. As sessile organisms, plants cannot escape from adverse constraints and, therefore, a complex array of physiological, biochemical, and molecular responses builds the self-defense against stress. These responses lead to changes in gene expression, protein activity, cellular metabolite, and ion levels and must be perfectly coordinated (Gong et al. 2013).

Phytohormones are a diverse group of growth regulators found in trace amounts in the cell. Among them, abscisic acid (ABA), jasmonates (JA), salicylic acid (SA), ethylene, auxins, gibberellins (GA), cytokinins (CK), brassinosteroids (BR), and strigolactones (ST) are of key importance for the development and plastic growth of plants (Kohli et al. 2013).

Research on mutants, particularly in *Arabidopsis*, has contributed substantially to the current knowledge of hormone action. While substantial progress has been made in understanding individual aspects of phytohormone perception and signal transduction, increasing evidence suggests that these signaling pathways are interconnected in a network, in which hormones not only coordinate developmental cues but also convey environmental inputs by means of synergistic or antagonistic actions referred to as signaling crosstalk. However, the understanding of the complexity of signal crosstalk is far from being resolved. In this sense, the underlying molecular mechanisms have yet to be elucidated and there is little information on the cellular responses to multiple hormone signals (i.e., the product of this crosstalk). Moreover, many studies of hormone crosstalk have used whole plants and it is possible that different mature cell types have distinct responses to hormones. Supporting evidence for this is provided by the differential responses to abiotic stress between roots and shoots (de Ollas et al. 2013) or, even more specifically, among different types of root cells (Dinneny et al. 2008; Dugardeyn et al. 2008). Simplified experimental systems are a good choice for improving our understanding of the mechanisms underlying these intricate interactions.

Throughout this chapter, we will revise how molecular mechanisms are conserved among different hormone signaling pathways, which accounts for their evolutive importance. One of these mechanisms is the ubiquitin-dependent protein degradation by the 26S proteasome, which is key in the signal transduction of JA, IAA (indole-3-acetic acid, the most biologically active auxin), and GA (Chini et al. 2009; Kim et al. 2009). Other common element of regulation is the existence of loops for the precise control of hormone response. Therefore, feedback regulation can be found in most of the hormone biosynthetic genes. What it is more striking is that the same gene can be both positively and negatively regulated by its gene product in different situations (Robert-Seilaniantz et al. 2011). Another important feature is the existence of responsive downstream genes induced or repressed by different hormone signaling pathways. Therefore, specific physiological processes can be regulated by different phytohormones through controlling the expression of a common set of downstream genes. As examples, JA repression of specific genes can be

relieved by DELLA proteins, the key negative regulator of GA signaling (Hou et al. 2010). Binding of DELLA to JA ZIM-domain (JAZ) proteins removes the repression on MYC2, and, subsequently, the downstream JA-responsive genes are expressed. Another node of crosstalk less understood so far is at the level of biosynthesis and/or metabolism. Thus, the signal from a hormone can modulate the metabolism of another. Some recent examples on this interaction are as follows: transient JA signal seems to be an early response of roots to drought, essential for subsequent ABA progressive accumulation (de Ollas et al. 2013); CK and auxins seem to modulate GA metabolism genes (Brenner et al. 2005).

Moreover, different pathways could share common components, leading to a more complicated hormone signaling than expected. It has been shown that the transcription factor MYC2 can be considered as a point of convergence of various hormonal pathways and a potential point of crosstalk between JA and ABA (Kazan and Manners 2012). Another point of crosstalk is the AUX/IAA gene SHORT HYPOCOTYL 2 (SHY2), expressed in the root meristem transition zone under the control of B-type *Arabidopsis* response regulator (ARR) transcription factors (Dello Ioio et al. 2007, 2008), which are the end points of CK signaling. Moreover, the transcription factor *Solanum lycopersicum* (Sl)DREB (a dehydration-responsive element-binding protein) induced under stress conditions has been found to play a negative role in tomato plant architecture, whereas enhances drought tolerance. SlDREB downregulates the expression of key genes required for GA biosynthesis and acts as a positive regulator in drought stress responses by restricting leaf expansion and internode elongation (Li et al. 2012a).

Molecular studies revealed that the crosstalk between different phytohormones represents a precisely coordinated web of nodes and lines. Considering the crosstalk among different hormone signaling pathways, roles of hormone signaling in regulating expression of the genome seem very complex.

Abscisic Acid Crosstalk Under Abiotic Stress Conditions

In the adaptation of plants to adverse environments, ABA plays an important role as regulator of stomatal closure (Eyidogan et al. 2012), progressive desiccation (Ye et al. 2012), growth (Nitsch et al. 2012), senescence (Kato et al. 2006), and organ abscission (Gómez-Cadenas et al. 1996, 1998, 2000). All these physiological adjustments are induced to avoid (or at least delay) the damaging effects of abiotic stress on plant physiology. As indicated in the introduction, it seems nowadays clear that most of the effects of ABA on plant metabolism are carried out in interaction with other effectors and/or growth regulators.

ABA accumulation in plant cells is one of the fastest responses to environmental stress and seems an essential factor that triggers stomatal closure that, in turn, reduces water loss through transpiration (Dodd et al. 2009). Actually, mutants impaired in ABA biosynthesis, perception, or signal transduction are also affected in their ability to regulate water loss even under non-stressful conditions exhibiting

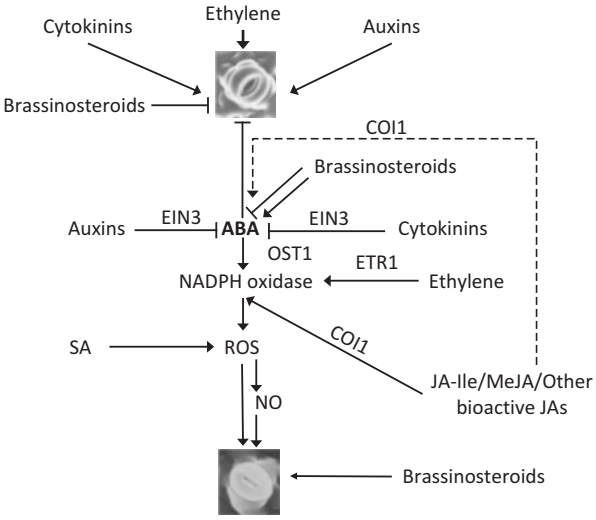

Fig. 1 Crosstalk of abscisic acid with other hormones in the regulation of stomatal aperture. Adapted from Acharya and Assmann (2009)

an evident *wilty* phenotype in *Arabidopsis* and tomato plants (Verslues and Bray 2006; Dodd et al. 2009). Nevertheless, new experimental evidence indicates that not only ABA regulates stomatal closing but other plant regulators such as BR, SA, JA, and nitric oxide (NO) have a similar effect in vivo (Peleg and Blumwald 2011). The model, as presented in Acharya and Assmann (2009), shows ABA in a central role promoting stomatal closure and inhibiting opening (see Fig. 1). ABA would induce a NADPH oxidase through open stomata 1 (OST1), an Snf1-related protein kinase 2 (SnRK2) that acts downstream of ABA-insensitive 2 (ABI2), a protein phosphatase 2C. At this point, ethylene and ABA may act synergistically, as the gaseous hormone also contributes to induce NADPH oxidase through the ethylene receptor 1 (ETR1) pathway. The mechanism also postulates that JA such as JA-Ile or methyl jasmonate (MeJA) activates NADPH oxidase. To this respect, it is quite likely that this is performed through ABA signaling (de Ollas et al. 2013). In turn, NADPH oxidase triggers reactive oxygen species (ROS) production that subsequently initiates stomatal closure (Acharya and Assmann 2009). It seems that NO could be a key intermediate in the ABA-mediated signaling network leading to stomatal closure (Hancock et al. 2011) since the accumulation of NO has been reported in stomatal guard cells linked to the ABA-regulated stomatal closure (Neill et al. 2002) and also in other processes where ABA is involved (Zhang et al. 2009). The generation of NO seems to be induced by ABA and associated to H_2O_2 production by NADPH oxidase (Bright et al. 2006). However, this requirement of NO does not seem to be necessary for stomatal closure in dehydrated leaves (Ribeiro et al. 2009). Therefore, the stress-induced production of ROS would be integrated within the signaling network including ABA as a modulator and the ROS-induced production of NO as an effector leading to physiological responses. Besides responses to

water deficit, ABA and NO have also been found to interact in the responses to UV-B radiation. Upon UV-B treatment, ABA concentration increases in exposed tissues along with H_2O_2 and NO. However, a viviparous maize mutant was found to be more sensitive to UV-B radiation as well as showed lower H_2O_2 and NO accumulation. The wild-type (WT) responses to UV-B were restored after ABA treatment (Hancock et al. 2011). Likewise, water stress and UV-B radiation responses seem to be regulated by ABA, H_2O_2, and NO.

In addition to hormonal regulation, there are other developmental factors that modulate the ability of ABA to regulate stomatal movement such as aging. In different plant species, it has been shown that ABA is less effective in terms of stomatal closure in aged leaves than in the young ones. Chen et al. (2013) found that this phenomenon was modulated by ethylene. Therefore, the inhibition of ethylene perception by 1-methylcyclopropene partially restored the ABA-induced stomatal closure in old wheat leaves. Strikingly, ethylene overaccumulation in the *Arabidopsis eto1-1* mutant suppressed ABA-induced stomatal closure (Tanaka et al. 2005). Indeed, this inhibitory effect seems to be specific of ABA signaling as it does not affect dark-induced stomatal closure. Nevertheless, since ethylene alone promotes stomatal closure a complex scenario of multiple interactions with hormones is likely to exist. In citrus, a different ABA/ethylene interaction was confirmed in roots upon exposition to severe desiccation. In this system, ABA accumulation was required for the initiation of ACC biosynthesis (Gómez-Cadenas et al. 1996).

Plants have to integrate growth and development to environmental cues. Then, it seems quite logical that ABA interacts with other hormones associated to the regulation of developmental processes such as GA, CK, or auxins. Traditionally, GA have been regarded as central growth regulators and, indeed, *Arabidopsis* mutants defective in GA synthesis (*ga1–3*) or signal transduction (*gai1*) exhibit an altered growth pattern and defective flowering. Under physiological conditions, active GA bind to the soluble GA receptor gibberellin-insensitive dwarf1 (GID1 in rice) or GID1-related proteins (*Arabidopsis*). This complex interacts with DELLA proteins that act as growth repressors at low GA concentration and promote their degradation (Golldack et al. 2013). In other systems, the interaction between ABA and GA seems more logical as both hormones are expected to regulate completely antagonistic processes; such is the case of seed ontogenesis and seed germination. In the process of seed production, fresh fecundated ovaries have to progressively reduce their water content and become quiescent in order to produce an autonomous plant structure able to endure the most aggressive cues. In this process of acquired desiccation tolerance, ABA plays a fundamental role (Gómez-Cadenas et al. 1999) and, indeed, in maturing seeds, ABA levels increase upregulating several ABA-responsive genes. Conversely, during germination, ABA levels decrease and GA take over control. In the cereal aleurone layer system, GA induce the expression of α-amylases by promoting the degradation of SLN1 (slender1, a DELLA protein) which acts as a repressor. In germinating cereal seeds, this production is inhibited by ABA through an ABA-induced protein kinase (PKABA1), a repressor of the GAMyb expression, a transcription factor that regulates α-amylase expression (Ho et al. 2003). Another point of interaction involving ROS has been recently

proposed in which GA induce H_2O_2 production whereas ABA represses it; H_2O_2 could, in turn, inhibit PKABA1 activity, but its role in GA signaling pathway is not yet known (Ishibashi et al. 2012). To add more complexity, GA metabolism and DELLA activity have been demonstrated to be affected by osmotic stress, and the cold-responsive transcription factor CBF1 controls DELLA accumulation. In addition, mutations affecting the DELLAs GAI and RGA suppressed freezing tolerance in *Arabidopsis* (Golldack et al. 2013).

Auxins, especially IAA, seem to be also interconnected with ABA at the signaling level. This interaction has been thoroughly described in the promotion of lateral root growth, which is an important response to several abiotic stress conditions (Saini et al. 2013). Upon ABA treatment, lateral root development is inhibited by suppression of the IAA-responsive lateral root formation. Furthermore, ABI3 has been shown to interact with the auxin-responsive factor (ARF) or Aux/IAA proteins. Indeed, *abi4* plants show an increased number of lateral roots, whereas overexpression of this transcription factor impairs their development. ABI4 represses the expression of the auxin-efflux carrier PIN1, showing that ABA signaling is also involved in the regulation of the auxin polar transport (Shkolnik-Inbar and Bar-Zvi 2010; Saini et al. 2013). To this respect, citrus plants subjected to prolonged soil waterlogging showed increased IAA levels along with a depletion of ABA concentration in roots which might account for the promotion of lateral root growth as part of the physiological responses to cope with severe soil flooding (Arbona and Gómez-Cadenas 2008). Under these conditions, ROP GTPases that have been described to regulate oxygen deprivation tolerance in *Arabidopsis* (Baxter-Burrell et al. 2002) are the target of RIC proteins that positively regulate IAA and negatively ABA signaling (Choi et al. 2012).

Further interactions between ABA and CK are described in the CK section in this chapter.

Jasmonates Crosstalk Under Abiotic Stress Conditions

Increased levels of JA are detected in plants challenged with certain biotic and abiotic stresses, such as wounding, herbivore feeding, and infections from necrotic fungi (Wasternack 2007; Wu and Baldwin 2010). Furthermore, ABA and JA signaling pathways can interact at several points in response to developmental or stress cues such as water stress, suggesting a role for JA in the response to water deficit. There is some overlap in the biological activities mediated by ABA and JA as both inhibit plant growth and germination, promote tuberization and senescence, and induce the expression of a number of the same genes. Hays et al. (1999) reported that napin and oleosin gene expression was dependent on both ABA and JA. Interestingly, one of the explanations to this hormonal interplay was that JA may stimulate an increase in ABA endogenous levels, and therefore, JA somehow uses ABA as an intermediate in JA-induced gene expression. This idea was previously considered by (Creelman et al. 1992). As indicated in the ABA section in this

chapter, there is also an interaction or a shared signaling pathway in the ABA- and JA-induced stomatal closure in guard cells.

