Signaling and Communication in Plants

Rujin Chen
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Polar Auxin Transport

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Preface

The plant hormone auxin plays essential roles in diverse plant developmental processes including morphogenesis, organogenesis, vascular tissue differentiation, and growth responses to biotic and abiotic signals. The involvement of auxin in the broad spectrum of biological processes is mainly mediated by its regulation of cell division, expansion, and differentiation. Experimental evidence indicates that local auxin gradients generated by three families of auxin transporter proteins are essential for auxin actions. The molecular cloning of auxin transporter proteins in the past two decades marks a milestone in auxin research. Importantly, the biological activity of auxin transport proteins is associated with their characteristic plasma membrane localization. The PIN-FORMED (PIN) family of auxin efflux proteins has been shown to exhibit asymmetric plasma membrane localization and determine the direction of auxin flow. The maintenance of PIN protein plasma membrane localization involves clathrin-coated vesicle endocytosis, a process that is evolutionarily conserved in plant, yeast, and animal cells, and intracellular trafficking targeting proteins to distinct plasma membrane domains or lytic vacuoles for turnover. Auxin reinforces its own transport by regulating transcription and endocytosis and recycling of its transport proteins. Recently, a group of new auxin transport proteins, the so-called "short" PINs, has been demonstrated to localize to the endoplasmic reticulum membrane and play a role in intracellular transport and homeostasis of auxin.

Experimental evidence supports that membrane microdomains regulated by the composition of membrane lipids and sterols play a role in endocytosis of auxin transport proteins. Intriguingly, differences exist in the clathrin-mediated endocytosis of auxin influx and efflux proteins. Within plant cells, sequence-specific phosphorylation by plant AGC kinases counterbalanced by protein phosphatase-mediated dephosphorylation is necessary to channel PIN proteins to distinct intracellular trafficking pathways destined to specific membrane domains. Auxin transport proteins are also targeted to lytic vacuoles for protein turnover. Interestingly, gravity and light, two prevalent environmental signals, have been shown to modulate intracellular trafficking and targeting of auxin transport proteins during plant tropic responses.

Amongst numeric milestones in the field of auxin research are the identification and characterization of auxin signaling pathways and the TIR1/AFB family of auxin receptors. Development of different auxin sensors and quantification methods with increased sensitivities has greatly facilitated auxin research in the model species *Arabidopsis thaliana*. The advanced molecular and genetic tools have gradually become available in other plant species with interesting biological processes, providing new insights into our understanding of the role of polar auxin transport in developmental processes such as plant–microbe interactions.

As sessile organisms, higher plants evolved various mechanisms to cope with changing environmental conditions. Biotic and abiotic stresses have profound effects on plant development and reproduction in part through manipulation of the level, response, and distribution of plant hormones. As summarized in this book series, polar auxin transport is a prime target/mediator of abiotic and biotic stress responses of plants, as well as a practical target for agronomic trait improvements in crop plants. While tremendous progresses have been made in auxin research, new insights of the role of auxin transport in diverse developmental processes await to be gained.

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Contents

Signaling in Polar Auxin Transport

Anindya Ganguly and Hyung-Taeg Cho

Abstract Polar auxin transport, directionally delivering auxin and generating local auxin gradients, is pivotal to fundamental developmental processes in plants. The directional auxin flow is attributable to polar-localized auxin transporters. In particular, over the past several years, the cytological and molecular mechanisms of how auxin transporters distribute asymmetrically in the plasma membrane have become greatly understood. The cellular polarity players for auxin transporters include intracellular trafficking components, cargo phosphorylation cues and corresponding kinases, membrane lipid composition, ubiquitylation, and extracellular auxin receptor-mediated modulation of cytoskeleton. In addition to these internal polarity regulators, other major plant hormones, signaling molecules, environmental stimuli, and nutrients have been implicated in regulation of auxin transporters, reflecting the communication between diverse developmental/environmental signals and auxin-mediated plant development. In this chapter, we review the recent studies that have elucidated the regulatory mechanisms of auxin transport.

1 Introduction

He got up early as 6:40 A.M. in the summer morning, walked to a quite sizable dark box on the table, and carefully opened the top. He slowly looked over the stems and leaves of a verbena plant inside the dark box and then cautiously placed the fifth dot on the glass plate precisely above the tiny bead affixed through a glass filament to the verbena stem apex. Over the night, the bead had traveled from the east to the southwest (Fig. 1).

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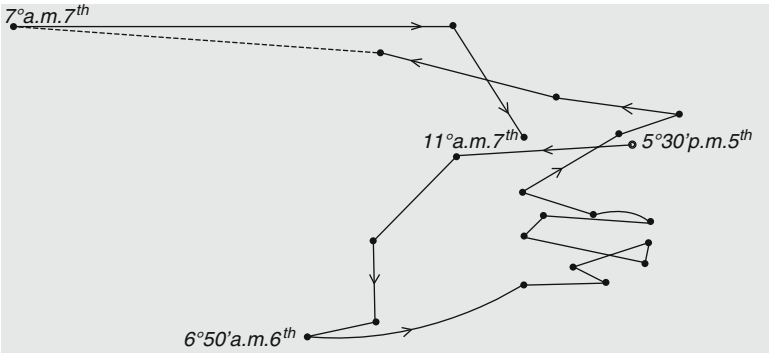

Fig. 1 *Verbena melindres*: circumnutation of stem in darkness, traced on vertical glass, from 5.30 P.M. on June 5 to 11 A.M. June 7. The *broken line* represents a nocturnal course of the movement (Darwin 1880)

Just a few years before he passed away, Charles Darwin with his son Francis had left pioneering insights on the field of auxin physiology. The Darwins reported 157 tracings (like Fig. 1) of movements from more than 40 plant species in their book "The Power of Movement in Plants" (Darwin 1880). Darwin's great contribution to the phytohormone physiology in this volume is the prediction of auxin and its directional transport. The polar auxin transport concept was suggested by Darwin as a mobile "influence" which necessarily translocates from the tips of stem and root, which sense the direction of light and gravity sources, respectively, to the bending or differentially growing part of the organs. Forty-eight years later, the moving "influence" materialized into "auxin" (Went 1928). Since Darwin's prediction, it took more than 100 years to identify the auxin-transporting proteins (Bennett et al. 1996; Chen et al. 1998; Gälweiler et al. 1998; Luschnig et al. 1998). However, over the past decade since the discovery of auxin transporters, ~350 research papers on the major auxin transporters have been published, and now we have a much more detailed and enriched picture of plant developmental mechanism, illustrating at the cellular and molecular level how the embryonic apical/basal axis forms, how the leaf regularly emerges from the plane shoot apical dome, how fate determination and emergence of a lateral root occur in the pericycle cell, how organs under tropism acquire auxin gradients across them, and many other auxin-mediated developmental processes.

The Darwinian auxin transport is achieved by cell-to-cell movement of auxin, which requires both influx and efflux carriers in the plasma membrane. Three major families of auxin-transporting proteins have been so far studied: AUXIN-RESISTANT1 (AUX1)/AUX1-LIKEs (LAXs) for auxin influx and PIN-FORMEDs (PINs) and several ATP-binding-cassette B (ABCB)/P-glycoprotein (PGP) members for auxin efflux (for a recent review, see Grunewald and Friml 2010). A recent study has added a new group of membrane proteins, PIN-LIKES (PILS), as auxin transporters, which mediate intracellular auxin transport (Barbez et al. 2012). As the major natural auxin, indole-3-acetic acid ($pK_a = 4.75$) is

predominantly ionized in the cytosol, the activity of efflux carriers is essential for the cell-to-cell auxin movement. In particular, PIN proteins play the critical role for the directionality of auxin movement (so the formation of local auxin gradients) because they asymmetrically localize in the plasma membrane and dynamically change their intracellular distribution in response to developmental and environmental signals (Grunewald and Friml 2010). In this chapter, we introduce the molecular properties of different auxin-transporting proteins and their regulatory mechanisms.

2 AUX1/LAXs

Although a portion of apoplastic auxin is lipid permeable, the majority are in their ionized form, indicating that influx carriers can facilitate uptake of auxin into the cytoplasm. AUX1/LAXs, a small conserved group of amino acid/auxin permease-like proteins with >60 % amino acid identity and 11 transmembrane spans (Kerr and Bennett 2007), have been identified as auxin influx carriers in plants and heterologous systems (Yang et al. 2006; Péret et al. 2012). AUX1/LAXs have been implicated in auxin-related biological functions such as root gravitropism, lateral root formation, root hair growth, and vascular patterning (Bennett et al. 1996; Cho et al. 2007b; Swarup et al. 2008; Jones et al. 2009; Péret et al. 2012). While the localization of AUX1 is either polar or nonpolar depending on cell type, for example, apical in the protophloem cells and basal in the lateral root cap cells but no polarity in the columella cells (Bennett et al. 1996; Swarup et al. 2001), LAXs do not show particular polar localizations (Swarup et al. 2008; Péret et al. 2012). AUX1 is thought to function for long-distance auxin transport from the shoot to the root through the vasculature, and LAXs seem to primarily maintain local auxin gradients (Swarup et al. 2002, 2004, 2008).