At the molecular level, Lackman et al. (2011) described how MeJA can modulate NtPYL4 and NtT172 (PP2C proteins) transcript levels in tobacco plants. In addition, genomic data indicate that the expression profile of the *Arabidopsis* PYL4/PYL5/PYL6 branch can also be modulated by JA. The induction of MYC2 by ABA seems to rely on the JA receptor coronatine insensitive (COI1) according to Lorenzo et al. (2004). In rice, the MYC-homolog OsbHLH148 interacted with OsJAZs in response to drought. Furthermore, transgenic rice plants overexpressing *OsbHLH148* showed a drought-tolerant phenotype. Recently, a model has been proposed in which ABA and JA act synergistically in response to stress with JA acting upstream of ABA (Seo et al. 2011).

JA-insensitive mutants such as coronatine-insensitive1-16 (*coi1-16*) and JA-resistant (*jar1*) showed higher sensitivity to exogenous ABA than wild-type (WT) plants. Furthermore, a synergistic effect was observed when ABA and JA were combined to inhibit seed germination in WT (Fernandez-Arbaizar et al. 2012).

Recent research performed by de Ollas et al. (unpublished data) points to an interaction between JA-dependent signaling and ABA biosynthesis in roots of *Arabidopsis* under water stress conditions. In this work, mutants impaired in JA biosynthesis do not accumulate ABA to the same extent that WT seedlings accumulate in the first stages of desiccation. Interestingly, this defect in ABA accumulation is only present in roots, as shoots are able to accumulate ABA to the same extent that WT seedlings.

The potential of JA to induce auxin biosynthesis was originally proposed by Devoto et al. (2005). Besides the similarity between auxin and JA signaling pathways, physiological and genetic studies have suggested a complex and little understood crosstalk (Kazan and Manners 2008). For example, treating *Arabidopsis* plants for 48 h with MeJA resulted in a significant increase of free IAA levels (Dombrecht et al. 2007). Plants overexpressing *ERF1* show both increased expression of genes encoding Trp biosynthetic enzymes and increased inhibition of root elongation by JA (Lorenzo et al. 2003), indicating that auxin homeostasis might also be altered in ERF1-overexpressing plants grown in the presence of exogenous JA. Interestingly, it was also shown that auxins increases the transcript levels of JA biosynthesis genes in *Arabidopsis* (Tiryaki and Staswick 2002). Conversely, ARF6 and ARF8 have been shown to promote JA production in *Arabidopsis* flowers (Nagpal et al. 2005), and according to Grunewald et al. (2009), JAZ1/TIFY10A expression is dependent of auxins and independent of JA signaling. MeJA-mediated IAA synthesis may be critical for the proper regulation of plant growth and development under biotic stress. Indeed, a study in insect-attacked tobacco plants suggested that JA signaling suppressed growth and contributed to apical dominance, a role expected from auxins (Zavala and Baldwin 2006). A similar role for auxins was also proposed for ethylene-mediated inhibition of root elongation (Rahman et al. 2001; Stepanova et al. 2005).

The increased JA levels are usually associated with an enhanced defense but also with an impaired growth (Baldwin 1998; Zhang and Turner 2008).

Recent studies have suggested that intensive crosstalk between GA and JA signaling mediates equilibrium between plant development and defense to biotic or abiotic stress. In particular, interactions between DELLAs and JAZ proteins, which are key repressors in GA and JA signaling pathways, respectively, play a central role in mediating the balance between plant growth and defense through modulating the activity of their interacting transcriptional factors in response to GA and JA signals. Also, according to Heinrich et al. (2013), increased levels of JA repress the biosynthesis of GA by inhibiting the transcription of several GA biosynthetic genes, including GA20ox, which encodes a key enzyme catalyzing the formation of bioactive GA. Furthermore, evidence suggests that suppressed GA production is likely largely responsible for the decreased plant growth, but not for the diverted resources for the biosynthesis of secondary metabolites (Heinrich et al. 2013).

Salicylic Crosstalk Under Abiotic Stress Conditions

Most of the research on SA has focused on its role in the local and systemic response against microbial pathogens. However, SA has been recognized as a regulatory signal mediating plant response to abiotic stress such as drought (Munné-Bosch and Peñuelas 2003), high salinity (Gémes et al. 2011), chilling (Kang and Saltveit 2002), heavy metal exposure (Metwally et al. 2005), and heat (Larkindale and Knight 2002).

As indicated in the specific section in this chapter, auxins are widely recognized as a key growth regulator and are emerging as a new candidate in mediating plant response to biotic and abiotic stresses (Wolters and Jürgens 2009). Auxin perception is due to members of a small family of F-box proteins, transport inhibitor response 1 (TIR1), and its paralog auxin signaling F-box 1 (AFB1–3). Auxin binding to SCF^{TIR}1-AFBs results in the targeted degradation of auxin/IAA transcriptional repressors via SCF E3-ubiquitin-ligase proteasome pathway. Thereafter, auxin/IAA degradation promotes activation of ARFs and the consequent expression of auxin-responsive genes. Work involving SA-inducible DNA-binding-with-one-finger (DOF) transcription factors OBP1, OBP2, and OBP3 unveiled that in addition to SA, these transcription factors are responsive to auxins (Kang and Singh 2000). *Arabidopsis cpr5*, *cpr6*, and *snc1* mutants with reduced apical dominance and stunned growth present a similar phenotype of mutant deficient or insensitive to auxins and elevated endogenous SA levels. Supporting this relationship, overaccumulating SA mutants have lower IAA levels and are partially insensitive to auxins. Interestingly, the breeding of those genotypes (SA accumulating and auxin overproducing) rescues the phenotype caused by the high auxin content. These facts point to an antagonism between SA and auxins, with SA interfering with auxin-dependent signaling but not with auxin accumulation. According to Iglesias et al. (2011), under salt stress, pathogenesis-related 1 (PR-1) was induced 3.5-fold in SA-treated *tir1 afb2* seedlings compared with SA-treated wild-type plants, indicating that auxin signaling might interfere with SA-regulated PR-1 induction. Coincidently, PR-1 was also significantly induced in *Arabidopsis* mutant plants with reduced IAA levels.

The relationship between JA- and SA-dependent signaling has often been shown to be antagonistic in the defense response to biotic threads. In *Arabidopsis*, pathogen-induced SA accumulation is associated with the suppression of JA signaling. In contrast, it was demonstrated that JA acts together with SA to confer thermotolerance in *Arabidopsis*. Plants have the capacity to ameliorate the effects of heat shock (HS) via a basal thermotolerance mechanism (Hong and Vierling 2000). In addition, lesser increases in temperature can acclimatize plants against high temperatures through a process known as acquired thermotolerance. The production of heat-shock proteins (HSPs) plays a vital role protecting proteins against heat damage (Hong et al. 2003). Recently, Clarke et al. (2009) indicated that SA signaling pathways promote basal thermotolerance but are dispensable for the acquired mechanism. Heat shock was found to induce SA-regulated PR-1 transcripts, and the ability of the nonexpressor of PR1 protein (*npr1-1*) to recover from heat stress was impaired. Also, the constitutive expresser of PR1 protein (*cpr5-1*) displayed an enhanced basal thermotolerance (Clarke et al. 2000) and, together with the activation of the SA pathway, the JA-inducible genes PDF1.2 and THI2.1 were constitutively expressed. According to Clarke et al. (2009), the enhanced thermotolerance observed in *cpr5-1* was not seen in the *cpr5-1 jar1-1* double mutant, implying a requirement for JA to accomplish a full tolerance. An additional signaling interaction within *cpr5-1* was likely to occur between SA and ethylene as *ein2-1* plants were less susceptible to heat stress.

PR proteins have been well defined as plant proteins that are induced not only during pathogen infection but also in response to abiotic stress. Recent studies have revealed that PR10 proteins are involved in various environmental stress conditions, such as drought, high salinity, low and high temperatures, wounding, and UV exposure. According to Takeuchi et al. (2011), there is an involvement of the JA and ethylene signaling pathways in RSOsPR10 induction in response to high salinity and wounding and the antagonistic inhibition by exogenous SA treatment at a transcriptional level.

Ethylene Crosstalk Under Abiotic Stress Conditions

The gaseous hormone ethylene has been implicated in many pathways that involve the regulation of different stages of plant growth and development, such as flower induction, fruit ripening, and organ senescence (Arteca and Arteca 2008). Ethylene also plays an essential role in plant adaptation and survival against different stress conditions, triggering mechanisms, or being the final effector of the response mediated by other hormones (Bleecker and Kende 2000).

One typical response to water deficit in plants is a massive abscission of leaves and fruits whose magnitude is directly correlated with stress intensity. This process is regulated by the crosstalk between ABA and ethylene (see ABA section in this chapter), but other hormones such as CK are also playing a role in this process (Dal Cin et al. 2009). Exogenous application of benzyladenine (BA, a CK)

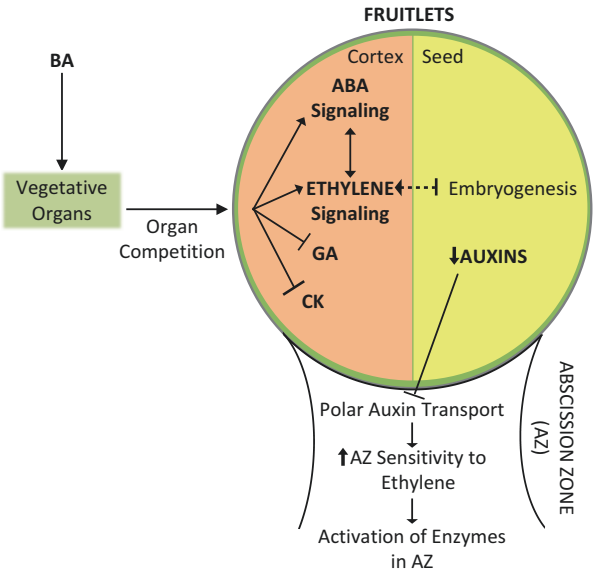

Fig. 2 Model involving hormonal crosstalk that regulates the fruit abscission of young apple. Exogenous application of benzyladenine stimulates vegetative growth, leading to a competition between shoots and developing fruitlet. This nutritional stress (mainly carbohydrate starvation) upregulates genes involved in GA (GA2-oxidase) and CK (cytokinin dehydrogenase) inactivation in the fruit cortex. ABA (AMP-MAPKinases) and ethylene (ERF) pathways are also upregulated. Under this situation, embryo development is arrested and levels of auxins decrease in the seed. Low auxin levels and a depolarization of auxin transport increase the sensitivity to ethylene in the abscission zone, promoting the activation of cell wall-degrading enzymes which is ended with the abscission of the fruitlet. Adapted from Botton et al. 2011. *Plant Physiol*. 155: 185-208

stimulates the nutrient competition between fruits and leaves and upregulates the expression of genes involved in ABA and ethylene signaling and in the inactivation of GA and CK (Botton et al. 2011). In this sense, it was reported that ERF1, an ethylene-responsive factor, physically interacts with mitogen-activated protein kinase 1 (MAPK1), being also induced by ABA and SA under different stress conditions. Moreover, overexpression of ethylene response factor (ERF1) in maize resulted in a higher sensitivity to exogenous ABA in transgenic plants (Xu et al. 2007). The model proposed by Botton et al. (2011) and recently reviewed by Estornell et al. (2013) involves the idea that during fruit abscission, CK should be perceived in the fruit cortex causing an ethylene accumulation that could be transported from this tissue to the developing seeds. The parallel decrease in auxin content in seeds enhances the sensitivity to ethylene inducing fruit abscission. Thus, the balance between auxins and ethylene has been pointed out as the main factor regulating abscission since the basipetal polar flow of auxins to the abscission zone of leaves and fruits determines the sensitivity to ethylene (Estornell et al. 2013) (see Fig. 2).

JA and ethylene crosstalk seems to affect each individual hormone signaling (Zhu et al. 2011) and also is involved in floral abscission in *Arabidopsis* (Patterson and Bleecker 2004; Butenko et al. 2006). *Arabidopsis ein2* ethylene-insensitive mutant (a pivotal component in ethylene signaling located downstream of ethylene receptors) showed an increased response to ethylene in flower abscission when JA level was reduced in a double mutant that apart from *ein2* mutation presented an impaired allene oxide synthase (AOS) activity, a key enzyme on JA biosynthesis. Hence, reduced levels of JA in ethylene-insensitive plants could be modifying plant sensitivity to this phytohormone. However, this was not evident when ethylene-insensitive *etr1-1* mutants that are affected in an ETR were used. Authors suggested that JA inhibits ethylene signal transduction downstream ETRs (Kim et al. 2013).

Roots surrounded by water are prone to accumulate ethylene in cells and air space inside the root due to the slow diffusion of ethylene in water (Nakano et al. 2006; Vandenbussche et al. 2012). One of the most common responses of flood-tolerant plants is a fast elongation of shoots that emerge out from water level to act as a "snorkel," thus improving the gas exchange to escape from the submergence conditions (Cox et al. 2004). In rice, ethylene promotes the expression of Snorkel 1 and 2 (SK1 and SK2) genes (Hattori et al. 2009; Nagai et al. 2010) that act directly or indirectly promoting GA accumulation or signal transduction, favoring shoots elongation (Hattori et al. 2009). This response is mediated not only by an increase in ethylene production but also by an enhanced plant sensitivity to this hormone (Hattori et al. 2009). Moreover, interplay between different hormones regulates shoot elongation, basically involving ABA and GA. In rice, it was demonstrated that ethylene induces a hormonal signaling cascade which regulates the cell elongation by affecting ABA/GA balance (Bailey-Serres et al. 2012). Regarding ABA, its concentration in internodes and leaves of deep-water rice as well as in other species decreases sharply after a few hours from the beginning of submergence. This decrease was caused by downregulation of the 9-*cis*-epoxycarotenoid dioxygenase (NCED) expression triggered by ethylene (Benschop et al. 2005; Saika et al. 2007). Moreover a concomitant increase in the expression of *OsABA8ox1* (a gene that encodes an ABA hydroxylase protein) accelerates ABA catabolism to phaseic acid (Benschop et al. 2005; Saika et al. 2007). The model proposes that under flooding, a decrease in ABA levels is necessary to trigger ethylene-induced mechanisms (Jackson 2008).

Apart from ABA and ethylene, GA and auxins play a key role in this plant system, interplaying with both ethylene and ABA. Exogenous ethylene stimulates the submergence-induced shoot elongation by an increase in GA_1 (a bioactive GA) in *Rumex palustris* within the first 24 h (Rijnders et al. 1997), while RpGA3ox1 transcripts increase after plant submergence or exogenous ethylene application (Benschop et al. 2005). GA application in submerged rice plants also induced an increment in ACC synthase *OsACS5* transcription (Van Der Straeten et al. 2001) suggesting also the existence of feedback regulation mechanisms. Overall, GA effect on shoot elongation is only possible after ethylene downregulation of ABA content (Benschop et al. 2006). The involvement of auxins appears to be tissue specific since in petioles of *R. palustris*, submergence induced a slight decrease in

endogenous IAA (Cox et al. 2004), whereas in the outer layers of the petioles, IAA levels increased (Cox et al. 2006). Interestingly, plants were unable to stimulate petiole elongation when leaf blade was removed, being restored when exogenous auxins were applied. However, GA or ethylene could not restore the petiole elongation. Thus, since ethylene and GA stimulate leaf elongation, it is proposed that the effect of both hormones under submergence is auxin dependent.

There are some plant species that are able to keep an effective quiescent tolerance to adverse conditions, avoiding shoot elongation. In lowland rice, a typical quiescent tolerant plant, the production and sensitivity to ethylene are limited (Hattori et al. 2009). It is suggested that the response to submergence is dependent not only on the genotype and ethylene per se but also on the ethylene interaction with several other phytohormones (Bailey-Serres et al. 2012; Kim et al. 2012). In this sense, Kim et al. (2012) described the existence of an alternative EIN3/EIL1 independent pathway in *Arabidopsis* based on the double mutants (*ein3 eil1-1*) behavior. This suggests that ethylene could affect GA metabolism genes in a way mediated by EIN3/EIL1 and also independently of these transcription factors (Kim et al. 2012).

Auxin Crosstalk Under Abiotic Stress Conditions

Auxins are a group of phytohormones that play a key role in plant metabolism and are often recognized as positive regulators of plant growth. There is a wide range of information available regarding how auxins and other phytohormones regulate plant growth and development in different organs and tissues, under diverse physiological conditions (Nemhauser et al. 2006). In this sense, the control of apical dominance and lateral bud sprouting is a process in which auxins play a key role (Gallavotti 2013). However, in the last few years, auxins have been also implicated in plant responses to different abiotic stress. These studies have revealed that auxins play an important role in mediating the response to adverse environmental conditions, interacting in processes where the main characters have been mainly attributed to other phytohormones (Popko et al. 2010). Under osmotic stress, ABA signal transduction affects auxin signaling, leading to a coordinate response that finally ends up in a decrease in shoot growth (Albacete et al. 2008). It is widely accepted that roots are more resistant to osmotic stress than shoots, being able to continue their growth under stress condition (Spollen and Sharp 1991). Thus, under reduced water availability, both hormones ABA and auxins act coordinately to minimize water loss in the shoots (Hansen and Grossmann 2000) and to induce reorganization of the roots (Popko et al. 2010). In *Arabidopsis*, ARF2, which negatively regulates the transcription of auxin-responsive genes (Lim et al. 2010), was demonstrated to be inducible by ABA (Wang et al. 2011).

As mentioned before, under water stress, most plants inhibit shoot growth while maintaining or even increasing root elongation to reach wetter zones by modulating primary, lateral, or even adventitious root (AR) growth (Van Der Weele et al. 2000;

Sharp and LeNoble 2002; Yamaguchi and Sharp 2010) and developing a new root architecture (Hong et al. 2013). ABA is the main hormone that influences the plant response at physiological, cellular, and molecular level when the water potential decreased near the roots. A rapid increase in ABA content is registered in the root tip, which conducts to further changes in root shape (Zhang and Tardieu 1996; Sengupta et al. 2011). However, auxins also display a role in this process, controlling root growth and development (Ribaut and Pilet 1994; Fu and Harberd 2003) by guiding root growth (Grieneisen et al. 2007; Robert and Friml 2009) and by the promotion of H^+ secretion, which regulates the activity of the plasma membrane (PM) H^+-ATPase (Rober-Kleber et al. 2003; Staal et al. 2011). It has been clearly shown that auxins increase the amount of H^+-ATPase protein in the plasma membrane (Hager et al. 1991), and this H^+ secretion mediated by PM H^+-ATPases plays a key role in primary root elongation or root hair development (Santi and Schmidt 2009). In *Arabidopsis* and rice, ABA accumulation triggered by osmotic stress (salinity or water deficiency) modulates auxin transport in the root tip (Xu et al. 2013), which results in a local accumulation and consequent redistribution of auxins within the root (Ottenschläger et al. 2003). Moreover, in ABA-deficient mutants subjected to moderate water stress, H^+ secretion, primary root elongation, root hair density, and PM H^+-ATPase activity were reduced; meanwhile, ABA or water stress application induced all these mechanisms in wild-type plants as well as induced transcript abundance of auxin influx and efflux transporters (Xu et al. 2013). Also, the fact that MYB96-mediated ABA signaling (MYB96: drought-induced transcription factor) is transduced through an auxin signal pathway during drought response in *Arabidopsis* (Seo et al. 2009) suggests a strong relationship between both phytohormones under drought.

Apart from ABA and auxin interactions, ethylene also interplays in this process, making the regulatory network existent under water stress conditions even more complex. A positive regulatory crosstalk between auxins and ethylene usually exists, since one phytohormone induces the biosynthesis of the other (Abel et al. 1995). However, in some cases a negative regulation between them could exist. For example, root growth is inhibited by ethylene in response to an increase in auxin biosynthesis in the shoots. Auxins are basipetally transported to the root tip and then redistributed to the root elongation zone. The presence of auxins in the root elongation zone causes a reduction in root growth by inhibition of root cell elongation (Růzicka et al. 2007). More information about the crosstalk between these hormones is available in the ethylene section in this chapter.

As occurred under water stress, a complex network is intertwined among several hormones in response to wounding (da Costa et al. 2013; Han et al. 2009). It is worthy to point out that the complexity of the network woven among phytohormones in response to wounding is quite similar among newly developed organs (adventitious roots, lateral roots, and shoots developed from the roots) with auxins playing a central role. An interesting example of the complex interactions among hormones is the mechanisms triggered in response to wounding that are able to release lateral buds from paradormancy, a hormone-regulated process, mainly determined by the balance between auxins and ABA (Fedoroff 2002; Anderson et al. 2012).

Indeed, auxins are the main phytohormones responsible for the inhibition of axillary bud sprout (Booker et al. 2003; Leyser et al. 1993) although this should be an indirect effect since auxins from the apical bud do not reach the lateral ones (Hall and Hillman 1975; Morris 1977). Under paradormancy, GA and CK biosynthesis genes are downregulated (Anderson et al. 2012) while ABA signaling and responsive genes such as DREB proteins are upregulated (Ruttink et al. 2007; Anderson et al. 2012). Moreover, the polar transport of auxins affects root levels of other hormones, such as ethylene and ST (Puig et al. 2012), responsible for bud outgrowth inhibition (Grossmann and Hansen 2001; Shimizu-sato and Mori 2001; Brewer et al. 2009; Beveridge and Kyozuka 2010). In this sense, it has also been suggested that ABA could regulate ST biosynthesis (López-Ráez et al. 2010). ST and auxins interplay to control adventitious root (AR) formation (Rasmussen et al. 2012) and bud outgrowth since basipetal auxin transport from apical bud stimulates ST production through the coordinated action of two carotenoid cleavage dioxygenases (MORE AXILLARY GROWTH, MAX3/CCD7, and MAX4/CCD8). In *Arabidopsis*, they act together with MAX1, a cytochrome P450 family member located downstream MAX3 and MAX4, to produce ST (Booker et al. 2005; Gomez-Roldan et al. 2008; Umehara et al. 2008). Moreover, in *Arabidopsis* and pea, ST signaling through MAX2, an F-box protein and the most downstream known component of ST signaling (Challis et al. 2013), results in an inhibition of AR initiation (Rasmussen et al. 2012). Indeed, ST are transported into the bud and through MAX2 action inhibited auxin transport, where repression of Pin-formed 1 (*PIN1*) plays a crucial role (Shinohara et al. 2013). Many of these MAX genes such as MAX1, MAX3, and MAX4 are regulated by auxins (Bennett et al. 2006; Simons et al. 2007; Gomez-Roldan et al. 2008). Also, it was suggested that auxins and ST could modulate each other's levels and distribution in a feedback loop that controls the axillary outgrowth (Hayward et al. 2009).

Genes involved in the $SCF^{TIR}1$ complex (ESM-2; auxin response processes) are upregulated after damage; meanwhile, AUX/IAA proteins involved in repressing ARFs (positive transcriptional regulators of auxins response and AR formation such as ARF6) are degraded (Gutierrez et al. 2009; Anderson et al. 2012). However, it is necessary to reach a certain auxin threshold to trigger downstream signaling pathway, below which this signaling is arrested by the action of the AUX/IAA repressor proteins that directly inhibit ARFs. On the contrary, high levels of auxins negatively affect AUX/IAA repressors, allowing the transcriptional induction of auxin-responsive genes by ARFs (Mockaitis and Estelle 2008; Han et al. 2009; Jain and Khurana 2009). The effect of auxins in the generation of new shoots from the roots is mediated also by auxin redistribution within the roots. PIN1 and PIN3 are essential for the polar auxin transport from shoot-to-root tip, being strongly upregulated after wounding, leading to new shoots from the undergrowth roots (Ding et al. 2011; Anderson et al. 2012).

Apart from auxins, levels of other hormones increase after wounding and are likely involved in the regulation of new tissue formation. That is the case of ethylene and JA, whose levels increase after sectioning tissues (Ahkami et al. 2009;

Anderson et al. 2012). Recently, it was suggested that the crosstalk between ethylene and ABA and the signals generated by the loss of the polar auxin transport induced by wounding (Grossmann and Hansen 2001) are central in the redistribution of auxins in roots that allow the new shoot growth (Anderson et al. 2012). However, ethylene can promote adventitious roots but at the same time inhibit lateral root development, by affecting auxin transport (Negi et al. 2010). Maybe those differences in ethylene and auxin crosstalk are ascribable to the specific role of ethylene during different stages of adventitious root development. In this sense, it was reported that after a stress, ethylene could stimulate auxin transport, which is accumulated in the stem and hence induces an additional ethylene synthesis, through the induction of *ACS* genes. Hence, this newly synthesized ethylene induces a new auxin flow to the stem that stimulates the growth of preformed root initials (Swarup et al. 2007; Vidoz et al. 2010).

Shortly after root wounding, JA levels transiently increased (Ahkami et al. 2009), a response that has been also reported as common to other different abiotic stress (de Ollas et al. 2013). After an initial peak in response to wounding, JA levels are reduced by conjugation with amino acids (Gutierrez et al. 2012), an effect that is related to ARF6 and ARF8 transcriptional factors, both positive regulators of AR (Gutierrez et al. 2009). Hence, ARF6 and ARF8 upregulate the transcription of GH3.3, GH3.5, and GH3.6 genes, responsible of the JA conjugation with amino acids. However, observations made with *Arabidopsis coi-1* mutants suggest that JA regulation should be acting through both auxin-dependent and auxin-independent pathways (Raya-González et al. 2012). Since initiation of AR was described to be mediated by COI1-dependent JA pathway (Sun et al. 2009) in a negative way, these mutants in contrast were able to promote lateral roots outgrowth only after JA application (Raya-González et al. 2012). Hence, a differential mechanism could be acting in adventitious root or lateral roots formation regarding JA. Therefore, more information is needed to elucidate the role of JA signaling and how it interplays with auxins in the control of adventitious root, lateral roots, and lateral shoot budbreak. In this sense, more attention should be focused on newly described phytohormones like BR, which could also be interplaying in this response since these compounds negatively regulate the JA-induced inhibition of primary root growth which is also related to the induction of lateral roots (Huang et al. 2010; Miller et al. 2010).

Similar to ST, CK also inhibit both lateral root development and adventitious root (Corrêa et al. 2005; Laplaze et al. 2007; Rasmussen et al. 2012; da Costa et al. 2013). However, axillary bud growth is promoted by CK whereas it is inhibited by SL (Dun et al. 2012). In the case of roots, CK inhibit lateral outgrowth by regulating auxin transport, through the downregulation of PIN1 and upregulation of MIZ1 (a gene involved in hydrotropism), which reduced auxins accumulation (Laplaze et al. 2007; Moriwaki et al. 2011). Recently, it was demonstrated that CK inhibit adventitious root independently of ST and vice versa (Rasmussen et al. 2012). However, this inhibition is mainly exerted during the first stages of induction (Corrêa et al. 2005; Ramírez-Carvajal et al. 2009).

Gibberellin Crosstalk Under Abiotic Stress Conditions

It is well known that GA regulates many aspects of plant growth and development, including germination, growth, and flowering. The key components of GA signaling include DELLA proteins, the GA receptor GID1, and the F-box proteins SLEEY1 (SLY1) and SNEEZY (SNZ). Once GID1 receptor binds to GA, it is able to capture a nuclear growth-repressing DELLA protein. This complex is subsequently polyubiquitinated and the DELLA protein finally degraded by E3 ubiquitin-ligase $SCF^{SLY1/GID2/SNZ}$ (Nakajima et al. 2006; Murase et al. 2008; Ariizumi et al. 2011). Thus, the DELLA proteins act to restrain plant growth, while GAs promote it by targeting them for destruction (Shimada et al. 2008).

However, the DELLA proteins are not exclusive from the GA signaling pathway and interact with other hormonal and environmental signaling molecules. Therefore, they are involved in different aspects of plant growth, development, and adaptation to stress situations (Achard et al. 2006; Hou et al. 2010). An example of this crosstalk is provided by how DELLA proteins regulate photomorphogenesis through their interaction with PIF3 and PIF4 bHLH domains and the blockage of their DNA-binding activity (de Lucas et al. 2008). Another example is the physical interaction of DELLA with JAZ proteins (the major repressors of JA signaling), which inhibit the activity of MYC2 as a transcriptional activator of the JA response. SCF^{COI1} degrades JAZ proteins to release MYC2 which, in turn, activates the expression of JA-responsive genes. Stabilized DELLA proteins compete with MYC2 for binding to JAZ proteins, thereby enhancing the capacity of MYC2 to regulate its target genes (Hou et al. 2010). In this way, DELLA proteins enhance plant tolerance to high salinity through JA signaling activation (Magome et al. 2004, 2008; Achard et al. 2006, 2008; Navarro et al. 2008). On the contrary, GA causes DELLA degradation, which potentiates the binding of JAZ1 to MYC2 and, therefore, the suppression of JA signaling. This is an example of a candidate mechanism by which JA signaling may be fine-tuned by other signaling pathways through DELLAs. Moreover, the cold-responsive transcription factor CBF1 controls accumulation of DELLA (Achard et al. 2008). In *Arabidopsis*, CBF1-activated GA 2-oxidases reduced the cellular GA content and caused enhanced accumulation of the growth-repressing DELLA protein RGA (Achard et al. 2008). Excitingly, loss of function mutation of GAI and RGA suppressed the freezing tolerance in *Arabidopsis* and provided evidence that DELLA proteins contribute to the survival of plants at low temperatures. Here, the DELLA-mediated growth restraint might allow the cellular reprogramming to activate stress adaptive mechanisms instead of cellular growth processes.