AUX1 exhibits subcellular localization not only in the plasma membrane but also in internal compartments such as Golgi and endosomes (Kleine-Vehn et al. 2006). A fungal toxin brefeldin A (BFA) inhibits recycling of PINs, which requires the BFA-sensitive ADP-ribosylation factor-GDP/GTP exchange factor (ARF-GEF)-mediated vesicle trafficking (Donaldson and Jackson 2000; Geldner et al. 2001). However, in the case of AUX1, BFA is inhibitory for its internal trafficking but not for its trafficking to the plasma membrane, suggesting that BFA-insensitive ARF-GEFs are implicated in AUX1 trafficking to the plasma membrane (Kleine-Vehn et al. 2006). The plasma membrane targeting of AUX1 requires AUXIN-RESISTANT 4 (AXR4), an endoplasmic reticulum (ER) chaperone, whereas PINs' trafficking does not need the protein (Dharmasiri et al. 2006). These studies collectively demonstrate the existence of different trafficking tools and pathways between AUX1/LAXs and PINs, reflecting the need of diversified regulatory mechanisms between auxin influx and efflux.

Although they all show auxin influx activities, AUX1/LAXs seem to have different regulatory mechanisms and biological functions (Péret et al. 2012). Not only their expression patterns and mutant phenotypes are unique to each member, but also the regulatory mechanism for their intracellular trafficking differs between

the members. For example, while they are properly targeted to the plasma membrane in their own expression domains, LAX2 and -3 fail to be targeted to the plasma membrane in AUX1-expressing tissues (Péret et al. 2012). This indicates that there are molecular cues in each influx carrier and some cell type-specific regulators operate for intracellular trafficking of each AUX1/LAX species. Péret et al. (2012) proposed that each AUX1/LAX-specific ER chaperone may exist for the specific trafficking as shown for the case of AXR4-mediated AUX1 trafficking (Dharmasiri et al. 2006).

A couple of recent studies have demonstrated that organ-level signals are implicated in regulation of AUX1-mediated auxin transport activity. Shoot-supplied ammonium inhibits lateral root initiation. Because AUX1 is required for lateral root formation and shoot-supplied ammonium suppresses AUX1 expression in vascular tissues, ammonium in the shoot seems to modulate lateral root number by controlling AUX1-mediated shoot-to-root auxin transport (Li et al. 2011). Shoot-applied iron also facilitates lateral root formation by inducing AUX1 expression in the lateral root, which increases auxin supply in the lateral root for its initiation and elongation (Giehl et al. 2012). The promoter analysis of the AUX1 gene and following search for the upstream factors would reveal diverse regulatory pathways for AUX1-mediated auxin transport in response to various internal and external signals.

3 ABCBs

Among 21 ABCB members, ABCB1, ABCB4, and ABCB19 have been implicated in auxin transport (Noh et al. 2001; Multani et al. 2003; Geisler et al. 2005; Terasaka et al. 2005; Cho et al. 2007b). In plant cells, these three ABCBs play mainly as auxin efflux transporters (Geisler et al. 2005; Cho et al. 2007b; Kubes et al. 2012). Auxin-transporting (AT) ABCBs consistently localize to the plasma membrane mainly with no polarity but with polarity in certain cells (Geisler et al. 2005; Terasaka et al. 2005; Blakeslee et al. 2007; Wu et al. 2007; Cho et al. 2007b, 2012; Mravec et al. 2008). Being predominantly nonpolar, the AT-ABCBs may control the auxin amount available for PIN-mediated polar auxin transport (Mravec et al. 2008). Several lines of evidence have pointed toward a direct interaction between AT-ABCBs and PINs in coordinating polar auxin transport (Bandyopadhyay et al. 2007; Blakeslee et al. 2007). Genetic interactions between AT-ABCBs and PINs also have been shown to be important for both embryogenesis and organogenesis (Mravec et al. 2008). These studies suggest that AT-ABCBs together with PINs may also play the major role for cellular auxin efflux or play alone for local auxin loading for PIN-mediated long-distance auxin transport (Bandyopadhyay et al. 2007; Blakeslee et al. 2007; Bailly et al. 2008).

4 ABCB Endocytosis

AT-ABCBs generally more stably reside in the plasma membrane than PINs. Among three AT-ABCBs, ABCB19 and ABCB4 show more stable plasma membrane localization compared with ABCB1, which shows some endocytic movement (Blakeslee et al. 2007; Titapiwatanakun et al. 2008; Cho et al. 2012). Fluorescence recovery after photobleaching (FRAP) experiments have revealed that ABCB4 and ABCB19, stably anchoring to the plasma membrane, seldom recycle between the endosome and the plasma membrane (Titapiwatanakun et al. 2008; Cho et al. 2012). In contrast to PINs whose polarity is maintained by dynamic recycling, the nonpolarity of AT-ABCBs may not require the active recycling. The difference in BFA sensitivity also shows the diverged intracellular trafficking pathways between different AT-ABCBs. ABCB19 was shown to be more resistant to BFA than did ABCB1 and ABCB4, consistently with the observation that ABCB19 takes GNOM-independent but GNL1-dependent trafficking mechanism (Titapiwatanakun et al. 2008; Cho et al. 2012; Wu et al. 2010). On the other hand, ABCB4 trafficking seems to be dependent on GNOM as well as other ARF-GEFs (Cho et al. 2012).

5 Phosphorylation-Mediated Regulation of ABCB Trafficking

Similarly to PIN proteins, AT-ABCBs also are regulated by AGC (protein kinase A, G, and C family) kinases (Christie et al. 2011; Henrichs et al. 2012). However, while the AGC kinases act as positive regulators for PIN activities, they either activate or deactivate the AT-ABCBs depending on the kinase-ABCB combinations and environmental conditions. For example, while PHOT1, the blue-light receptor AGC kinase, inhibits ABCB19 auxin efflux activity in the hypocotyl by directly phosphorylating ABCB19 in response to light (Christie et al. 2011), the PINOID (PID) AGC kinase directly phosphorylates ABCB1 to increase its efflux activity in protoplast and yeast assay systems (Henrichs et al. 2012). The inactivation of ABCB19 by PHOT1 increases auxin levels in and above the hypocotyl apex to halt vertical hypocotyl growth in response to blue light (Christie et al. 2011). PHOT1 also regulates the direction of auxin flow during the hypocotyl phototropism where PHOT1 in response to light decreases PID transcription and in turn may affect the lateral PIN3 polarization as will be mentioned in the later section for PINs (Ding et al. 2011). Altogether, it seems that PHOT1 regulates simultaneously the ABCB19 activity and the PIN3 polarization in order to channelize auxin to the elongation zone of the hypocotyl upon phototropic stimuli and promote the hypocotyl bending toward the light source.

TWISTED DWARF1 (TWD1), an immunophilin-like protein, was found to directly interact with the C-terminal end of ABCB1 and ABCB19, and the loss-of-function *twd1* mutant phenotypes are similar to those of the *abcb1*/*abcb19* mutant (Murphy et al. 2002; Geisler et al. 2003). Based on these results, TWD1 was

proposed to be an activator of the membrane-localized ABCB complexes by causing conformational changes in the ABCB proteins (Geisler et al. 2003; Bouchard et al. 2006; Bailly et al. 2008). In the *twd1* mutant, the plasma membrane localizations of ABCB1, ABCB4, and ABCB19 were significantly compromised, suggesting that TWD1 is also implicated in the trafficking of AT-ABCBs to the plasma membrane (Wu et al. 2010). As TWD1 has been demonstrated to be localized both in the plasma membrane and ER (Geisler et al. 2003; Wu et al. 2010), it is conceivable that ER-localized TWD1 functions for ABCB trafficking and plasma membrane-localized TWD1 modulates the ABCB activity.

It has been lately shown that TWD1 directly binds to PID, and this TWD1–PID interaction modulates the ABCB1-mediated auxin transport activity (Henrichs et al. 2012). In the absence of TWD1, PID (but not WAG1, a PID paralog) phosphorylates a single serine residue (634) in the ABCB1 linker domain and enhances ABCB1-mediated auxin efflux activity. Corresponding phospho-defective (S634A) and phospho-mimic (S634E) versions of ABCB1 also showed reduced and elevated auxin export activities, respectively. Conversely, the presence of TWD1 inhibits PID-induced enhancement of ABCB1-mediated auxin transport activity most likely by inhibiting PID-mediated phosphorylation of the S634 residue in the ABCB1 linker domain. PID phosphorylates the NSVSSPIMTR motif of ABCB1, and this motif bears no sequence similarity to the known phosphorylation motifs of PINs as will be mentioned later.

6 PINs

PIN genes have been found throughout the land plant lineage consistently with the presence of auxin signaling components and auxin responses in land plants (Krecek et al. 2009; Lau et al. 2009). The Arabidopsis PIN family consists of eight members, and their homologs in angiosperms can be clustered roughly into four subgroups: PIN1 and 2 subgroups; PIN3, 4, and 7 subgroups; PIN6 subgroup; and PIN5 and 8 subgroups. On the other hand, PINs from moss and Lycopodiopsida form outgroups in the molecular phylogeny (Krecek et al. 2009), indicating that the functional diversification among different PIN subgroups could have occurred during the angiosperm evolution.

All PINs have two sets of five transmembrane helices, each in the N-terminal and the C-terminal regions, which are connected by a varying length of central hydrophilic loop depending on PIN species (Krecek et al. 2009; Ganguly et al. 2010, 2012a, b). PIN1–4, 6, and 7 and their orthologs have a long hydrophilic loop (>300 residues, so-called long PINs), and PIN5 and 8 and their orthologs have a short hydrophilic loop (<50 residues, so-called short PINs). Long PINs generally localize in the plasma membrane in diverse cell types, whereas short PINs have been shown to localize predominantly in internal compartments such as ER (Krecek et al. 2009; Mravec et al. 2009; Ganguly et al. 2010; Dal Bosco et al. 2012; Ding et al. 2012). However, PIN8, when ectopically expressed in Arabidopsis root hair

cells and tobacco Bright Yellow (BY)-2 cells, showed a dual localization pattern in both the plasma membrane and ER-like compartments, which is contrasted with the observation that PIN5 consistently localized only to the ER-like compartment in both cell types (Ganguly et al. 2010). These observations suggest that the hydrophilic loop of PINs includes certain molecular cues for intracellular PIN trafficking and those cues could be operational in a cell type-dependent manner. Because the short PINs show the partial plasma membrane targeting depending on cell type, the short hydrophilic loop of short PINs might carry limited molecular code for plasma membrane trafficking. On the other hand, the hydrophilic loop of long PINs includes diverse molecular cues for clathrin-mediated endocytosis, ubiquitylation, and phosphorylation which collectively modulate trafficking, stability, and subcellular polarity of long PINs (Grunewald and Friml 2010; Kleine-Vehn et al. 2011; Ganguly et al. 2012b; Leitner et al. 2012).

Most long PINs localize asymmetrically to certain plasma membrane side of the cell, which necessarily generates the directional flow of auxin and the formation of auxin gradients. Most long PINs such as PIN1, 3, 4, and 7, localizing at the basal (toward the root tip) side, contribute for the downward auxin flow in the root (Friml et al. 2002a, b; Blilou et al. 2005). Apically localized PIN2 in root epidermal cells transports auxin from the tip to the upper part of the root (Luschnig et al. 1998; Müller et al. 1998). Laterally localized PIN3 in the pericycle and basally localized PIN2 in the cortex contribute for redirecting auxin back to the root meristem (Blilou et al. 2005). Redistribution of PIN3 and PIN7 in the columella upon gravity vector changes plays a role for gravitropic bending of the root (Friml et al. 2002b; Kleine-Vehn et al. 2010). During phototropism of the hypocotyl, the abundance of PIN3 in the outer lateral membrane of the endodermal cell in the illuminated hypocotyl side is decreased so as to transport auxin to the shaded side of the hypocotyl (Ding et al. 2011). PIN1 in the shoot apical cells localizes at the membrane toward the incipient leaf primordium, establishing auxin maxima for the initiation of leaf development (Reinhardt et al. 2003). Mutant phenotypes of PINs reflect the biological role of each PIN's subcellular polarity in plant development. The Arabidopsis *pin1* mutant, as the name of PIN-FORMED originated, lacks lateral organs in the inflorescence stem (Okada et al. 1991). Loss of PIN2 causes defects in root gravitropism and root hair growth (Chen et al. 1998; Luschnig et al. 1998; Müller et al. 1998; Utsuno et al. 1998; Cho et al. 2007a). Consistently with their expression and dynamics in polarity changes responding to gravity, PIN3 and PIN7 are required for root gravitropism (Friml et al. 2002b; Kleine-Vehn et al. 2010), and phototropism is partially defective in the *pin3* mutant (Friml et al. 2002b).

Internally localized short PINs seem to be implicated in internal relocation of auxin such as sequestration, nuclear transport, and compartmentalization of active auxin or auxin derivatives (Mravec et al. 2009; Ganguly et al. 2010; Dal Bosco et al. 2012). In contrast to long PINs, the mutant phenotypes of short PINs are difficult to be inferred from their internal localization and probable function. *pin5* mutants are defective in hypocotyl and root growth as well as lateral root formation (Mravec et al. 2009), and loss of PIN8 causes defects in pollen germination but with normal pollen tube growth (Ding et al. 2012). Ectopic overexpressions of PIN8 (PIN8ox)

and PIN5 (PIN5ox) show opposite effects: auxin export, longer hypocotyl and root, and inhibition of root hair growth in PIN8ox versus auxin import, shorter hypocotyl and root, and stimulation of root hair growth in PIN5ox (Mravec et al. 2009; Ganguly et al. 2010; Ding et al. 2012). These results are difficult to be explained because both PIN5 and PIN8 seem to be localized mainly in the ER, but the auxin responses mediated by these two short PINs are opposite. In the root hair cell, because PIN8 is also localized in the plasma membrane, PIN8ox could decrease the auxin response of the root hair by lowering cellular auxin levels (Ganguly et al. 2010). In contrast, higher auxin responses by PIN8ox in the root meristem and hypocotyl (Ding et al. 2012) may take place via more complicated processes.

7 Modulators for the Regulation of PIN Polarity

The subcellular polarity of PINs is not determined by polar trafficking of newly synthesized proteins but by polar recycling of preexisting proteins in the plasma membrane (Dhonukse et al.2008). There are three major intracellular PIN trafficking pathways (1) ER → Golgi → trans-Golgi network (TGN)/early endosome (EE) → plasma membrane, for the exocytic pathway of newly synthesized PINs, (2) plasma membrane → TGN/EE → recycling endosome (RE) → plasma membrane, for recycling of preexisting PINs, and (3) plasma membrane → TGN/EE → pre-vacuolar compartment (PVC)/multivesicular body (MVB) → vacuole, for the vacuolar lytic pathway (Grunewald and Friml 2010). Here, we will focus on the several regulatory players for the polar recycling pathway of PINs.

7.1 ARF-GEFs and Their Modulators

The first characterized regulators for polar PIN trafficking are ARF-GEFs. The fungal toxin BFA, an ARF-GEF inhibitor, blocks RE → plasma membrane exocytic and TGN/EE → PVC/MVB lytic PIN trafficking resulting in PIN accumulation in "BFA compartments" inside the cell (Geldner et al. 2001; Kleine-Vehn et al. 2008a). Conversely, the plasma membrane → TGN/EE endocytic process of PIN is not affected by BFA (Geldner et al. 2003). The basal PIN targeting is regulated by GNOM, a BFA-sensitive ARF-GEF (Steinmann et al. 1999; Geldner et al. 2001, 2003). The basal PIN1 localization was compromised in the *gnom* loss-of-function mutant, while apically localized PIN2 and AUX1 remained mostly unaffected (Steinmann et al. 1999; Kleine-Vehn et al. 2006, 2008a). Moreover, the response to BFA was comparatively much stronger in PIN1 than PIN2 and AUX1, suggesting that the apical localization of certain membrane proteins is mainly controlled by BFA-resistant ARF-GEFs (Kleine-Vehn et al. 2006, 2008a). GNOM-like (GNL) ARF-GEFs also are implicated in PIN trafficking. GNL1, a BFA-insensitive ARF-GEF, is important for ER → Golgi trafficking (Richter et al. 2007) and

interestingly mediates PIN2 endocytosis rather than exocytosis (Teh and Moore 2007). GNL2 is required for germination and tip growth of the pollen (Jia et al. 2009; Richter et al. 2011) and functionally compatible with GNOM in PIN1 recycling from endosome to (basal) plasma membrane (Richter et al. 2011).

A small GTPase ARA7 (a Rab5-GTPase) and its regulator VPS9A play for endocytosis-mediated PIN polarization (Dhonukshe et al. 2008). In contrast to GNOM, which is predominantly implicated in basal PIN recycling, ARA7 and VPS9A seem to regulate the endocytosis of both apical and basal PINs. ARA7 has been demonstrated to localize in several intracellular compartments such as early/late endosomes, multivesicular bodies, and tonoplast but not in the plasma membrane (Ueda et al. 2004; Haas et al. 2007; Ebine et al. 2011). Hence, it may not be directly involved in PIN endocytosis from the plasma membrane. However, because the PIN polarity, but not its PM localization, is defective in the dominant negative ARA7-expressing plant, ARA7 may indirectly regulate PIN endocytosis (Dhonukshe et al. 2008).

7.2 PIN Phosphorylation

Protein phosphorylation has long been implicated in regulation of auxin transport. In tobacco suspension cells, protein kinase inhibitors inhibited cellular auxin efflux and phosphatase inhibitors inhibited both efflux and influx of auxin (Delbarre et al. 1998). Arabidopsis protein phosphatase 2A (PP2A) was shown to have a negative effect on auxin transport (Rashotte et al. 2011), and the mutations of PID have been related with defects in auxin transport (Bennett et al. 1995; Benjamins et al. 2001). Consistently with these previous studies, PID was shown to play as a positive effect or for auxin efflux transport in Arabidopsis root hair cells and tobacco BY-2 cells (Lee and Cho 2006).

7.2.1 A Subset of AGC Kinases Phosphorylate the Hydrophilic Loop of PINs

A series of recent studies on phosphorylation of PINs have provided cellular and molecular mechanisms to understand auxin-mediated plant growth and development (for a recent review, see Ganguly et al. 2012a). The hydrophilic loop of PINs has recently been shown to be the direct phosphorylation target by a subgroup of AGC kinases (AGC1 or AGCVIIIa subgroup, Bögre et al. 2003). In vitro and transient protoplast phosphorylation assay systems, four members of AGC1 kinases, PID, WAG1, WAG2, and D6PK, were able to phosphorylate the hydrophilic loop of PINs (Michniewicz et al. 2007; Zourelidou et al. 2009; Huang et al. 2010; Dhonukshe et al. 2010; Ding et al. 2011; Ganguly et al. 2012b). PID-mediated phosphorylation of PIN1 was antagonized by PP2A (Michniewicz et al. 2007).