Quadruple mutants in *rga*, *gai*, *rgl1*, and *rgl2* (coding for DELLA proteins) show impaired salt tolerance demonstrating a role of DELLAs on ABA-dependent tolerance (Achard et al. 2006). The RING-H2 zinc finger factor XERICO is a convergent downstream target of both DELLA proteins and ABA, and the function of XERICO in modulating GA and ABA signaling pathways has been suggested (Zentella et al. 2007; Golldack et al. 2013). Intriguingly, RGL proteins have a regulatory function

in connecting and balancing crosstalk of GA and ABA in *Arabidopsis* seeds (Piskurewicz and Lopez-Molina 2009). These findings indicate that DELLA proteins are a regulatory hub that integrates endogenous developmental signals with adverse environmental conditions. DELLA proteins modulate the dynamics of hormone signaling and contribute to the ability of plants to survive.

Transcriptional regulators SCR (SCARECROW) and SHR (SHORTROOT) could also have a role as an interface of developmental and stress signaling. SCR and SHR are functionally related to hypersensitivity to ABA and sugar in *Arabidopsis*, and involvement of SCR in plant drought adaptation has been hypothesized (Cui et al. 2012). GRAS-type proteins can have different functions in signaling and cellular adaptation as indicated by the distinct roles of SCL14 (SCARECROW-like 14) in plant responses to xenobiotic stresses and involvement of SCL13, SCL14, and PAT1 in phytochrome A signal transduction (Torres-Galea et al. 2006, 2013). Therefore, it seems that DELLA-mediated growth restraints are modulated by competition and interaction with other nuclear transcriptional regulators of the GRAS-type family of proteins to permit flexible responses of plant development to changes in environmental conditions (Golldack et al. 2013).

It is also well known that ABA and GA are the primary factors that regulate (antagonistically) the transition from dormancy to germination. These hormones interact at both the signal transduction level (see ABA section in this chapter). Furthermore, GA synthesis is enhanced in the *aba2* mutant, indicating that ABA is involved in the suppression of GA biosynthesis (Seo et al. 2006). It has been recently shown that ABI4 (an AP2/ERF transcription factor, involved in the ABA signal transduction pathway in seeds) could be the molecular switch that balances ABA and GA biosynthesis (Shu et al. 2013).

GA interaction with other hormone signaling pathways under abiotic stress conditions has been recently shown in Alonso-Ramírez et al. (2009). In this work, exogenous application of gibberellic acid (GA_3) was able to reverse the inhibitory effect of salt, oxidative, and heat stresses in the germination and seedling establishment of *Arabidopsis* plants, this effect being accompanied by increases in SA concentration, and in the expression levels of the isochorismate synthase 1 and nonexpressor of PR1 genes, involved in SA biosynthesis and action, respectively. In the same work, it was proved that transgenic plants overexpressing a GA-responsive gene from beechnut (*Fagus sylvatica*), coding for a member of the GASA family (GA_3-stimulated in *Arabidopsis*), showed a reduced GA dependence for growth and improved responses to salt, oxidative, and heat stress at the level of seed germination and seedling establishment. In the seeds of these transgenic plants, the improved behavior under abiotic stress was accompanied by an increase in SA endogenous levels. All these data taken together suggested that GA are able to counteract the inhibitory effects of adverse environmental conditions in seed germination and seedling growth through modulation of SA biosynthesis.

Recently, it has been identified that the transcription factors ERF5 and ERF6 act as master regulators that adapt leaf growth to environmental changes. ERF5 and ERF6 gene expression is induced specifically in actively growing leaves by water stress conditions. ERF6 inhibits cell proliferation and leaf growth by a process

involving GA and DELLA signaling. It has also been demonstrated that ERF6 induces the expression of the GA-degrading enzyme GA2ox6 and, consequently, DELLA proteins are stabilized. ERF6 also activates the expression of a plethora of osmotic stress-responsive genes, including the well-known stress tolerance genes STZ (salt tolerance zinc finger), MYB51, and WRKY33 (Dubois et al. 2013).

Cytokinin Crosstalk Under Abiotic Stress

As key regulators of root system architecture, CK play a main role in abiotic stress adaptation. Decreases in the CK levels retard differentiation of the root meristem (Werner et al. 2003) and lead to a larger root system and a higher root-to-shoot ratio. CK also play a role in delaying leaf senescence under stress conditions, antagonizing the effect of other hormones such as ABA (Jia et al. 2013), ethylene (Zhang and Zhou 2013), JA (Yan et al. 2012), and SA (Miao and Zentgraf 2007). These features make these nitrogenous compounds, derived from nucleotides, a key hormone in controlling morphological adaptation to abiotic stress.

Therefore, CK overproduction in transgenic plants led to a significant tolerance to water deficit (Zhang et al. 2010). In early studies, a correlation between nitrogen nutrition and stomatal response was found and, surprisingly, addition of kinetin influenced this response (Radin et al. 1982). In these experiments, kinetin had no significant effect on stomatal movement but appeared to modulate stomatal response to exogenous ABA treatment. It is known that abiotic stress increases endogenous ABA levels and concomitantly decreases overall CK concentration in a way correlated with the downregulation of cytokinin oxidase expression as well as the activity of other enzymes involved in their catabolism. This coregulation appeared to be dependent on ABA signaling (Wilkinson et al. 2012). In tomato, transformation of plants with isopentenyl transferase (IPT)-encoding gene under the control of the *HSP70* promoter leads to increased zeatin and zeatin riboside levels upon salt stress exposure along with higher root temperature. In addition, these plants showed lower ABA levels in all tissues including roots, xylem sap, and leaves, improving relative growth rate (Ghanem et al. 2011). The advantages of plants expressing *IPT* gene over wild-type plants growing in a salinized medium might be associated to the maintenance of cell division, improved carbon status, and delayed stomatal closure probably associated to low ABA levels. It could be expected that decreasing ABA levels (and subsequently allowing a higher stomatal aperture) would increase NaCl uptake (Gómez-Cadenas et al. 2002). Nevertheless, elevated levels of CK apparently increased K^+/Na^+ ratio in leaves, reducing the damaging effects of salinity.

In wheat, levels of CK were increased after EBR (24-epibrassinolide, an active BR) treatment through the inhibition of CK oxidase-/dehydrogenase-encoding *CKX* gene expression. These data indicated that BR are involved in the regulation of CK metabolism. In addition, it could be suggested that the physiological effects of BR could be partially due to a direct effect on CK biosynthesis (Yuldashev et al. 2012). In line with this, it was recently reported that exogenous application of BR had an

effect on the expression of CK primary response genes such as *ARR5* (Kudryakova et al. 2012). In this work, several BR molecules such as brassinolide, EBR, homobrassinolide, and 6-*O*-carboxymethyloxohomocastasterone were tested on transgenic *Arabidopsis* plants carrying the *pARR5::GUS* construct. In these plants, application of benzyladenine increased β-glucuronidase activity about threefold. Enhanced *GUS* expression was also observed with BR application although to a more moderate level (Kudryakova et al. 2012). In addition, application of BR also increased GUS activity in transgenic plants expressing the *uidA* gene under the control of CK-dependent histidine kinases (AHKs, Kudryakova et al. 2012), indicating that BR also influenced CK signaling, probably through the regulation of their metabolism.

Brassinosteroid Crosstalk Under Abiotic Stress

The BR are a group of plant growth regulators that show a close structural similarity to steroid hormones from arthropods and mammals (Müssig and Altmann 1999). In plants, BR are synthesized form campesterol, a membrane sterol, and are highly abundant in young growing tissues, in pollen, and in immature seeds (Bartwal et al. 2012). This new class of phytohormones not only is known to elicit a series of plant responses associated to normal growth and development but also is involved in the adaptation of plants to adverse environmental conditions (directly or through the modulation of other plant growth regulators). To this respect, it has been shown that BR regulate IAA long-distance transport by modulating *PIN* gene expression and ROS signal, thereby influencing systemic stress responses (Xia et al. 2011). Hence, exogenous treatment of cucumber plants with EBR not only prevented photooxidation after paraquat treatment but, conversely, also induced systemic H_2O_2 accumulation, leading to an increase in the expression of several genes, such as the cytosolic *APX*, *PR-1*, and *WRKY6* that are involved in defense (Xia et al. 2011). Furthermore, this effect on stress tolerance in cucumber seemed to be associated to NO production since the application of EBR along with the NO quencher PTIO failed to increase the activity of antioxidant enzymes catalase, superoxide dismutase, ascorbate peroxidase, and glutathione reductase (Cui et al. 2011). Under different stress conditions, the exogenous application of BR increased stress tolerance in different plant species (Bartwal et al. 2012). For instance, radish plants grown on Cu^{2+} contaminated media reduced by 50 % the uptake of this heavy metal after treatment with BR. However, the combined treatment with BR and spermidine recovered control Cu^{2+} concentration in tissues (Choudhary et al. 2012a).

BR have also been reported to interact with GA in rice (Wang et al. 2009). In this plant species, the gene *OsGSR1* was induced by GA application but repressed after BR treatment. By silencing the expression of *OsGSR1*, it was possible to associate the resulting dwarf phenotype to BR deficiency. This altered phenotype was rescued by exogenous BR application, suggesting the involvement of OsGR1 in BR biosynthesis (Wang et al. 2009). To this respect, it has been recently reported that

brassinazole-resistant 1 (BZR1), a transcription factor activated upon BR signaling, interacts with RGA (repressor of GA1–3), a member of the DELLA protein family which inhibits GA signaling in *A. thaliana*. Overexpression of DELLA proteins reduced BZR1 activity, suggesting an antagonistic relationship between BR and GA signaling pathways (Li et al. 2012b). In rice, GA- and SA-mediated immunity against the oomycete *Pythium graminicola* was antagonized by BR, showing a negative crosstalk among these hormonal factors. This crosstalk occurred downstream of SA biosynthesis but upstream of OsNPR1 and OsWRKY45, whereas BR negative crosstalk affected GA metabolism, subsequently preventing DELLA degradation (De Vleesschauwer et al. 2012). Recently, it has been reported that GAI protein, the major negative regulator of the GA signaling pathway, physically interacts with BZR1 resulting in the deactivation of its transcriptional regulatory activity (Gallego-Bartolomé et al. 2012).

Interactions between BR and ABA have been also reported in seed germination, for instance, BR-related *Arabidopsis* mutants *det2-1* (de-etiolated 2-1) and *bri1-1* (brassinosteroid-insensitive 1-1) showed increased sensitivity to the inhibitory effects of ABA compared to WT (Choudhary et al. 2012b). It is generally known that brassinosteroid-insensitive mutants show severe pleiotropic effects associated to developmental processes; hence, *bri* mutants show dwarfism, de-etiolation, male sterility, and altered leaf morphology. Characterization of these mutants has allowed the identification of the BR receptor BRI1 (Bartwal et al. 2012). In tomato, this receptor has been found to act as a receptor for the peptide hormone systemin that mediates responses to wounding and insect predation. At the whole-plant level, co-application of ABA and EBR had a synergistic effect toward drought protection over that observed after application of ABA alone. Nevertheless, in *Arabidopsis* BR-deficient mutants, application of ABA alone had an enhanced effect on stomatal closure, and EBR application increased expression of drought-responsive genes RD29A, ERD10, and RD22 (Acharya and Assmann 2009).

BR are known to influence ethylene biosynthesis through the regulation of ACC synthase and ACC oxidase activities (Hansen et al. 2009). The characteristic hyponastic growth is associated to soil flooding and mediated by ethylene which, in turn, also regulates the expression of *ROTUNDIFOLIA3*/*CYP90C1*, a gene that encodes a protein involved in C23 hydroxylation of several BR. The inhibition of BR biosynthesis as well as the influence of ethylene on the expression of BR-dependent genes was tested indicating that BR was involved in hyponastic cell expansion induced by ethylene (Polko et al. 2013). Moreover, response to submergence in rice is mainly regulated by ethylene through *SUB1A* gene that encodes an ERF protein. This transcription factor differentially regulates genes involved in BR biosynthesis during submergence, and pretreatment with EBR increased tolerance to submergence. Besides, it was found that BR reduced GA levels and induced *SLR1* expression. Together, these results indicate that BR might mediate ethylene-regulated responses through modulation of GA signaling (Schmitz et al. 2013). This mechanism is in line with the abovementioned findings about the antagonistic crosstalk between BR and GA signaling pathways.

Auxins induce *DWF4* expression, a gene that encodes a steroid 22 α-hydroxylase which is rate limiting for BR biosynthesis, whereas BR repress the expression of this gene, as a negative feedback mechanism that limits its own signaling. In turn, BR signaling influences sublocalization of both influx PINs and efflux AUX1/LAXs auxin carriers (Saini et al. 2013). Plants carrying mutations in *AUX/IAA* genes showed altered sensitivity to BR, measured as the ability to develop new roots. In addition to this, BIN2 is activated by auxins (although the exact mechanism is still unknown) but repressed by BR. This repressor mediates ARF2 phosphorylation that results in a loss of DNA-binding repression activity.

Strigolactone Crosstalk Under Abiotic Stress

The ST are a new class of plant hormones first reported in 1966 as stimulators of the germination of parasitic plant species such as *Orobanche* and *Striga* (from which the main ST took their names: orobanchol and strigol). These molecules have been identified as signaling compounds that mediate symbiotic interactions between plant roots and arbuscular mycorrhizal fungi (AMF) as root exudates, regulate the above- and belowground plant architecture, and also might be involved in other developmental and stress response processes (Marzec et al. 2013). These molecules are synthesized from b-carotene in plastids by isomerization, carried out by D27, followed by desaturation catalyzed by MAX3 dioxygenase in *Arabidopsis* and the subsequent synthesis of carlactone catalyzed by MAX4 and, finally, the synthesis of 5-deoxystrigol carried out by the cytochrome P450 monooxygenase MAX1. Most of the information on the physiological role of ST comes from the study of its biosynthetic *max* mutants (Marzec et al. 2013).

In the induction of shoot and root branching, ST are known to act downstream the auxin signaling pathway. Indeed, ST have been considered as second messengers in this signaling pathway that interact with IAA in a dynamic feedback loop (Bartoli et al. 2013). To this respect, the environmental control of primary root growth seems to be independent of ST. Nevertheless, ST could affect IAA levels by regulating its biosynthesis and/or polar transport as evidenced by higher auxin levels in ST-deficient mutants. This increased auxin transport in ST mutants might inhibit primary root growth and exogenous ST application, therefore, could revert this phenotype (Rasmussen et al. 2013).

In rice, treatment with NCED-specific inhibitors abamine and abamine-SG decreased ST release in exudates. This is in line with the results reported in López-Ráez et al. (2010) where ABA-deficient tomato mutants showed significantly decreased amounts of endogenous ST respect to their wild types. Similarly, inhibition of ABA biosynthesis with abamine-SG reduced not only root ABA level but also that of ST in phosphate-starved tomato plants. Taken together, these results suggest that ABA could be involved in ST biosynthesis, although the exact point of interaction is not yet known. It was proposed that ST could influence ROS levels,

which are secondary messengers in many different hormone signaling pathways. In this sense, *max2* mutant plants exhibit a delayed senescence phenotype associated to a higher tolerance to oxidative stress compared to wild type (Marzec et al. 2013). In response to high light, most of the upregulated transcripts in *Arabidopsis* cell suspensions were specifically associated to singlet oxygen production. These transcript levels remained unchanged in *aba1* and *max4* mutants, suggesting a relationship between ABA and ST signaling and the expression of these genes (González-Pérez et al. 2011), although further lines of evidence are needed to ascertain this crosstalk.

Conclusions and Prospects

All data revised in this chapter support the fact that under environmental constrains, plants display many mechanisms to avoid, tolerate, or adapt to the new conditions. Abiotic stress triggers responses at the whole plant level, involving an intricate crosstalk mechanism among hormones. Thus, interactions among these compounds integrate diverse input signals to cope with highly variable environmental conditions. Multiple and redundant responses from hormone-dependent signalings seem to be a part of a strategy to adapt to a vast array of unfavorable conditions.

It is also expected that more findings contribute to enrich the already important amount of data explaining the molecular mechanisms that regulate these hormonal crosstalks. In this sense, it is important to understand some of the hormonal interactions at the biosynthesis level, where catabolism must play an important role. Elucidation of cellular processes governing hormone homeostasis seems essential for understanding developmental and defense-related processes mediated by different group of hormones.

Finally, it will be important to find divergent experimental systems. On one hand, future work must focus on specific hormonal crosstalk in particular tissues and for this simplified plant systems are a good choice. On the other hand, considering the plant as a whole will provide further information among interactions that can be hidden so far due to the massive use of model plants in early stages of growth or cultivated in artificial conditions. This kind of systems will also help to appreciate the physiological significance of the putative interactions to avoid their overestimation. In addition, new experiments based on whole-plant responses will help to establish the bases for genetic manipulation to improve crop performance and yield. Specific hormone interactions could represent targets for breeding/managing for yield resilience under multiple stress situations.

Finally, it is proposed that models will be continuously revised avoiding generalization based on limited experimentation. This is crucial when dealing with hormonal interactions where only combined work at molecular, genetic, and physiological levels will provide solid models.

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Plant Hormone Crosstalks Under Biotic Stresses

Hiroshi Takatsuji and Chang-Jie Jiang

Abstract Plants have developed defense signaling systems to protect themselves from invading pathogens. Plant hormones such as salicylic acid, jasmonates, and ethylene act as signals to trigger and mediate a diverse array of defense responses. Other hormones such as abscisic acid, auxin, gibberellic acids, cytokinins, and brassinosteroids, which were previously implicated in developmental and abiotic stress responses, also play important roles in defense signaling against pathogens. These hormone signaling pathways interconnect in an antagonistic or synergistic manner, providing plants with a vast regulatory potential to adapt rapidly to their biotic environment and to use their limited resources for growth and survival in a cost-efficient manner. On the other hand, pathogens have developed strategies to manipulate the signaling network and increase their virulence. This chapter reviews recent progress in research on the roles of hormone signaling pathways and their interactions in plant defense, mainly focusing on the salicylic acid signaling pathway and its interactions with other pathways. In addition to studies on *Arabidopsis* and other dicots, we also discuss some of the studies on rice, a monocot model plant, because such studies have provided some additional insights into the effects of signaling crosstalks on resistance to abiotic and biotic stresses. We also discuss some of the biotechnological and pharmaceutical strategies to manipulate defense hormone signaling to improve the disease tolerance of crops.

Keywords Rice • *Arabidopsis* • Induced resistance • Signaling • Salicylic acid • Biotechnology

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Introduction

In nature, plants are continuously threatened by a wide range of pathogens and pests, including viruses, bacteria, fungi, oomycetes, nematodes, and insect herbivores. Plants have an array of structural barriers and preformed antimicrobial metabolites to prevent invasion by these potential attackers; however, some of them occasionally succeed in breaking through this preinvasive layer of defense. To counteract these attackers, plants have developed a broad spectrum of inducible defense strategies to translate the perception of the attackers into effective immune responses. Plant hormones such as salicylic acid (SA), jasmonates (JAs), and ethylene (ET) act as signals to trigger and mediate a diverse array of defense responses. In the past decade, other hormones that have previously been implicated in plant development and abiotic stress responses, such as auxin, gibberellic acids (GAs), brassinosteroids (BRs), abscisic acid (ABA), and cytokinins (CKs), have also emerged as critical factors that are actively involved in plant immunity and play roles in fine-tuning immunity and growth/development (Bari and Jones 2009; Grant and Jones 2009). There is mounting evidence that these hormones influence disease outcomes by feeding into the SA–JA–ET backbone of the immune signaling circuitry (Robert-Seilaniantz et al. 2007, 2011a). Such interplay or crosstalks among the signaling pathways of individual hormones presumably enable plants to tailor their inducible defense system to the type of invader encountered under particular environmental conditions and to use their limited resources in a cost-efficient manner.

Most of our current knowledge about hormone-based defense signaling pathways and the interactions among them was obtained from studies on *Arabidopsis thaliana*. However, studies on monocots such as rice have provided additional important insights into the role of phytohormones in defense responses. Rice is not only one of the most important staple foods worldwide but also an excellent model monocot plant because of its fully sequenced genome, its ease of transformation, and the wealth of genetic and molecular resources that are available for it. Although information about hormone crosstalks in rice is still limited, there are increasing efforts to elucidate the roles of various hormones in its immune responses and to identify the regulatory components involved.

In this chapter, we review recent advances in research on the roles of hormones and their crosstalk in the immune responses of plants, with an emphasis on the SA-signaling pathway and its interactions with other hormone signaling pathways. As well as reviewing important studies on *Arabidopsis*, we also discuss some recent studies on other plants, especially rice. We also discuss some of the ways in which hormone signaling pathways could be modified to improve the disease tolerance of crops.

Salicylic Acid Signaling Pathway

Sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) initiates PAMP-triggered immunity (PTI) to prevent pathogen colonization (Nurnberger et al. 2004; Ausubel 2005; Boller and Felix 2009;

Schwessinger and Ronald 2012; Zipfel 2009). As a second layer of induced defense, resistance (R) proteins in plants recognize effector proteins secreted by microbial pathogens and trigger strong disease-resistance responses (effector-triggered immunity, ETI). The ETI is usually associated with hypersensitive responses (HR) characterized by rapid programmed cell death at the sites of infection (Jones and Dangl 2006; Dodds and Rathjen 2010; Spoel and Dong 2012). The SA-mediated immune responses are important components of both PTI and ETI (Tsuda et al. 2009). Both PTI and ETI activate secondary immune responses in distal uninfected tissues, resulting in systemic acquired resistance (SAR) (Durrant and Dong 2004), in which SA also plays a pivotal role. Pathogen infection often induces the accumulation of SA in infected leaves of various plant species. SA also accumulates in distal leaves that develop SAR and often parallels or precedes the increase in expression of *Pathogenesis-related* (*PR*) genes and the development of SAR (Malamy et al. 1990; Métraux et al. 1990). Application of exogenous SA or its functional analogs, such as 2,6-dichloroisonicotinic acid (INA), benzothiadiazole S-methyl ester (BTH), and probenazole, has been shown to activate the expression of the *PR* genes and resistance against viral, bacterial, oomycete, and fungal pathogens in a variety of dicots (Malamy et al. 1990; Métraux et al. 1991; Friedrich et al. 1996; Lawton et al. 1996; Yoshioka et al. 2001) and monocots (Iwata et al. 1980; Görlach et al. 1996; Pasquer et al. 2005; Makandar et al. 2006; Iwai et al. 2007). These chemicals have no direct effect on pathogens; therefore, they are less likely to lead to drug resistance in pathogens, a side effect that is often problematic for fungicides and bactericides. Because of such favorable activities, they are commercially produced and broadly used in agriculture as chemical defense inducers (also known as plant activators). Blocking SA accumulation by expressing an SA-degrading enzyme in transgenic tobacco (*Nicotiana tabacum*) and *Arabidopsis* abolished SAR (Gaffney et al. 1993; Delaney et al. 1995). Mutations in SA biosynthetic genes were shown to enhance susceptibility to pathogens, and resistance could be restored by exogenous SA application (Mauch-Mani and Slusarenko 1996; Nawrath and Metraux 1999; Wildermuth et al. 2001). Collectively, the results of these and other studies showed that the SA-signaling pathway is central in the defense mechanism of plants and is also the major target for disease control in agriculture.

There are a number of regulators that act upstream of SA and affect SA accumulation (Tsuda et al. 2009; An and Mou 2011). A transcriptional co-activator NONEXPRESSOR OF PR1 (NPR1) is a key regulator of the SA-mediated defense signaling pathway, acting downstream of SA in *Arabidopsis* (Cao et al. 1997; Dong 2004) and other plant species (Chern et al. 2005; Malnoy et al. 2007; Endah et al. 2008; Le Henanff et al. 2009). In the absence of SA or pathogen challenge, NPR1 is retained in the cytoplasm as an oligomer through redox-sensitive intermolecular disulfide bonds. Upon induction, the NPR1 monomer is released to enter the nucleus, where it activates defense gene transcription (Mou et al. 2003). This process is regulated by the sensing of cellular redox changes by NPR1 after its S-nitrosylation (Tada et al. 2008). As a transcriptional cofactor, NPR1 interacts with members of the TGA family of transcription factors (TFs), thereby directly regulating defense genes such as *PR1* (Despres et al. 2003; Johnson et al. 2003; Durrant and Dong 2004). Members of the WRKY TF family also act downstream

of NPR1 (Wang et al. 2006). A negative regulator of NPR1 (NIM1-INTERACTING1) antagonizes the NPR1-dependent SA pathway through direct binding to NPR1 (Weigel et al. 2001, 2005). An SA-dependent but NPR1-independent signaling pathway(s) is also present and operates during early phases of SA pathway activation (Li et al. 2004; Uquillas et al. 2004; Blanco et al. 2005). NPR1 undergoes degradation by the ubiquitin–proteasome system (UPS) in the nucleus (Spoel et al. 2009). It has been proposed that NPR1 is regulated by the UPS in two ways: first, the UPS constitutively degrades NPR1 to suppress spurious activation of defense responses in the absence of pathogen attack; and second, SA-induced degradation of NPR1 by the UPS results in full-scale activation of the transcriptional activity of NPR1. Recently, it was reported that NPR1 itself, as well as NPR3 and NPR4 are SA receptors. Wu et al. (2012) found that SA binds to NPR1 and induces a conformational change that relieves the repression of its transcriptional activation domain by its autoinhibitory N-terminal domain. Meanwhile, Fu et al. (2012) proposed that NPR3 and NPR4 modulate the immune response by controlling the proteasomal degradation of NPR1 in an SA-dependent manner.

The importance of the SA pathway was controversial during the early phases of research on defense signaling in rice. This is because basal SA levels in rice leaves are very high (8–37 μg/g fresh weight), and the levels do not change significantly, either locally or systemically, upon pathogen attack (Silverman et al. 1995). In contrast, in tobacco and *Arabidopsis*, basal levels of SA are low (<100 ng/g fresh weight), but they can markedly increase upon pathogen infection (Malamy and Klessig 1992). In rice, SA at high levels functions as an antioxidant that protects plants from oxidative damage caused by aging, pathogen attack, or abiotic stress (Yang et al. 2004). However, there is increasing evidence for the importance of the SA-signaling pathway in mediating defense signaling in rice. Despite the high endogenous levels of SA in rice, exogenous application of SA and SA analogs leads to activation of defense against pathogens (Shimono et al. 2007). The SA levels increased in response to probenazole, a chemical defense inducer acting upstream of SA, in adult rice plants, but not in juvenile ones (Iwai et al. 2007). Like the SA-signaling pathway in *Arabidopsis*, the SA-signaling pathway in rice also involves an NPR1 protein (OsNPR1/NH1) that acts as a signaling component downstream of SA (Chern et al. 2001; Fitzgerald et al. 2004; Yuan et al. 2007; Sugano et al. 2010). Unlike *Arabidopsis* NPR1, proteasome degradation of OsNPR1 was not observed (Matsushita et al. 2013). Whereas *NPR1* overexpression in *Arabidopsis* did not provoke defense reactions until induction by chemicals or pathogen infection (Cao et al. 1998), overexpression of *OsNPR1* in rice induced constitutive activation of *PR* gene expression, resulting in strong resistance to the leaf blight bacterial pathogen *Xanthomonas oryzae pv. oryzae* (*Xoo*) and the blast fungus *Magnaporthe oryzae* (Chern et al. 2005; Sugano et al. 2010). The rice protein WRKY45 was identified as a TF that is essential for resistance to *M. oryzae* and *Xoo* induced by the chemical inducers BTH, probenazole, and tiadinil (Shimono et al. 2007, 2012; Takatsuji et al. 2010). While NPR1 in *Arabidopsis* regulates nearly all (>99 %) of the BTH-responsive genes, the rice SA pathway appears to branch into OsNPR1-mediated and WRKY45-mediated sub-pathways (Shimono et al. 2007;

Sugano et al. 2010). Besides upregulating the genes directly involved in defense reactions, OsNPR1 downregulates several genes involved in photosynthesis and protein synthesis, suggesting that this protein functions to relocate energy and resources from housekeeping cellular activities, such as photosynthesis, to defense reactions (Sugano et al. 2010). WRKY45 proteins are degraded by the UPS in the nucleus (Matsushita et al. 2013). Furthermore, similar to *Arabidopsis* NPR1, WRKY45 also appears to be regulated by the UPS in two ways, that is, (1) constitutive degradation to suppress spurious defense activation in the absence of pathogens and (2) SA-induced degradation to enhance the transcriptional activity of WRKY45 (Matsushita et al. 2013). Rice transformants overexpressing *WRKY45* (*WRKY45*-ox) showed extremely strong resistance to both *M. oryzae* and *Xoo*, but not to *Rhizoctonia solani*, the causal agent of sheath blight disease (Shimono et al. 2007, 2012; Takatsuji et al. 2010). There was only a small fitness cost of *WRKY45* overexpression in terms of the trade-off between growth and resistance level (reduced growth for enhanced resistance). Presumably, this is in part because of its degradation by the UPS to suppress spurious defense activation in the absence of a pathogen. A synergistic interaction between SA and CKs is also likely to contribute to the reduction of the fitness cost (see below). Nevertheless, the lines overexpressing *WRKY45* under the control of a strong constitutive promoter showed substantial growth retardation in the field trials. Additionally, growth retardation was exacerbated by low temperature and high salinity, presumably because of currently unknown signaling crosstalks (Goto et al., unpublished). Recently, however, we successfully improved crop yield while retaining strong resistance to *M. oryzae* and *Xoo* by using a lower-activity promoter or pathogen-inducible promoters to drive *WRKY45* expression (Goto et al., unpublished).