These studies have identified multiple phosphorylation motifs in the PIN hydrophilic loop. The serine residue in the "TPRXS" (where X is any amino acid residue)

motif of the PIN1 and PIN2 hydrophilic loop was shown to be phosphorylated by PID, WAG1, and WAG2 in vitro (Huang et al. 2010; Dhonukshe et al. 2010). The hydrophilic loop of long PINs carries three repeats of TPRXS motif, and phosphorylation of any one of these motifs seems to be enough for the biological function of PIN, indicating the functional redundancy among three TPRXS motifs (Huang et al. 2010). Recently, another phosphorylation motif, RKSNASRRSF(/L) (called "3m1" motif), has been identified from the PIN3 hydrophilic loop by in vitro and *in planta* phosphorylation assays (Ganguly et al. 2012b). The 3m1 motif is located a few residues ahead of the first TPRXS motif (the "M3" motif together with 3m1), and the capacity for phosphorylation and biological function of the 3m1 motif was augmented when it operated together with the neighboring TPRXS motif. The 3m1 or M3 motif was shown to be the target of PID but not of WAG1. This study also showed that PID phosphorylates other residues in the PIN3 hydrophilic loop in addition to the M3 motif. The third phosphorylation motif, Ser337/Thr340, was biologically identified which is necessary for proper polarity and biological function of PIN1 (Zhang et al. 2010). However, this Ser337/Thr340 motif was not phosphorylated by PID, suggesting that other kinases are implicated in the phosphorylation of this motif.

Multiple phosphorylation motifs in the PIN hydrophilic loop and the target specificity of their kinases suggest the possibility that diverse combinatorial phosphorylation codes are operational to determine subcellular polarity of different PINs under different cellular conditions. These functionally characterized phosphorylation motifs in the hydrophilic loop have been largely conserved among long PINs even in moss and Lycopodiopsida (Huang et al. 2010; Ganguly et al. 2012b; our unpublished multiple-alignment analysis), implying that the phosphorylation-mediated regulatory tools for PIN polarity and directional auxin transport had emerged from the beginning of land plant evolution.

7.2.2 Phosphorylation of the PIN Hydrophilic Loop Modulates the Subcellular Polarity of PINs

Overexpression of PID led to the basal-to-apical polarity switch of PIN1, PIN2, and PIN4 in the Arabidopsis root (Friml et al. 2004). Consistently, PINs are preferentially targeted to the basal membrane in *pid* loss-of-function mutants (Friml et al. 2004), and the loss of PP2A leads to preferential apical PIN targeting in Arabidopsis embryos and roots (Michniewicz et al. 2007). These lines of evidence suggest that phosphorylated PINs are primarily recruited to the apical targeting pathway, whereas the dephosphorylated PINs are recruited to the basal targeting pathway (Michniewicz et al. 2007). PID, WAG1, or WAG2 is localized in the membrane with no particular polarity. These kinases phosphorylate the TPRXS motifs of PINs to lead them to the apical membrane, and phospho-defective mutations of these motifs result in basal targeting of PINs (Huang et al. 2010; Dhonukshe et al. 2010).

Prolonged BFA treatment causes apical-to-basal transcytosis of PIN1 (in the root epidermis) and PIN2 (in the root meristematic cortex) (Kleine-Vehn et al. 2008a, 2010; Rahman et al. 2010). These results suggest that distinctive ARF-GEFs are

implicated in apical-basal transcytosis of PINs: BFA-sensitive for basal and BFA-resistant for apical transcytosis (Kleine-Vehn and Friml 2008). Therefore, phosphorylation-mediated apical targeting and BFA-sensitive GNOM-mediated basal targeting are seemingly antagonistic to each other. A supporting observation for this notion is that loss of the phosphatase PP2A enhances the BFA-mediated apical targeting of cortical PIN2 (Rahman et al. 2010), indicating that both the defect of PIN dephosphorylation (thus more phosphorylated PINs) and the blocking of BFA-sensitive basal trafficking cause apicalization of PINs. Although the PIN localization had not been directly shown, AGC1 kinases are likely to redundantly operate for antagonizing BFA-sensitive ARF-GEF-mediated basal trafficking because the quadruple mutation of AGC1 kinases (PID, PID2, WAG1, and WAG2) was able to reduce the BFA sensitivity for root growth and gravitropism (Rahman et al. 2010). AGC1 kinases phosphorylate PIN proteins at the plasma membrane to lead them to the BFA-resistant apical targeting pathway (Kleine-Vehn et al. 2009; Dhonukshe et al. 2010), and compromised AGC1 activities lead to basal PIN targeting (Dhonukshe et al. 2010).

Three recent studies have suggested that PID also modulates lateral PIN polarity (Ding et al. 2011; Rakusová et al. 2011; Ganguly et al. 2012b). During phototropism of the Arabidopsis hypocotyl, PIN3 gradually disappears in the outer lateral membrane of the endodermal cell of the illuminated hypocotyls side, whereas PIN3 in the endodermal cell of the shaded side maintains apolar localization (Ding et al. 2011). PID is inhibitory to this light-mediated polarization of PIN3. Overexpression of PID strongly inhibits light-mediated inner lateral polarization of PIN3 in the endodermal cell of the illuminated side thereby making the hypocotyl nonresponsive to the light (Ding et al. 2011). Similarly, during gravitropism of the Arabidopsis hypocotyl, PIN3 polarizes to the lower side of the hypocotyl endodermal cell, and PID overexpression inhibits this PIN3 polarization (Rakusová et al. 2011).

In the Arabidopsis root pericycle cell, PIN3 localizes to the inner (toward the vasculature) and basal (toward the root tip) membranes (Blilou et al. 2005). Phospho-defective mutations of the 3m1 (RKSNASRRSF(/L)) or M3 (RKSNASRRSF(/L) + TPRPSNL) motif of the PIN3 hydrophilic loop, which are the targets of PID, made PIN3 apolar in the pericycle cell and failed to rescue the *pin3* mutant phenotypes such as shorter roots and defects in root gravitropism (Ganguly et al. 2012b). These observations demonstrate that PID is inhibitory in polar localization of PIN3 in the hypocotyl endodermis, whereas it is promotive for polarization of PIN3 in the root pericycle. It would be interesting to know whether the phosphorylation motif like 3m1 or M3 is also implicated in lateral PIN3 localization in the hypocotyl endodermis.

Phosphorylation-mediated intracellular trafficking and polarity determination of PINs seem to depend on certain cell type-specific factors. Phospho-defective mutations in the M3 motif of the PIN3 hydrophilic loop led PIN3 localization to the tonoplast in the root hair cell, whereas the same mutations led PIN3 localization normally to the plasma membrane but with no polarity in the root pericycle cell (Ganguly et al. 2012b). Interestingly, the basal PIN3 localization in the root vasculature remained unaffected by the M3 mutations (Ganguly et al. 2012b). These results

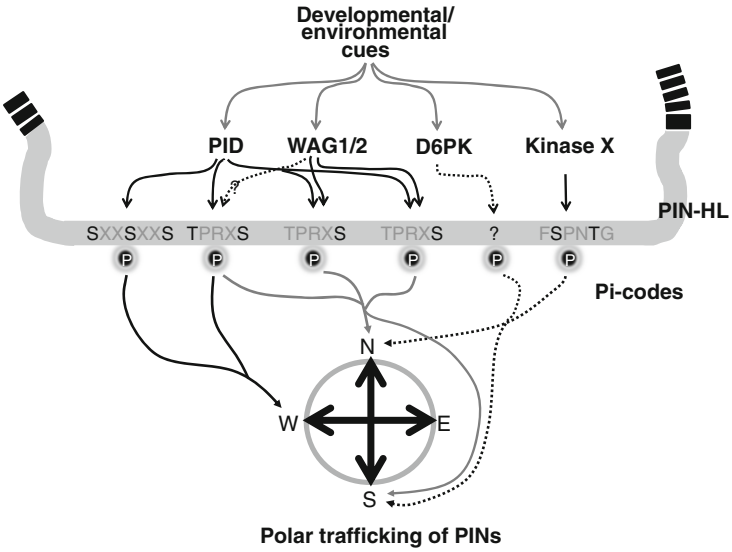

Fig. 2 Phosphorylation codes in the PIN hydrophilic loop (HL) modulate PIN polarity. Developmental and environmental cues may regulate different protein kinases which in turn generate different phosphorylation codes on the PIN-HL for the specific PIN polarity depending on cell type and PIN molecule

suggest that the M3 phosphorylation code of PIN3 is interpreted as a polarity determinant in the root pericycle cells but is recognized primarily as a plasma membrane-targeting cue in the root hair cell.

As mentioned previously, multiple phosphorylation sites have been functionally identified in the PIN hydrophilic loop, and more sites would possibly exist. Some phosphorylation sites have been conserved among different PIN species, but those phosphorylation sites also show some diversity in their sequence structures. Different PINs show different subcellular polarity. Furthermore, cell-type specificity, stimuli responsiveness, and the existence of multiple protein kinases are added to this complexity. Those multiple phosphorylation motifs with some contextual diversity and multiple kinases may play combinatorially to generate a specific phosphorylation code for cell type-, stimuli responsiveness-, or molecule-specific trafficking and polarization of PINs (Fig. 2). So far, the downstream trafficking or polarity factors of phosphorylated (or dephosphorylated) PINs have remained to be identified. These downstream factors, together with the upstream components, would additionally contribute for the diversity of PIN polarization signaling.

7.2.3 Upstream Regulatory Pathways for PIN Phosphorylation

Two PID-interacting proteins have been identified: TCH3 (TOUCH3, a calmodulin-like protein) and PBP1 (PID-BINDING PROTEIN1, an EF-hand motif protein) (Benjamins et al. 2003). The interaction between these proteins and PID requires

calcium ions, and these proteins are likely to function upstream of PID. The autophosphorylation activity of PID was shown to be enhanced by PBP1 (Benjamins et al. 2003). However, the *in planta* functions of these PID-interacting proteins in PID-mediated regulation of auxin transport still remain to be elucidated. The PID kinase activity is also boosted by 3-phosphoinositide-dependent protein kinase-1 (PDK1) which directly phosphorylates PID in vitro (Zegzouti et al. 2006). In this context, a recent study has implicated the inositol triphosphate (InsP3)-mediated calcium signaling in regulation of PIN polarity (Zhang et al. 2011). Increases of InsP3-mediated calcium signaling disrupted basal polarity but did not affect apical polarity of PIN localization. High levels of InsP3 and calcium also reduced PID overexpression-induced root collapse and strengthened cotyledon-defective phenotypes of the *pid* loss-of-function mutant. Although the mechanism how InsP3-mediated calcium signaling regulates the PID activity remains to be elucidated, this observation adds another evidence for the connection between InsP3-mediated calcium signaling and PID-regulated PIN polarity.

7.3 Membrane Lipid Composition

The lipid composition of the plasma membrane is important for movement and polar localization of membrane proteins (Brown and London 2000). Defects in sterol-biosynthetic enzymes such as sterol methyltransferases (SMTs) and cyclopropylsterol isomerase1 (CPI1) disrupt particularly the polar localization of PINs and cause defects in auxin-related development such as cell polarity and embryogenesis (Willemsen et al. 2003; Men et al. 2008; Carland et al. 2010). PIN proteins localize to the newly formed cell plate of the dividing cell (Geldner et al. 2001; Dhonukshe et al. 2006). After cell division, the polar PIN protein like PIN2 needs to be removed from one side of the membrane in order to retain its usual apical polarity. In the *cpi1-1* mutant, this removal of PIN2 from one side of the daughter membrane is inhibited, and PIN2 is retained on both sides of the newly formed daughter cell plasma membrane (Men et al. 2008). Moreover, sterols co-localize with PIN2-positive endosomes and undergo BFA-sensitive and actin-mediated endocytic trafficking thereby indicating a sterol-mediated process in PIN polarity regulation (Grebe et al. 2003). Likewise in animals, the plant plasma membrane also harbors certain microdomains which are rich in sterols, detergent resistant, and implicated in plasma membrane-related signaling for endocytosis (Mongrand et al. 2004; Borner et al. 2005; Sharma et al. 2002; Titapiwatanakun and Murphy 2009). Although sterols are obviously involved in regulation of PIN polarity, the mechanism remains to be elucidated.

In addition to sterols, the metabolism of membrane phospholipids also is implicated in PIN trafficking. Phospholipase A2 (PLA2) is a fatty acyl hydrolase of membrane phospholipids. Pharmacological or genetic suppression of PLA2 interrupts the plasma membrane localization of PINs, causing their internalization (Lee et al. 2010). Although the mechanism how PLA2 regulates PIN trafficking is

currently speculative, the phospholipase, localizing in the Golgi, might be involved in modification of membrane phospholipids for the cargo-vesicle trafficking from Golgi to the plasma membrane.

7.4 Ubiquitylation

In addition to synthesis, secretion, phosphorylation, endocytosis, and recycling/transcytosis, degradation has been another orchestrating member for regulation of polar PIN localization. So far only PIN2 degradation by ubiquitin-mediated proteolysis has been studied in detail. A proteasome inhibitor MG132 is capable of blocking both internalization and degradation of PIN2, indicating that the proteasome activity is involved in these processes (Abas et al. 2006). Ubiquitylated PIN proteins in the plasma membrane have been shown to destine to the lytic vacuole (Abas et al. 2006; Laxmi et al. 2008; Kleine-Vehn et al. 2008b). A recent study has directly demonstrated that lysine63-linked polyubiquitin chain formation is involved in the vacuolar targeting and proteolytic degradation of PIN2 (Leitner et al. 2012). This study tested 22 out of 28 lysine residues in the PIN2 hydrophilic loop and found that multiple mutations (Lys to Arg) of 12 or 17 residues together were required to generate ubiquitylation-defective PIN2. Mutations of these lysine residues stabilized PIN2 in the plasma membrane and interfered with its vacuolar targeting and degradation (Leitner et al. 2012).

7.5 ABP1 and ROPs

Auxin promotes its own efflux from the cell by inhibiting endocytosis of PINs and AT-ABCBs, thereby increasing the abundance of these efflux transporters in the plasma membrane, and this process is independent of the TRANSPORTER INHIBITOR RESISTANT1/AUXIN-BINDING F-BOX-mediated nuclear auxin signaling (Paciorek et al. 2005; Cho et al. 2007b). Recent studies have shown that this auxin effect on PIN endocytosis is mediated by another auxin receptor AUXIN BINDING PROTEIN1 (ABP1). ABP1 mainly localizes in the ER but also partly localizes in the apoplast (Jones and Herman 1993; Tian et al. 1995; Henderson et al. 1997). Recently, apoplastic ABP1 has been found to regulate clathrin-mediated PIN endocytosis. Auxin binding to ABP1 inhibits the clathrin-dependent PIN endocytosis thereby leading to a high PIN abundance in the plasma membrane (Robert et al. 2010).

Currently, it remains to be elucidated how apoplastic ABP1 can regulate the clathrin-mediated endocytosis which basically occurs in the cytosolic side. It has recently been speculated that ABP1 might act through a transmembrane protein to transmit the signal from the extracellular space to the intracellular cytosol (Shi and Yang 2011). ABP1 may regulate endocytosis by affecting the phosphorylation

status of cargo proteins such as PINs or core components for the clathrin-mediated endocytic process. Future studies to identify certain ABP1-interacting proteins in the apoplast should shed light on the process of auxin-mediated inhibition of endocytosis. On the other hand, the role of ER-localized ABP1 remains unknown. Given the fact that membrane proteins like PINs are synthesized in the ER and secreted to the plasma membrane, it is tempting to speculate that ER-ABP1 may also play a role for PIN trafficking from ER.

Although the direct interacting partner of ABP1 remains elusive, several ABP1 downstream targets have been recently characterized. Xenbiao Yang's group has been studying the function of ROP (Rho of plant) small GTPases during interdigitated cell expansion of the Arabidopsis leaf epidermal pavement cells. Formation of lobes (tip) and indentations in the characteristic jigsaw puzzle-shaped pavement cells is coordinated by ROP2 and ROP6 (Fu et al. 2002, 2005). ROP2 is locally activated at the lobe-forming site and promotes cortical diffuse F-actin formation and lobe outgrowth by working with its effector ROP-interactive CRIB motif-containing protein 4 (RIC4) (Fu et al. 2005). In the lobe tip, ROP2 suppresses well-ordered cortical microtubule (MT) arrays by inactivating another effector RIC1, thus relieving MT-mediated outgrowth inhibition (Fu et al. 2002, 2005). In the opposing indenting zone, ROP6 activates RIC1 to promote MT organization and to suppress ROP2 (Fu et al. 2005, 2009).

Recently, ROP2 and ROP6 have been found to antagonistically control the formation of lobes and indentations in response to asymmetric auxin distributions (Xu et al. 2010). PIN1 proteins are polar-localized or more concentrated in the lobing plasma membrane than in the indenting membrane part of the pavement cell. This polar-localized PIN1 is thought to create local auxin accumulation in the lobe apoplast so as to cause binding of auxin to ABP1. Auxin-bound ABP1 is thereafter likely to activate the ROP2-RIC4 pathway in the lobe side to promote lobe formation. Conversely, auxin-free ABP1 activates the ROP6-RIC1 pathway in the indentation side of the adjacent cell, resulting in the development of interdigitated leaf pavement cells. ABP1-mediated activation of ROP2 seems to increase PIN1 abundance at the plasma membrane of the lobe side, which in turn leads to a positive feedback on ROP2-dependent formation of cortical diffuse F-actin.

Even though several lines of evidence have pointed toward an actin-dependent trafficking of PIN proteins (Geldner et al. 2001; Grebe et al. 2003), the question still remains how ROPs affect polar PIN1 localization in the pavements cells. It has been recently shown that the ROP2/RIC4-dependent auxin signaling pathway induces localized accumulation of cortical fine F-actin which inhibits clathrin-dependent PIN1-endocytosis and leads to PIN1 polarization (Nagawa et al. 2012), supporting the previous study where ROP2 was shown to regulate PIN2 polarization during gravitropic response (Li et al. 2005). However, the mechanism how local accumulation of actin can inhibit PIN endocytosis remains yet to be elucidated. In addition to the inhibition of PIN1 endocytosis, ROP2 signaling may also promote polar PIN1 recycling (Nagawa et al. 2012).