Some of the enzymes that are directly or indirectly related to the SA pathway can significantly modify SA signaling. For example, OsSGT1 (*Oryza sativa* UDP-glucose:SA glucosyltransferase 1) promoted probenazole-inducible resistance by catalyzing the conversion of free SA into SA-O-ß-glucoside (Umemura et al. 2009). *OsSSI2*, the ortholog of *Arabidopsis SSI2* (*suppressor of SA insensitivity 2*), which encodes a fatty acid desaturase, was shown to act upstream of WRKY45 to negatively regulate WRKY45-dependent resistance to *M. oryzae* and *Xoo* (Jiang et al. 2009).

Other Plant Hormones and Their Interactions with SA

Jasmonic Acids

Jasmonic acid and its metabolites, including methyl jasmonate (MeJA), are lipid-derived hormonal molecules that regulate many aspects of plant growth and development, as well as plant responses to biotic and abiotic stresses (Bowles 1997; Enyedi et al. 1992; Koda 1992). In biotic stress responses of many dicots, the JA signaling pathway is primarily induced by, and mediates resistance against,

herbivores and necrotic pathogens, while the SA pathway is induced by, and defends against, biotrophic pathogens (Glazebrook 2005). Jasmonic acid alone activates plant responses to wounding and herbivory, but the presence of ET enhances defenses against necrotrophic pathogens. The interaction between SA and JA signaling pathways can have a synergistic effect to enhance resistance against pathogen attacks (Schenk et al. 2000; van Wees et al. 2000). However, the interaction between these hormones is more often antagonistic, and the induction of one pathway attenuates the other (Feys and Parker 2000; Kunkel and Brooks 2002). This JA–SA antagonism has been observed in as many as 17 plant species in various taxonomic groups, suggesting that this interaction is evolutionarily conserved in angiosperms or that it evolved even before the split of gymnosperms and angiosperms (Thaler et al. 2012).

Many biotrophic pathogens exploit the JA–SA antagonism to attenuate host defenses (Glazebrook 2005). For instance, some strains of *Pseudomonas syringae* produce a polyketide phytotoxin known as coronatine (Bender et al. 1999) that structurally resembles a JA derivative, JA–isoleucine (Fonseca et al. 2009b). The pathogen-derived coronatine suppresses SA signaling through the SA–JA antagonistic signaling networks, leading to disruption of plant immune responses and a fitness advantage for the pathogens (Brooks et al. 2005; Laurie-Berry et al. 2006). In the presence of coronatine, JAZ (jasmonate ZIM-domain) proteins that repress JA responses are ubiquitinated by the F-box component COI1 (coronatine insensitive 1) of the E3 ubiquitin ligase complex. The ubiquitinated proteins are subsequently degraded by the 26S proteasome to activate JA signaling (Chini et al. 2007; Thines et al. 2007; Fonseca et al. 2009a), which in turn suppresses SA signaling. While SA–JA crosstalk can be exploited by pathogens to enhance virulence, its true function in plants is presumably to establish a hormonal balance that favors host defense and survival in response to biotic stress; that is, plants use SA signaling to suppress the JA-pathway-mediated virulence strategy of pathogens. This strategy seems to be effective in fine-tuning plants' responses against single biotrophic pathogens. However, the resistance trade-off in which infection by biotrophs renders plants more susceptible to necrotrophs (or vice versa) may be detrimental when plants are attacked simultaneously by biotrophic and necrotrophic pathogens. Spoel et al. (2007) and Spoel and Dong (2008) proposed a threshold model that included spatial (local and systemic) and temporal gradients of SA and JA concentrations. They proposed that antagonistic SA–JA crosstalk operates transiently at infection sites but not in systemic tissues. This model was supported by the observation that SA and JA acted synergistically when applied to plants at low concentrations, whereas a high concentration of one hormone antagonized the other (Mur et al. 2006).

In *Arabidopsis*, suppression of JA signaling by SA requires NPR1 (Spoel et al. 2003). Nuclear localization of NPR1, which is essential for SA-mediated defense gene expression, is not required for the suppression of JA signaling. Therefore, NPR1 modulates SA–JA crosstalk in the cytosol, but it probably has a different function in the nucleus (Spoel et al. 2003). Interestingly, ET also modulates SA–JA crosstalk and NPR1 was shown to play a role in this regulation (Leon-Reyes et al. 2009). The ET potentiated SA- and NPR1-dependent *PR1* transcription, while

it rendered the antagonistic effect of SA on methyl jasmonate-induced *PDF1.2* (*PLANT DEFENSIN 1.2*) and *VSP2* (*VEGETATIVE STORAGE PROTEIN2*) expression NPR1 independent (Leon-Reyes et al. 2009). Li et al. (2004) proposed that the transcription factor WRKY70 plays a role in controlling JA–SA crosstalk in *Arabidopsis*. In *WRKY70*-overexpressing plants, *PR* genes were constitutively activated in an SA- and NPR1-independent manner, whereas JA-regulated genes were repressed in an NPR1-dependent manner. *WRKY70* antisense lines showed reduced induction of *PR* genes but constitutively activated expression of JA-inducible genes. Recently, it was reported that an R2R3 MYB TF of *Arabidopsis*, AtMYB44, modulates the JA–SA antagonistic interaction through direct transcriptional control of *WRKY70* (Shim et al. 2013). A mitogen-activated protein (MAP) kinase, MPK4, was shown to act as a negative regulator of SA signaling and a positive regulator of JA signaling by suppressing *PAD4* (*PHYTOALEXIN DEFICIENT4*) and *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY1*), which also act as SA activators/JA repressors (Brodersen et al. 2006). The JAZ proteins mediate JA crosstalk with SA, ET, auxin, and GA (Kazan and Manners 2012). In the absence of JA, JAZ proteins repress the JA-responsive EIN3 (ETHYLENE INSENSITIVE 3) and EIL1 (ETHYLENE-INSENSITIVE3-LIKE 1) TFs, which suppress SA synthesis (Kazan and Manners 2012). Future research should clarify which component(s) is the node of convergence of the SA and JA signaling pathways.

The roles of JAs in defense responses appear to differ between rice and dicots. Application of JA to rice plants induces resistance to necrotrophs, e.g., *R. solani* (Taheri and Tarighi 2010), consistent with the effects of JA against necrotrophs in dicots. However, application of JA to rice also enhances resistance to the hemibiotrophic pathogens, e.g., *M. oryzae* and *Xoo* (Mei et al. 2006; Yamada et al. 2012; Riemann et al. 2013). Rice plants overexpressing the pathogen-responsive *WRKY30* gene showed resistance to *R. solani* and *M. oryzae*, concomitant with increased JA accumulation and induction of JA-responsive defense genes (Peng et al. 2012). The JA pathway also plays a pivotal role in rice defenses against root-knot nematodes (Nahar et al. 2011) and herbivores (Zhou et al. 2009; Ye et al. 2012). OsJAZ8 acts as a repressor of JA signaling and is degraded by the 26S proteasome pathway in a JA-dependent manner, similar to JAZ proteins in *Arabidopsis* (Yamada et al. 2012), indicating conservation of the JA signaling pathway between rice and *Arabidopsis*. However, the generally accepted theory that JA-mediated defense is effective against necrotrophs but not biotrophs does not hold true for rice, because in rice, JA can also induce resistance to hemibiotrophic pathogens.

Although the role of JAs in defense responses in rice is not easy to predict based on the lifestyles of pathogens, there is some evidence that SA–JA antagonism is also conserved in rice. In rice roots, SA inhibited the induction of *RSOsPR10*, a root-specific rice *PR* gene (Takeuchi et al. 2011). JA levels rose while SA levels declined during early stages of the wounding response (Lee et al. 2004). These observations are consistent with antagonistic SA–JA crosstalk. Similar to *Arabidopsis* NPR1, rice OsNPR1 appears to regulate SA signaling positively and JA signaling negatively. Overexpression of OsNPR1 was characterized by strong activation of SA-responsive genes and concomitant suppression of JA marker genes (Yuan et al.

2007). Moreover, different subcellular localizations of OsNPR1 appear to be necessary for antagonistic regulation of the two signaling pathways, as is the case for *Arabidopsis* NPR1 (Yuan et al. 2007). *OsNPR1* antisense plants showed elevated JA levels and increased expression of JA biosynthetic genes upon insect infestation (Li et al. 2013). A role of OsWRKY13 in SA–JA crosstalk in activating the SA pathway while suppressing the JA pathway by acting upstream of OsNPR1 has also been suggested (Qiu et al. 2007, 2008, 2009). Positive interactions between the SA and JA pathways appear to be more common in rice than in *Arabidopsis*. In rice plants with a mutated hydroperoxide gene *OsHPL3* (*Oryza sativa hydroperoxide lyase 3*), activation of JA synthesis was accompanied by increased SA accumulation and increased SA-responsive *PR* gene transcription (Liu et al. 2012; Tong et al. 2012). In *OsPLD* (*Oryza sativa Phospholipases D*) *α3*/*α4* antisense rice, in which the genes for phospholipase D were silenced, activation of JA biosynthesis led to increased SA levels after infestation by rice striped stem borer (Qi et al. 2011). Microarray analysis showed that more than 50 % of the BTH-/SA-upregulated genes were also upregulated by JA (Garg et al. 2012; Tamaoki et al. 2013). Overall, the SA–JA antagonism in rice appears to be weaker than that in dicots, or it may be limited to particular tissue or conditions. Instead, positive interactions between SA and JAs are more prevalent in rice than in dicots. If it is true that the JA-induced susceptibility to biotrophs in *Arabidopsis* results from the suppression of SA signaling by JAs, then the weak SA–JA antagonism in rice could explain why JAs can induce resistance to hemibiotrophic pathogens in this monocot. Alternatively, the ability of JAs to induce resistance to hemibiotrophic pathogens in rice could be attributable to the presence of short necrotrophic phases during the infection process of these hemibiotrophic pathogens.

Ethylene

A bacterial PAMP flagellin triggers PTI in *Arabidopsis* after being perceived by FLS2, a plasma membrane receptor for flagellin. Plants with mutations in the key ET-signaling protein EIN2 showed impaired FLS2-mediated responses (Boutrot et al. 2010), demonstrating a pivotal role of ET in plant immune responses. Ethylene is generally thought to work together with JA in the resistance responses to necrotrophic pathogens and herbivore pests (Derksen et al. 2013). There is also increasing evidence that ET can both positively and negatively affect SA-mediated defense responses, depending on the different lifestyles of the pathogens (van Loon et al. 2006; Derksen et al. 2013). Ethylene and SA showed cooperative effects in the potentiation of *PR-1* gene expression in *Arabidopsis* (Lawton et al. 1994; De Vos et al. 2006) and SAR development in tobacco plants (Verberne et al. 2003). The EIN3 and EIL1 TFs repress SA biosynthesis. Accordingly, *ein2-1* single and *ein3-1*/*eil1-1* double mutants showed enhanced resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Chen et al. 2009).

Rice seedlings showed an increase in ET emission after infection by *M. oryzae* (Iwai et al. 2006). The increase in ET emission was much more rapid in

blast-resistant rice cultivars than in blast-susceptible cultivars, and in resistant cultivars there was a concomitant appearance of small necrotic lesions due to the HR and induction of *PR* genes (Iwai et al. 2006). Elevation of endogenous ET levels by exogenous application of ethephon (which is converted to ET in plant cells) enhanced blast resistance, while inhibitors of ET biosynthesis compromised it (Singh et al. 2004; Iwai et al. 2006). Overexpression of *OsACS2*, which encodes a key enzyme (1-aminocyclopropane-1-carboxylic acid synthase) in ET biosynthesis, under the control of a pathogen-inducible promoter, resulted in a large increase in ET and significantly enhanced resistance to *M. oryzae* and *R. solani* (Helliwell et al. 2013). Silencing of ET biosynthetic genes or *OsEIN2b* by RNAi rendered rice plants more susceptible to *M. oryzae* infection (Bailey et al. 2009; Seo et al. 2011). Thus, in rice, ET plays an important role in defenses against various pathogens including *M. oryzae*. Interestingly, upregulation of ET biosynthesis in rice was responsible for the partial *M. oryzae* resistance of plants grown under flooded or anaerobic soil conditions (Lai et al. 1999; Singh et al. 2004), which may be a strategy to evade pathogen infection after flooding. Seo et al. (2011) reported that the *Pi-i* gene-mediated resistance to blast fungus was compromised in transgenic rice lines with silenced ET biosynthetic genes. This resistance was restored by exogenous application of cyanide, a by-product of ET biosynthesis which inhibits the growth of blast fungus in vitro and in planta, explaining the mechanism of ET action in rice resistance to blast fungus.