7.6 More Regulatory Signals for Auxin Transport

We have described diverse regulatory factors for auxin transport. In addition to these regulators, auxin transport is also modulated by many other factors such as plant hormones and other signaling molecules, nutrients, and even pathogens.

High cytokinin signaling in the root transition zone enhances *SHY2* (*SHORT HYPOCOTYL2*, encoding an AUXIN/INDOLE-ACETIC-ACID repressor) expression which in turn inhibits *PIN3* expression (DelloIoio et al. 2007, 2008). *PIN3* downregulation reduces auxin transport in the transition zone, ultimately leading to cell elongation and differentiation in the transition zone. Cytokinin regulates auxin transport not only through transcriptional regulation of *PIN* but also by promoting selective endocytosis and proteasome-mediated degradation of PIN1 in the Arabidopsis root and tobacco BY-2 cells (Marhavý et al. 2011). Cytokinin is also implicated in vascular development by promoting bisymmetric distribution of PIN proteins (Bishopp et al. 2011). Several studies have further demonstrated that cytokinin regulates the expression of *PIN* genes during root and other organ development (Laplaze et al. 2007; Ruzicka et al. 2009; Pernisová et al. 2009). Ethylene affects transcription as well as polar localization of PINs during Arabidopsis apical hook development (Zádníková et al. 2010). Brassinosteroid (BR) extends the *PIN2* expression domain from the root meristem to the elongation zone, resulting in enhanced root gravitropism (Li et al. 2005). This BR effect on root gravitropism might be achieved by way of ROP2 action because BR regulates the expression and subcellular localization of ROP2 and ROP2 modulates PIN2 polarity by affecting F-actin (Li et al. 2005; Lanza et al. 2012). Gibberellin has recently been shown to play as a positive factor to maintain PIN abundance most likely by modulating vacuolar degradation of PIN proteins (Willige et al. 2011). Jasmonic acid (JA) also has been known to regulate PIN trafficking. Low methyl JA (5 μM) inhibited PIN2 endocytosis through the TIR1/AFB-mediated auxin signaling, whereas higher (50 μM) methyl JA reduced PIN2 targeting to the plasma membrane in an auxin-independent manner (Sun et al. 2011).

Nitric oxide (NO), a regulator of plant development, decreases PIN1 protein levels in a proteasome-independent manner in the root meristem, which results in decrease of cell division and primary root growth (Fernández-Marcos et al. 2011). Furthermore, as previously mentioned, shoot-supplied ammonium and iron are involved in lateral root formation by affecting AUX1-mediated auxin transport (Li et al. 2011; Giehl et al. 2012). During the infection, plant-parasitic nematodes utilize the host PIN molecules, by modulating their gene expression and polarity, to direct auxin flow to the initial syncytial cell (Grunewald et al. 2009). A small group of transcription factors also regulate PIN expression for the proper leaf organogenesis in the shoot apex. Defects in three related PLETHORA-LIKE (PLT) AP2 transcription factors (PLT3, 5, and 7) cause alterations of phyllotaxis in Arabidopsis, and this is largely attributable to decreases in PIN1 levels in the shoot apical meristem (Prasad et al. 2011).

The diverse internal and external signaling pathways are operational in regulation of auxin transport, local auxin concentrations, and thus auxin-mediated developmental processes by affecting synthesis, trafficking, polarity, modification, and degradation of auxin-transporting proteins. Considering the broad and fundamental role of auxin in plant growth and development, it is natural to expect that such diverse signals interact to influence auxin transport and there could be more regulators to be identified. The regulatory process for the subcellular polarity determination of auxin transporters not only is important to understand how internal or environmental signals coordinate to affect plant development but also provides a model system to study how cellular polarity can be established in plants.

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Polar Auxin Transport: Cell Polarity to Patterning

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Abstract Auxin is a signaling molecule with profound influence on plant morphogenesis. Because of its activity gradient-related effects on plant development and response programs, it is considered as a plant morphogen. Auxin displays a spectacular ability to mobilize in a cell-to-cell and polar fashion. Auxin efflux carrier PIN proteins direct this intercellular flow of auxin and thus bear a rate-limiting effect on the formation of auxin activity gradients. With this influence on directionality and amount of auxin transport, PINs play crucial roles in plant body organization and connect cell polarity to plant patterning. As a consequence, mechanisms regulating the localization of PINs are widely investigated. Recent work uncovers the roles of vesicle trafficking regulator ARF–GEF GNOM, a kinase PINOID, a SNX1–VPS29 retromer complex, ROP-GTPases, Rab-GTPases, endocytosis regulator clathrin, membrane sterol composition, and cytoskeleton for subcellular PIN trafficking and their polar localization. In this chapter, we cover the state of the art of polar auxin transport and its impact on plant morphogenesis.

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1 Auxin: A Plant Signaling Molecule with Morphogen-Like Characters

The forms and functions of multicellular organisms are not possible without effective communication between cells, tissues, and organs. Due to cell immobility, the regulation of morphogenesis in plants occurs in a coordinated fashion, and it predominantly depends on spatially instructive chemical signals called hormones. Auxin was the first hormone to be discovered in plants with some morphogen-like characteristics. In a classical book "The Power of Movement in Plants" in 1880, Charles Darwin described the effects of light on movement of canary grass (*Phalaris canariensis*) coleoptiles. Upon application of unidirectional light on the coleoptile, it bends in the direction of the light. Darwin's experiment suggested that the tip of the coleoptile was the tissue responsible for perceiving the light and producing some signal, which was transported to the lower part of the coleoptile where bending occurred. In 1926, Fritz Went isolated a plant growth substance by placing agar blocks under coleoptile tips and then removing and placing them on decapitated Avena stems (Went 1926).

Indole-3-acetic acid (IAA) is the most important member of signaling molecules from the auxin family. The production and persistence of IAA in the plants are controlled in many ways. By functioning as a mobile signal that connects cells, tissues, and organs, auxin coordinates plant morphogenesis and response programs. It plays crucial roles in many growth and developmental processes and thus shapes plant architecture. Interestingly, auxin is the only hormone transported in a polar manner. The pattern of auxin distribution within the plant navigates plant growth (Friml et al. 2003; Benkova et al. 2003). The long-distance auxin transport occurs via the stream of fluid in phloem vessels, and the short-distance auxin transport exhibits cell-to-cell movement. Auxin regulates transcription of various downstream genes in auxin-signaling pathway (Abel and Theologis 1996; Hagen and Guilfoyle 2002). Auxin binds and activates the TIR1 F-box component of the SCFTIR1 E3 ubiquitin ligase, which then ubiquitinates the auxin-signaling repressor AUX/IAA proteins, targeting them for destruction by the proteasome (Dharmasiri et al. 2005; Kepinski and Leyser 2005). This releases the auxin response factor (ARF) from the repressing influence of AUX/IAA to activate transcription of downstream genes (Tiwari et al. 2001). Plant, such as *Arabidopsis*, possesses 29 AUX/IAAs and 23 ARFs representing a complex matrix of auxin-signaling-dependent transcriptional network. In addition, functions of the ARF proteins are variable and also a high degree of functional redundancy is seen among the family members (Rademacher et al. 2011). Together, this poses tremendous challenges to untangle the circuitry of auxin-signaling machinery. A transcription-based synthetic reporter, DR5, consisting of multiple tandem repeats of ARF binding site (TGTCTC) is generally used to detect auxin activity in plant cells (Ulmasov et al. 1997a, b). Recently, more sensitive and repression-based auxin sensor has been developed, exploiting the auxin perception-related degradation properties of IAA (Vernoux et al. 2011;

Brunoud et al. 2012). Additional experimental methods such as microdissection, mass spectroscopy, and immuno-localization are used to measure the auxin level, and its distribution can be correlated with DR5 reporter.

Auxin regulates cell expansion, cell growth, and cell division, in a concentration and context-dependent manner. Auxin concentration along with other local factors contributes to cell specification, differentiation, and dedifferentiation. Depending on the specific tissue, auxin may stimulate axial elongation, lateral expansion, or isodiametric expansion. In addition to the differential distribution of auxin-signaling machinery, auxin biosynthesis is also spatiotemporally regulated. Once auxin is synthesized, it is transported to sites of its action. Auxin is generally transported from shoot apex to root apex. For a long-distance auxin transport, phloem vessels act as the highway, but for a short-distance auxin transport, a unique system of cell-to-cell polar transport is exploited. As auxin signaling relates to auxin distribution patterns and as auxin distribution patterns are regulated by the landscape of cell-to-cell polar auxin transport canals, it is considered that polar auxin transporters directly feed on the auxin-regulated programs. Thus, the directional signaling of auxin depends on the subcellular localizations of plasma membrane-associated auxin efflux and influx carriers. As auxin can enter into the cell either with the help of influx carrier or in a passive manner and as auxin requires efflux carriers for its exit out of the cell, the efflux carrier bears critical rate-limiting and directional influence on auxin transport. Efflux carrier PIN-FORMED (PIN) proteins have been established to be the main actors for the directional auxin transport. The localization of PIN proteins determines the direction of auxin flow and the choreographed relay of PIN activity along the auxin passage generates auxin gradients (Benkova et al. 2003; Petrásek et al. 2006; Paponov et al. 2005).