As in dicots, ET has also been implicated in negative regulation of defense responses to some pathogens in rice (De Vleesschauwer et al. 2010; Shen et al. 2011). *OsEDR1* (*Oryza sativa ENHANCED DISEASE RESISTANCE 1*) is a rice ortholog of *Arabidopsis EDR1*, which encodes a MAPKK whose transcription is inducible by various environmental stresses and phytohormones including ABA, JA, ET, and SA (Kim et al. 2003). In transgenic plants in which this gene was silenced, the ACC synthase gene family was suppressed and ACC and ET levels were decreased, accompanied by enhanced resistance to *Xoo*. The resistance was associated with increased SA and JA levels and upregulated SA- and JA-responsive genes. These results suggested that, in the rice–*Xoo* interaction, OsEDR1 promotes ET synthesis, which in turn suppresses SA- and JA-mediated defense signaling. The antagonistic interaction between ET and JA signaling together with the simultaneous suppression of SA signaling are characteristic features of hormone signaling in rice. This pattern of interactions has not been observed in *Arabidopsis*, in which ET acts together with JA to negatively affect the SA pathway. In rice, ET was also implicated in negatively regulating resistance to the brown spot pathogen *Cochliobolus miyabeanus* (Xu et al. 2013).

Abscisic Acid

Abscisic acid has emerged as a key signaling molecule in plant–pathogen interactions, in addition to its roles in mediating abiotic signals and developmental regulation (Mauch-Mani and Mauch 2005; Asselbergh et al. 2008b). Exogenous ABA

application or increased endogenous ABA accumulation resulted from genetic defects enhances the susceptibility of various plant species to bacterial and fungal pathogens (Henfling et al. 1980; Matsumoto 1980; Ward et al. 1989; Mohr and Cahill 2003; Koga et al. 2004; Achuo et al. 2006; Fan et al. 2009). In contrast, enhanced resistance to various pathogens has been reported for ABA-deficient mutants of tomato (Audenaert et al. 2002; Achuo et al. 2006; Asselbergh et al. 2007, 2008a) and *Arabidopsis* (Mohr and Cahill 2003; de Torres-Zabala et al. 2007; Asselbergh et al. 2008b). The SA-dependent defense responses of these mutant plants are stronger than those of wild-type plants, suggesting that an antagonistic interaction between ABA- and SA-signaling pathways underlies the negative effect of ABA on plant defense responses. This idea is supported by studies showing antagonistic crosstalk between SA-dependent activation of SAR and ABA-mediated abiotic stress responses in *Arabidopsis* (Yasuda et al. 2008). Profound and drastic negative effects of ABA on plant defense pathways have been observed in many cases (Asselbergh et al. 2008b). For example, ABA pretreatment rendered potato slices vulnerable to infection by an incompatible isolate of the oomycete pathogen *Phytophthora infestans*, and even to infection by the fungal pathogen *Cladosporium cucumerinum*, which is normally nonpathogenic to potato (Henfling et al. 1980). Treatment with ABA also compromised the resistance of soybean to incompatible isolates of *Phytophthora sojae* (McDonald and Cahill 1999). On the other hand, several studies have shown that ABA can play positive roles in plant defense, e.g., via regulating stomatal closure (Melotto et al. 2006) and priming callose deposition (Ton and Mauch-Mani 2004; Flors et al. 2008). Thus, ABA appears to play different roles in defense depending on the lifestyles and infection stages of the pathogens (Mauch-Mani and Mauch 2005; Asselbergh et al. 2008b).

Many fungal and bacterial phytopathogens have developed mechanisms to disturb the balance of hormones in host plants as virulence strategies. This is achieved either by producing hormones themselves or by altering hormone synthesis in the host plants (Robert-Seilaniantz et al. 2007; Grant and Jones 2009). For example, *P. syringae* delivers type III effectors into host plant cells to induce ABA synthesis *in planta* during the interaction with *Arabidopsis*, thereby suppressing SA production and the basal defense in host plants (de Torres-Zabala et al. 2007). Several fungal pathogens such as *B. cinerea*, *Ceratocystis coerulescens*, *Fusarium oxysporum*, and *R. solani* are able to produce ABA (Dörffling et al. 1984; Kettner and Dörffling 1987; Oritani and Kiyota 2003), although its relevance in their pathogenicity remains unknown.

In rice, exogenous application of ABA compromised rice resistance to *M. oryzae* (Matsumoto 1980; Koga et al. 2004; Bailey et al. 2009; Jiang et al. 2010), *Xoo* (Xu et al. 2013), and the migratory nematode *Hirschmanniella oryzae* (Nahar et al. 2012). Pretreatment of rice seedlings with fluridone, an inhibitor of ABA biosynthesis, induced resistance to *M. oryzae* (Koga et al. 2004) and *Xoo* (Xu et al. 2013). Consistent with those results, rice plants with decreased ABA levels caused by transgenic expression of *OsABAox1*, and those in which ABA signaling was inhibited by the transgenic expression of a dominant negative mutant form of the *OsABI* gene, showed significantly decreased blast lesion numbers (Yazawa et al. 2012).

Abiotic stresses such as low temperature and drought render rice plants more susceptible to blast disease (Kahn and Libby 1958; Bonman et al. 1988; Gill and Bonman 1988; Koga et al. 2004). Given the role of ABA in mediating abiotic stress signaling, these findings strongly suggest that ABA is involved in the exacerbation of disease damage under certain abiotic stress conditions.

Recent studies have provided evidence that antagonistic crosstalk between SA and ABA signaling underpins the negative effect of ABA on rice immune responses (Jiang et al. 2010; Sugano et al. 2010; Yazawa et al. 2012; Xu et al. 2013). Abscisic acid suppressed SA/BTH- or pathogen-induced transcriptional upregulation of *WRKY45* and *OsNPR1*, the two key components of the rice SA-signaling pathway (Jiang et al. 2010). On the other hand, SA/BTH suppressed ABA-responsive gene expression (Jiang et al. 2010; Sugano et al. 2010). Overexpression of *OsNPR1* or *WRKY45* largely eliminated the increase in blast susceptibility induced by ABA, suggesting that ABA acts upstream of WRKY45 and OsNPR1 in the rice SA pathway (Jiang et al. 2010; Xu et al. 2013). Consistent with antagonistic crosstalk between SA and ABA, the expression of marker genes for the SA-signaling pathway was inversely correlated with that of marker genes for the ABA-signaling pathway during blast infection (Jiang et al. 2010). Our recent results indicated that a MAP kinase, OsMPK6, which phosphorylates WRKY45 in an SA-dependent manner (Ueno et al. 2013), is the node of convergence of antagonistic SA–ABA crosstalk in rice (Ueno et al., unpublished).

Exogenous application of ABA has shown to drastically reduce ET levels in rice, accompanied by enhanced susceptibility to *M. oryzae* (Bailey et al. 2009). In addition, RNAi-mediated suppression of *OsEIN2b* resulted in ABA hypersensitivity, reduced defense gene expression, and enhanced *M. oryzae* susceptibility. These observations suggest that ABA antagonizes the ET-signaling pathway in rice. Both the SA- and ET-signaling pathways positively affect rice resistance to *M. oryzae*; therefore, we presume that both ABA–SA and ABA–ET antagonistic crosstalks are responsible for the increased susceptibility to *M. oryzae* caused by ABA.

Rice genes responsive to ABA and dehydration stresses were induced during infection by *M. oryzae* (Ribot et al. 2008; Jiang et al. 2010; Sugano et al. 2010) and *Xoo* (Xu et al. 2013), suggesting that these pathogens affect cellular ABA levels or ABA signaling in plants. ABA was detected in the fungal body of *M. oryzae* and in its culture medium, indicating that the fungus is able to produce and secrete ABA (Jiang et al. 2010). These results imply that *M. oryzae* may use its own ABA to trigger ABA signaling in host cells, thereby suppressing the SA- and ET-signaling pathways to alleviate hosts' defense responses.

The finding that ABA negatively affects rice disease resistance has important agricultural value, leading to the development of a new method to control rice blast disease. Combinations of abamine, a highly specific ABA-biosynthesis inhibitor (Han et al. 2004), and BTH or BIT (benzisothiazole), chemical inducers that act on the SA pathway, were able to markedly increase the efficiency of blast control and reduced the amount of chemical inducers required to prevent the disease (Yoshida et al. 2006).

A positive effect of ABA on disease resistance has also been reported for rice. Exogenous ABA enhanced basal resistance against the necrotrophic brown spot pathogen *C. miyabeanus* (De Vleesschauwer et al. 2010). The resistance was the result of restricted fungal progression in the mesophyll and was dependent on an antagonistic interaction between ABA- and the ET-signaling pathways (De Vleesschauwer et al. 2010). The ABA-induced resistance to *C. miyabeanus* was compromised in transgenic knockdown lines of *OsMPK5*, which encodes a protein that mediates antagonistic crosstalk between ABA and ET signaling. Together, these findings suggested that the ABA effect is based on OsMPK5-dependent suppression of pathogen-induced ET signaling (Xiong and Yang 2003; Bailey et al. 2009; De Vleesschauwer et al. 2010).

Cytokinins

Cytokinins are well-known developmental hormones; however, recent studies have implicated CKs in various plant–pathogen interactions. Their effects are often manifested as morphological anomalies known as CK disorders (Walters and McRoberts 2006; Grant and Jones 2009; Choi et al. 2011). For example, infection of dicotyledonous plants by *Agrobacterium tumefaciens* causes crown gall tumors, which result from the overproduction of CKs and auxins via the products encoded by *IPT* (*isopentenyl transferase*) and *iaaM*/*H* (for tryptophan-2-monooxygenase and indoleacetamide hydrolase) genes, respectively, which are located on the bacterial T-DNA that is delivered into plant cells (Jameson 2000). The fungal pathogen *Plasmodiophora brassicae*, the causal agent of *Brassicaceae* clubroot disease, downregulates the CK degradation pathway during infection of *Arabidopsis*. Transgenic overexpression of CK oxidase/dehydrogenase suppressed clubroot development, indicating the importance of CKs in the pathogenicity of *P. brassicae* (Siemens et al. 2006). CKs are also associated with disease symptoms, such as fasciation, senescence, and the formation of "green islands" in plants (Jameson 2000; Walters and McRoberts 2006; Grant and Jones 2009; Choi et al. 2011; Stes et al. 2011). Thus, CKs appear to promote pathogen virulence in some pathosystems.

On the other hand, CKs have also been shown to play important roles in defense responses to some pathogens (Choi et al. 2011). In *Arabidopsis*, CKs modulate the SA-signaling pathway, thereby enhancing resistance to the hemibiotrophic bacterial pathogens *Pst* DC3000 and the biotrophic oomycete pathogen *Hyaloperonospora Arabidopsis* isolate Noco2 (Choi et al. 2010; Argueso et al. 2012). The action of CKs is mediated by a CK-activated TF, the ARR2 (*Arabidopsis* response regulator 2), which interacts with TGA3 (TGA1a-related 3), an SA-responsive TF to form a complex. The resulting complex binds directly to the promoters of *PR1* and *PR2* genes to induce their transcription, thereby positively regulating defense responses (Choi et al. 2010, 2011). Meanwhile, another group of ARRs (type A) negatively regulate SA-dependent basal immunity (Argueso et al. 2012). Transgenic tobacco plants expressing a bacterial *ipt* gene driven by a pathogen-inducible promoter

displayed enhanced resistance to virulent *P. syringae pv. tabaci* (Grosskinsky et al. 2011). The CK-mediated resistance was correlated with upregulated synthesis of two major antimicrobial phytoalexins, scopoletin and capsidiol (Grosskinsky et al. 2011). Interestingly, the CK action in the tobacco system was independent of SA, unlike that observed in the *Arabidopsis* system (Choi et al. 2010). Elevated levels of endogenous CKs in tobacco plants expressing *rgp1* (*ras-related GTP-binding protein*), a rice gene encoding a small GTP-binding protein, was associated with increased SA accumulation upon wounding and increased levels of acidic PR-1 proteins, leading to enhanced resistance to tobacco mosaic virus (Sano et al. 1994). Cytokinins were also implicated in resistance to necrotrophic pathogens. Transgenic tomato plants with increased CK levels showed delayed leaf senescence and attenuated disease symptoms after *Botrytis cinerea* infection (Swartzberg et al. 2008), and transgenic *Arabidopsis* with increased CK levels showed enhanced resistance to *Alternaria brassicicola* KACC40036 (Choi et al. 2010). By contrast, in tobacco, increased CK levels did not affect resistance to *Sclerotinia sclerotiorum* and even enhanced susceptibility to *B. cinerea* (Grosskinsky et al. 2011). Taken together, these results and observations indicate that the role of CKs varies among different pathosystems, reflecting the outcomes of coevolutionary interactions between pathogens and their hosts. Interestingly, CKs have also been implicated in plant resistance to insects by stimulating wound-inducible gene expression and by inducing the accumulation of insecticidal compounds (Giron et al. 2013).

In rice, CK treatment induces production of the major diterpenoid phytoalexins, momilactones and phytocassanes (Ko et al. 2010). The levels of these phytoalexins increase significantly in rice leaves in response to blast infection. Momilactone A treatment suppressed *M. oryzae* growth in planta and in vitro, indicating that these phytoalexins play an important role in blast resistance (Hasegawa et al. 2010). More recently, we showed that the levels of N^6-(Δ^2-isopentenyl) adenine (iP), iP riboside (iPR), and iPR 5′-phosphates (iPRP) in rice leaf blades increased during blast infection (Jiang et al. 2013). Consistent with CK accumulation, CK signaling was activated around the infection sites as shown by histochemical staining of β-glucuronidase expressed under the control of the CK-responsive *OsRR6* (*Oryza sativa response regulator 6*) promoter (Jiang et al. 2013). Interestingly, co-treatment of leaf blades with CKs and SA, but not with either one alone, strongly induced expression of the defense genes *OsPR1b* and *PBZ1* (*probenazole-inducible protein 1*), suggesting a synergistic interaction between the two hormones. The induction of these defense genes was diminished by RNAi knockdown of *OsNPR1* or *WRKY45*, indicating the dependence of the synergistic hormonal action on these central regulators of the SA pathway. These data imply a coevolutionary rice—*M. oryzae* interaction, wherein *M. oryzae* infection elevates CK levels in rice as a virulence strategy to alter physiological mechanisms such as nutrient translocation, while rice plants perceive the change in CK levels as an infection signal and activate defense reactions via the synergistic action with SA. “Priming” is the induction of the physiological condition in which plants can mount a more rapid or more effective defense response upon pathogen attack (Conrath et al. 2002). Recently, we proposed that the SA–CK interaction underlies the priming-based defense mechanism in rice, based on an

expression analysis of diterpenoid phytoalexin biosynthetic genes. That is, the signal of pathogen infection via CKs triggered the expression of diterpenoid phytoalexin biosynthetic genes in plants that had been primed by chemical inducers or previous pathogen infection through SA signaling (Akagi et al., unpublished). Various CK species were also detected in the hyphae (mycelia), conidia, and culture filtrates of blast fungus, indicating that *M. oryzae* is capable of producing and secreting CKs (Jiang et al. 2013). Whether or not the blast fungus-derived CKs are involved in its pathogenesis remains to be clarified.