Auxin biosynthesis-, auxin conjugation-, and polar auxin transport-achieved auxin accumulation provides spatial coordinates for navigating plant organ formation, organ growth, and organ response programs (Benjamins and Scheres 2008). Auxin contributes to apical dominance. The apical bud synthesizes auxin and it diffuses downward to suppress lateral bud dominance. As shoot tip forms an auxin source and root tip an auxin sink, cutting of shoot tip impairs auxin supply to the roots and leads to inhibition of root growth and the formation of lateral roots (Sassi et al. 2012). In contrast, shoot decapitation leads to the development of lateral stems that allows gardeners to practice pruning in order to promote formation of extra shoots. Further, auxin participates in phototropism, geotropism, hydrotropism, and other developmental modifications. Cell division increases the number of cells and cell expansions, and growth is ultimately reflected in the tissue morphology, organ shape, and plant architecture. Differential auxin distribution modifies the coordination between cell division and expansion and as a consequence leads towards differential growth, triggering the shoots bending towards light or the root bending towards gravity (Peer et al. 2011). Auxin is necessary for fruit development and it delays fruit senescence. Exogenous application of auxin in fruits with removed seeds initiates fruit growth. When polar auxin transport is disrupted, it leads to abnormal

fruit morphologies. Auxin also plays a role in flower initiation and development of reproductive organs (Sundberg and Østergaard 2009). Auxin also plays an instrumental role in regeneration process (Duclercq et al. 2011). Furthermore, auxin promotes organization and development of xylem and phloem (Ye 2002).

1.1 Morphogenic Properties of Auxin

A morphogen is generally thought of as a chemical whose concentration varies in space, and for which varying threshold concentrations direct qualitatively different cellular responses or fates. Auxin acts in the micro- to nano-molar range. Graded concentration of auxin is essential for embryonic patterning and root and shoot organogenesis. Total amount of auxin arriving from the shoot to the root influences the degree of root growth. Auxin appears to dictate cell fates in the embryo in a concentration-dependent manner. Through regulated transport, it can accumulate in a spatially asymmetric concentration gradient and acts as a transcriptional regulator. Varying concentrations of auxin could result in different degrees of AUX/IAA degradation, thus releasing variable amounts of ARF proteins that could then activate downstream targets in an ARF concentration-dependent manner. However, a direct correspondence between the cellular auxin concentration gradient and the development of discrete cell types or regions in the embryo has yet to be proved (Bhalerao and Bennett 2003; Friml et al. 2003).

1.2 Auxin Efflux Carrier PINs

The PIN proteins have been identified as the key regulators of auxin-mediated developmental processes including growth, tropism, embryogenesis, and organogenesis (Friml et al. 2002, 2003; Friml 2003). PIN proteins are plasma membrane-located proteins that act as efflux carriers (Petrásek et al. 2006; Paponov et al. 2005). The polar localization of PIN proteins determines the direction of auxin flow (Wisniewska et al. 2006). There are eight PIN genes in the genome of Arabidopsis and encode for protein between 351 and 647 amino acids. PIN1 and PIN4 are involved in organogenesis; PIN1, PIN3, PIN4, and PIN7 are involved in embryogenesis; PIN2 and PIN3 are involved in gravitropism; and PIN1 and PIN3 are involved in phototropism (Paponov et al. 2005). The Arabidopsis PIN proteins are functionally characterized and found to be localized in a polar fashion either at different sides of various cell types or within the cell organelle (Blilou et al. 2005; Vieten et al. 2005; Mravec et al. 2009; Ding et al. 2012). The PIN proteins constitute prominent cell polarity markers in plants. The analysis of intron–exon structures of Arabidopsis thaliana (At) AtPIN family members reveals the relationship between AtPIN1, AtPIN4, and AtPIN7. Five of the eight AtPINs are located in the duplicated blocks. AtPIN3 and AtPIN7 share the same location. AtPIN1 is more closely related

Table 1 Members of the PIN protein family and their respective roles for various plant developmental and response programs

Auxin efflux carrier	Role in development
AtPIN1	Phyllotaxy, vein formation. Embryogenesis, lateral organ formation. Vascular development
AtPIN2	Organ development, root gravitropism
AtPIN3	Gravitropism, phototropism, organ development
AtPIN4	Root patterning, embryogenesis
AtPIN5	Regulation of intracellular auxin metabolism
AtPIN6	Transport activity
AtPIN7	Embryogenesis, root development

to AtPIN3, AtPIN4, and AtPIN7 as compared to AtPIN2 (Paponov et al. 2005). The diversity of hydrophilic central regions of AtPIN shows that there is a degree of functional variation in this family. Genes homologous to Arabidopsis PIN family are identified in most of the plants. With the divergence of both monocot and dicot plants, there have been significant changes in the number and the structure of PINs. The plants *Medicago* and potato contain five PIN sequences similar to one of the eight PINs of Arabidopsis. The low identity between PIN5 genes and the hydrophilic domains in the proteins reveals that PIN5 diverged from the ancestral PIN gene from early stage of development. Not surprisingly, unlike other PIN proteins, PIN5 is not localized at the PM and is not involved in cell-to-cell polar auxin transport. Instead, it resides at the endoplasmic reticulum and regulates intracellular auxin homeostasis and metabolism (Mravec et al. 2009; Table 1).

Auxin efflux is proportional to the degree of PIN expression and its polar localization, and the entire process is sensitive to polar auxin transport inhibitors. Auxin abundance regulates *PIN* gene expression, localization, and degradation, forming a complex feedback loop between auxin and its transport amount and directionality. Besides the changes in PIN expression and localization in response to developmental cascades, PIN polarity switches can also occur in response to environmental stimuli (Friml et al. 2002, 2003; Benkova et al. 2003; Reinhardt et al. 2003; Scarpella et al. 2006).

2 Role of PINs in Plant Development

2.1 Auxin and Embryogenesis

Polar auxin transport has long been suggested to play a principal role in plant embryogenesis. It has been shown that the hypocotyl of mature embryos of both angiosperms and gymnosperms transports auxin in the direction of the root (Greenwood and Goldsmith 1970; Fry and Wangermann 1976). Fry and Wangermann

(1976) were the first to propose that the commencement of polarized auxin transport in globular embryo might facilitate the morphological polarity expressed in subsequent stages of plant embryogenesis. The probable role of polar auxin transport in somatic embryogenesis was confirmed by Schiavone and Cooke (1987) who treated different phases of carrot somatic embryos by TIBA and NPA. Both auxin transport inhibitors at a concentration of 1 μM are capable of blocking the ability of somatic embryos to go through morphogenetic change to the successive phases: globular embryo goes through persistent spherical expansion, oblong embryos (an intermediary phase in somatic embryogenesis) carry on axis elongation devoid of any cotyledon initiation, and heart embryo grows additional growth axis on their hypocotyls. The embryonic pattern formation is in fact well maintained by two coinciding mechanisms:

1. A positional mechanism that rises as a maternal consequence from the ovular tissue surrounding the zygotic embryo or as a result of the polarized location of the egg cell and/or early embryo inside the embryo sac
2. Auxin-mediated mechanisms that are established right since beginning of polar auxin transport in the late globular embryo

The route of auxin transport is determined by the polar plasma membrane localization of PIN proteins. Even before the identification of the PIN proteins, it was revealed that pharmacological inhibition of auxin transport obstructs normal embryo patterning in numerous plant species (Liu et al. 1993; Hadfi et al. 1998), indicating a role for auxin transport in embryo patterning. In *Arabidopsis*, four PIN proteins are dynamically expressed throughout the embryogenesis (Friml et al. 2003). Once the first division of the zygote is over, PIN7 is confined to the apical side of the basal cell and probably driving auxin transport into the apical cell. At the 32-cell phase, PIN7 polarity reverses to the basal membranes of the suspensor cells, possibly causing transport of auxin away from the suspensor cells. PIN1 gets expressed from the two-cell onwards to the 16-cell stage well before the establishment of its polarity in the embryo. At the 32-cell stage, PIN1 becomes polarly confined to the basal membranes in the provascular cells adjacent to the hypophysis and helps the transport of auxin into the hypophysis (Fig. 1). At the transition stage of embryogenesis, PIN1 gets polarly localized near the flanks of the apical embryo domain, which possibly results in auxin maxima at these convergent points. The PIN4 protein gets expressed in the hypophysis cell and following division, in its topmost daughter cell. The expression of PIN3 commences fairly late at the heart stage in the columella precursors.