Gibberellic Acids

Gibberellic acid was originally identified in the fungal pathogen *Gibberella fujikuroi*, the causal agent of the "foolish seedling" disease in rice, which is characterized by abnormal elongation of diseased rice plants (Kurosawa 1926). Until recently, most studies on GAs focused on their growth-promoting activities. However, recent studies have revealed the importance and mechanism of GA signaling in plant–pathogen interactions. In rice, infection by rice dwarf virus (RDV) repressed the expression of GA biosynthetic enzymes, causing dwarf phenotypes similar to those of GA-defective mutants, while exogenous GAs restored the normal phenotype (Zhu et al. 2005). These observations suggested that RDV modulates GA metabolism to promote disease development in rice. Hyper-accumulation of bioactive GAs in rice as a result of mutations to the gene encoding a GA-degrading enzyme (*Eui1*) led to compromised resistance against *Xoo* and *M. oryzae*; meanwhile, plants overexpressing *Eui1* showed increased resistance (Yang et al. 2008); thus, it is clear that GAs negatively affect resistance to these hemibiotrophic pathogens. Other studies showed that GA signaling also plays a negative role in rice immune responses. The *gid1* rice mutant, which is defective in GA perception, showed enhanced resistance to *M. oryzae* (Tanaka et al. 2006). DELLA proteins are negative regulators of GA signaling. Based on their studies on a quadruple loss-of-function *DELLA* mutant and a constitutive active mutant of *DELLA* in *Arabidopsis*, Navarro and coworkers (2008) proposed that DELLAs promote resistance to necrotrophs and susceptibility to biotrophs. These results are consistent with the negative role of GAs in defense against (hemi) biotrophic pathogens described in other studies. Expression patterns of SA and JA marker genes in the mutants suggested that the effects of the *DELLA* mutation were partly because of changes to the SA/JA balance in favor of JAs. It will be interesting to evaluate the effects of *DELLA* mutations on disease resistance in rice, given the considerable differences in SA–JA crosstalks between rice and *Arabidopsis*.

Recently, antagonism between JA and GA signaling was reported (Yang et al. 2012). In that study, *Arabidopsis* and rice *coi1* mutants with a defective JA receptor exhibited GA hypersensitivity. Jasmonic acid delays GA-mediated degradation of DELLA, and the *DELLA* mutant was less sensitive to JAs in terms of growth inhibition. These observations were interpreted as a mechanism to prioritize JA-mediated defense over GA-dependent growth.

Auxin

The most well-known activity of auxin is to regulate plant growth and development. However, recent studies have also highlighted the importance of auxin homeostasis in plant–pathogen interactions. Endogenous auxin levels are regulated in part through negative feedback by a group of auxin-inducible *GH3* (*Gretchen Hagen 3*) family genes that encode auxin-conjugating enzymes (Staswick et al. 2005). *Arabidopsis* lines overexpressing *GH3.5*, which encodes an indoleacetic acid (IAA)–amido synthetase that conjugates amino acids to IAA, showed enhanced SA accumulation and increased resistance to the virulent *Pst* DC3000. Conversely, mutation of this gene resulted in hyper-accumulation of free IAA upon pathogen infection and partially compromised SAR (Park et al. 2007; Zhang et al. 2007). AvrRpt2, a type III effector of *P. syringae*, promoted auxin production in *Arabidopsis*, thereby facilitating pathogen colonization of host plants (Chen et al. 2007). Flg22, a flagellin-derived peptide, induced the microRNA miR393, which negatively regulates mRNAs for the F-box auxin receptors TIR1 (transport inhibitor response 1), AFB (auxin signaling F-box) 2, and AFB3 (Navarro et al. 2006). This repression of auxin signaling restricted *Pst* DC3000 growth, implicating auxin in disease susceptibility (Navarro et al. 2006; Robert-Seilaniantz et al. 2011b). Auxin-mediated disease susceptibility is often associated with a mutually antagonistic interaction between the auxin and SA pathways (Kazan and Manners 2009; Pieterse et al. 2012). Salicylic acid inhibits auxin responses by stabilizing Aux/IAA repressor proteins, which are components of the SA-mediated disease-resistance mechanism (Wang et al. 2007). In contrast to the auxin-mediated susceptibility to biotrophs, auxin signaling is important for plant resistance to necrotrophic fungi. The *Arabidopsis* auxin signaling mutants *axr* (*Arabidopsis auxin-resistance*) *1*, *axr2*, *and axr6* all showed increased susceptibility to the necrotrophic fungi *Plectosphaerella cucumerina* and *B. cinerea* (Llorente et al. 2008). Similarly, pharmacological inhibition of auxin transport or proteasome function compromised necrotroph resistance (Llorente et al. 2008). Considering the opposite effects of SA and JA on biotrophs and necrotrophs, respectively, SA–JA crosstalk presumably intervene the actions of auxin.

The effects of overexpressing auxin-conjugating enzymes on disease resistance of rice have also been reported. Overexpression of rice *GH3.8*, which encodes IAA–amido synthetase, reduced the level of free IAA and enhanced rice resistance to *Xoo* (Ding et al. 2008). Overexpression of *GH3.2*, encoding IAA–amido synthetase, also resulted in resistance to a broad spectrum of pathogens including *M. oryzae*, *Xoo*, and *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*, the causal reagent of bacterial streak disease) in an SA- and JA-independent manner (Fu et al. 2011). *GH3.1* overexpression also resulted in disease resistance (Domingo et al. 2009). In contrast, pretreatment of rice with IAA increased its susceptibility to *Xoo*, *Xoc*, and *M. oryzae* and induction of a gene encoding expansin, which loosens the cell wall, suggesting that expansin was partly responsible for the enhanced sensitivity (Ding et al. 2008; Domingo et al. 2009). Collectively, these results showed that auxin

negatively affects resistance to various pathogens in rice. These findings may provide a genetic strategy for breeding rice with broad-spectrum disease resistance using *GH3* family genes. However, *GH3*-mediated resistance is usually accompanied by reduced plant growth and abnormal morphologies because of the decreased IAA levels (Ding et al. 2008; Domingo et al. 2009; Fu et al. 2011). Thus, the use of a pathogen-inducible promoter to drive *GH3-2* might be a more effective way to improve disease resistance (Fu et al. 2011).

Many bacterial and fungal pathogens can produce auxin themselves or can manipulate auxin signaling in the host during their infection processes (Kazan and Manners 2009). The rice pathogens *Xoo*, *Xoc*, and *M. oryzae* produce and secrete IAA (Fu et al. 2011; Jiang et al. 2013). These pathogens may use IAA as a virulence factor to facilitate their infection of rice tissues.

Brassinosteroids

Brassinosteroids regulate many developmental and physiological processes, such as cell elongation and vascular differentiation (Choudhary et al. 2012). However, they also have roles in modulating plant immunity. Treatment with BRs induces resistance against various viral, bacterial, and fungal pathogens in tobacco and rice in an SA-independent manner (Nakashita et al. 2003), indicating a positive role of BRs in pathogen defense responses. However, a recent study by De Vleesschauwer et al. (2012) showed that BR signaling rendered rice hypersensitive to the root pathogen *Pythium graminicola*. This effect was due to negative crosstalk of BR-signaling pathway with the SA- and GA-signaling pathways. Thus, the authors suggested that *P. graminicola* uses the plant BR pathway to inflict disease by antagonizing SA- and GA-mediated defenses. It was also reported that BR induced susceptibility of rice to the root-knot nematode *Meloidogyne graminicola* partly by antagonizing the JA pathway (Nahar et al. 2013).

The leucine-rich repeat receptor-kinase BRI1 (BRASSINOSTEROID INSENSITIVE 1), which is localized at the plasma membrane, functions as a BR receptor in *Arabidopsis* (Li 1997). Binding of BR to BRI1 induces phosphorylation of BAK1 (BRI1-associated receptor kinase 1), a cytoplasmic receptor kinase, thereby modulating BR signaling (Li et al. 2002). BAK1 also interacts with FLS2 and EFR (EF-Tu receptor), both of which are PRRs with a leucine-rich repeat receptor-kinase structure similar to that of BR1, and transduces defense signals to induce resistance against bacterial pathogens (Chinchilla et al. 2007). Thus, BAK1 appears to function as a common co-receptor in developmental regulation and innate immunity. Indeed, BRs modulate PTI antagonistically or synergistically through BAK1 (Albrecht et al. 2012; Belkhadir et al. 2012). Xa21 is rice PRR with a leucine-rich repeat receptor-kinase structure that confers resistance against most *Xoo* strains (Song et al. 1995). In rice cells, binding of BRs to the BRI1 extracellular LRR domain activated the BRI1–XA21 chimeric receptor kinase to induce the

XA21-mediated defense response (He et al. 2000). Knockdown of *OsBAK1* by RNAi decreased not only BR sensitivity but also *M. oryzae* resistance in rice (Park et al. 2011). Thus, BAK1 plays a dual role in development and innate immunity, suggesting that there is some interplay between these two signaling pathways in both dicot and monocot plants.

Concluding Remarks and Future Perspectives

Salicylic acid, JAs, and ET mediate core-signaling pathways for plant defense to pathogens of different lifestyles. Auxin, GAs, CKs, ABA, and BRs mainly regulate plant growth and development as well as abiotic stress responses but also have various effects on plant–pathogen interactions, with negative effects on plant resistance being more common. As illustrated in this chapter, the past few years have witnessed significant progress in elucidating the crosstalks among the different hormone signaling pathways that form complex networks. As plants are sessile organisms, such crosstalks are presumably important for plants to adapt to their changing environment. Antagonistic crosstalks would prioritize plant responses to one stress over those to other stresses. A specific stress response could also be prioritized over growth/development, or vice versa. Such trade-offs would allow cost-effective use of limited energy and resources. Positive (synergistic) interactions among hormone signaling pathways can be interpreted as reinforcement or fine-tuning of one signaling pathway by others.

Studies on hormone crosstalks have been extended from a few model dicots to many other plant species including rice, a model monocot. These studies have revealed similarities and differences in the actions of hormones and crosstalks between different hormone signals in defense responses. The current status of knowledge on hormone crosstalks, mostly gained from studies on *Arabidopsis* and rice, is summarized in Fig. 1. One of the factors that makes it difficult to understand hormone crosstalks is that the same combination of hormones can result in different outcomes, even in the same plant species. For example, the antagonistic SA–JA interaction has been reported in many studies on dicots, but positive interactions that occur in a concentration-dependent manner have also been reported. Both antagonistic and synergistic interactions have also been reported for rice, with synergistic interactions being prevalent.

Crosstalks are affected by many factors, including hormone concentrations and the age and condition of the plants. Further understanding of crosstalks would require identification of a molecule that functions as a bona fide node of convergence between different pathways. *Arabidopsis* NPR1, which regulates SA signaling in the nucleus and JA signaling in the cytosol (Spoel et al. 2003), is a candidate for such a molecule. It will be interesting to mutate such a key molecule so that it retains its function to mediate one signal but loses its function to mediate another. By using such mutants, researchers will be able to experimentally test whether

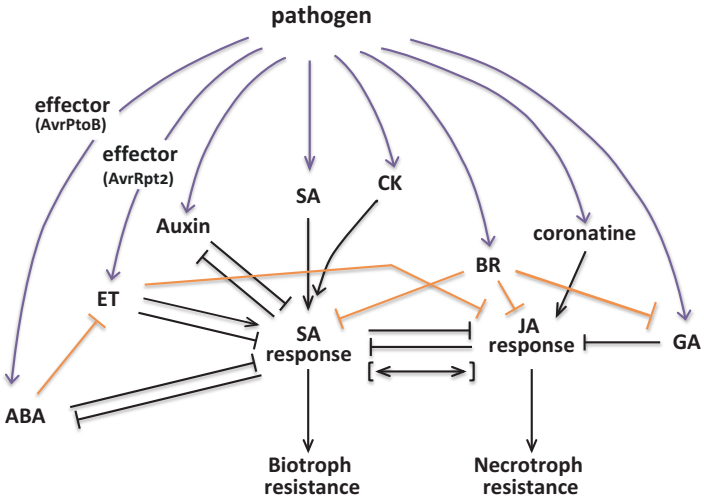

Fig. 1 Overview of hormonal crosstalks involved in plant defense against pathogens. Positive and negative interactions are indicated by *arrows* and *lines with bars*, respectively. Interactions observed only in rice are shown in *orange*. Interactions observed less frequently or under specific conditions are shown in *parentheses*

crosstalks are really beneficial for plants and provide clues as to why crosstalks are evolutionarily conserved among diverse plant groups.

Hormone signaling crosstalks can be a target for crop improvement to increase disease resistance using pharmaceutical, genetic, or transgenic approaches. Such strategies include strengthening resistance induced by a particular signaling pathway via suppressing its antagonistic pathway or exploiting synergistic interactions. However, fortifying one signaling pathway to improve crop resistance could have negative side effects. For example, strengthening the SA pathway by overexpressing *Arabidopsis* NPR1 made the plants more resistant to biotrophic pathogens, but more sensitive to salt and drought stresses (Quilis et al. 2008), presumably because of the antagonistic interaction between SA and ABA. Overexpression of *OsNPR1* in rice rendered the plants more sensitive to light (Chern et al. 2005) and that of *WRKY45* made them more sensitive to abiotic stresses (Shimono et al. 2007; Tao et al. 2011) (Goto et al., unpublished). Signaling crosstalks presumably underlie these results, although the details are yet to be elucidated. Overexpression of *OsNPR1* in rice conferred resistance to *M. oryzae* and *Xoo*, but enhanced sensitivity to herbivore damage, presumably as a result of SA–JA antagonism (Yuan et al. 2007). These are important factors to be considered when using these strategies to improve disease resistance of crops. One of the possible strategies to solve problems associated with unfavorable signaling crosstalks is to disconnect the crosstalk by modifying the molecules that function as the nodes of the interaction; therefore, it is particularly important to identify these molecules.

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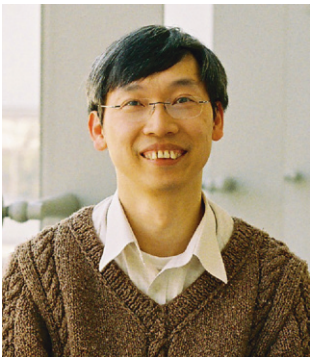

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