The direction of auxin flow indicated by the localization of PIN proteins corresponds well to the expression pattern of the auxin response reporter, suggesting that auxin response maxima are likely to reflect the concentration of auxin and that the auxin response pattern is a result of active transport. Certainly, *pin7* mutant embryos are affected in the DR5 action in the early embryo and show related cell division perturbations, signifying that an appropriate auxin circulation and response is required for exact cell specification in the early embryo. *pin1* mutant shows defects in patterning of apical half of embryo, eventually resulting either into fused cotyledon or into creation of tri-cotyledon or single-cotyledon seedlings

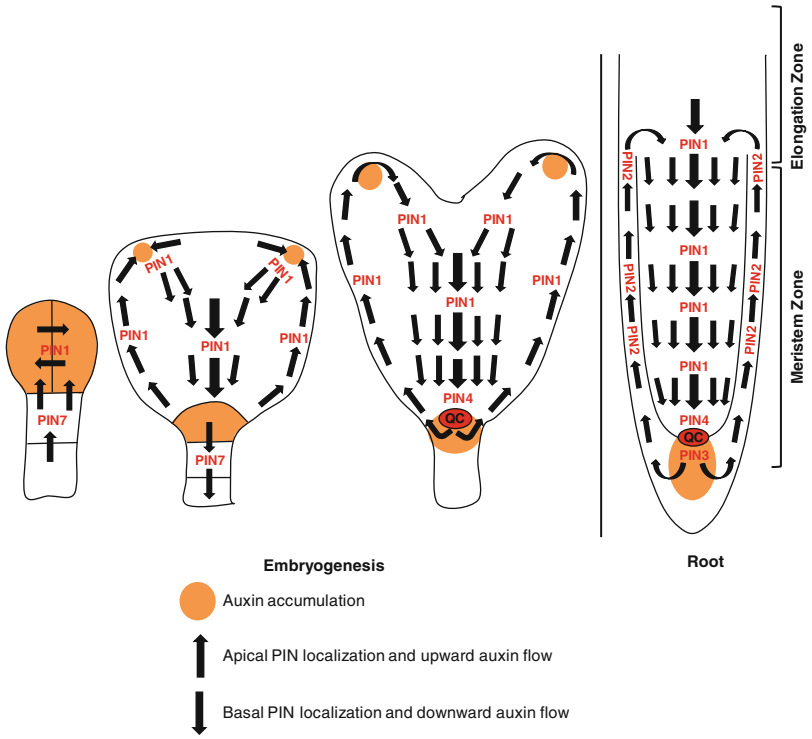

Fig. 1 Polar-localized auxin efflux carrier PIN proteins direct auxin flow during embryogenesis and root meristem growth to generate local auxin accumulation foci responsible for organ growth. This figure is adapted from Scientific World Journal (2012) 981658

(Aida et al. 2002). Single *pin* mutant does not display dramatic defects in embryonic patterning, suggesting redundant roles of PIN gene family during embryogenesis. Indeed, multiple *pin* mutant combinations show severe root and shoot pole defects (Friml et al. 2003; Blilou et al. 2005).

2.2 *Auxin and Root Development*

Auxin plays major roles in root development. Its concentration determines various aspects of root growth such as length of the epidermal-derived root hairs, the increase in quantity of lateral root primordia, and the response to gravity (Pitts et al. 1998; Rahman et al. 2002; Ishida et al. 2008; Péret et al. 2009). Auxin is synthesized in young leaves and cotyledons (Ljung et al. 2005) and transported to the root tip which represents the major sink tissue. Sorting of the root cells and measuring auxin concentration among them provide the most direct evidence of auxin gradients in the root, including the expected maxima in the quiescent center

(Petersson et al. 2009). In silico modeling of diffusion and PIN-facilitated auxin transport in and across root cells suggests that a robust auxin gradient associated with the maximum is able to explain the formation, maintenance, and growth of meristematic and elongation zones (Fig. 1) (Grieneisen et al. 2007). The local control of auxin levels creates regional concentration gradients and local maxima that are vital for establishing and sustaining a root primordium (Reviewed by Benjamins and Scheres 2008). The cellular auxin level in turn dictates the regulation of gene expression, which defines cell fate. Pharmacological or genetic interruptions of auxin transport intensely impact root patterning.

Several lines of experimental evidence support the idea that root-derived auxin contributes to establishment and maintenance of auxin gradient in the root and thus root growth. First, evidence for the role of auxin biosynthesis pathways in roots came from the characterization of the weak ethylene-insensitive (*wei2* and *wei7*) and transport inhibitor response 7 (*tir7-1*) mutants (Ljung et al. 2005; Stepanova et al. 2005). These mutants suppress the high-auxin phenotypes of the auxin-overproducing superroot (*sur1 or sur2*) and interrupt one of the two subunits of anthranilate synthase, an enzyme that catalyzes the rate-limiting step of anthranilate from chorismate during tryptophan synthesis. The failure of specific cells to yield tryptophan lowers their capacity to yield indole-3-acetic acid, which impairs root growth (Ljung et al. 2005; Stepanova et al. 2005).

2.3 Auxin and Lateral Root Development

Auxin is the key regulator of lateral root development (reviewed by Benkova and Hejatko 2009; Fukaki and Tasaka 2009). In many dicot plants as well as *Arabidopsis*, lateral roots originate from root pericycle cells adjacent to the protoxylem poles of the parent root (Beeckman et al. 2001). Initial events of lateral root formation are the divisions of a few pericycle cells positioned adjacent to a protoxylem pole. These cell division events are commonly designated as "lateral root initiation." Because the whole protoxylem pole pericycle displays a strong cell proliferation capability (Beeckman et al. 2001) and every pericycle cell adjacent to a xylem pole shows the ability to divide in response to elevated auxin levels (Himanen et al. 2002, 2004; Dubrovsky et al. 2008), it is believed that spatiotemporal control exists to limit lateral root initiation to certain sites and time points for the duration of root growth.

The even spacing and arrangement of lateral root primordia draws a parallel with a priming event that targets only a few pericycle cells as they depart the basal meristem (De Smet et al. 2007). These cells become primed due to an auxin response maximum that arises in the adjacent protoxylem cells. The auxin response maximum in the basal meristem and the simultaneous priming is not continuous but oscillates with a period of 15 h, which is in turn reflected in the regular spacing of lateral root along the root axis. The uniform spacing of lateral root primordia arises from pulses of auxin signaling in the basal meristem. The basal meristem includes

the set of cells that transit from the meristematic zone into the elongation zone and thus comprises of cells that undergo division as well as elongation. In seedlings that are grown in constant light, pulses of auxin signaling occur with a periodicity of 15 h. The response to auxin signaling in the xylem cells primes the adjacent pericycle cells so that they are competent to develop lateral root founder cells upon a second, auxin-dependent signal in the differentiation zone (De Smet et al. 2007). As such, the pulses of auxin in the basal meristem together with the uninterrupted growth of the root lead to the observed regular arrangement of lateral root primordia.

Auxin derived from both root and shoot is essential for the initiation and development of lateral roots. Passage of IAA via phloem from the leaf to root at the seedling stage is essential for emergence but not initiation of lateral roots in *Arabidopsis*. Genetic and pharmacological manipulation of this auxin movement interrupts lateral root formation (Reed et al. 1998; Bhalerao et al. 2002; Wu et al. 2007). Furthermore, experiments with the IAA transport inhibitor NPA demonstrate that IAA movement through the root tip is important for lateral root initiation, whereas shoot-derived transport is necessary for lateral root emergence (Casimiro et al. 2001).

Mutations or transgenes disturbing auxin biosynthesis, auxin metabolism, auxin transport, and auxin signaling affect the ability to form lateral roots. A gain-of-function mutation in a member of Aux/IAA protein family, IAA14/SOLITARY-ROOT, blocks the lateral root initiation (Fukaki et al. 2002). The presence of several auxin-related mutations specifically inhibiting lateral root initiation, lateral root morphogenesis, or lateral root emergence shows that auxin is essential not only for lateral root initiation but also for lateral root primordium development and morphology (Fukaki and Tasaka 2009). Mutations in several auxin efflux carrier *PIN* family members interrupt auxin-induced lateral root primordium development (Benkova et al. 2003). In addition, auxin influx carrier *AUX1/LAX* family members play important roles in lateral root initiation (Swarup et al. 2001; De Smet et al. 2007). These studies indicate that the regulation of distinct dynamic auxin transport systems is crucial for lateral root formation and development.

2.4 Auxin and Shoot Morphogenesis

Recent cellular and genetic data point towards the importance of auxin transport (Reinhardt et al. 2000, 2003) along with mechanical strains (Heisler et al. 2010; Peaucelle et al. 2011) in shaping phyllotactic patterns. These data allow incorporating in vivo PIN dynamics and auxin distribution details into computational models to theoretically test their relevance for phyllotaxis. Many models that attempt to understand the ability of auxin transport and mechanical stresses to capture leaf and even floral (van Mourik et al. 2012-considered for its similarity to purely phyllotactic models) patterns arising from cell-level simulations have

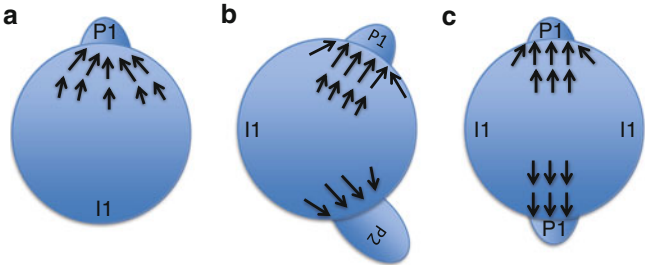

Fig. 2 Polar auxin transport based schematic representation of different patterns arising at the shoot apex; (**a**) Distichous (alternate), (**b**) Spiral, (**c**) Decussate (opposite) pattern. I (Incipient primordium), P (Primordium). The schematic representation is based on Reinhardt et al. (2003)

been proposed (de Reuille et al. 2006; Heisler et al. 2010; Stoma et al. 2008; Smith et al. 2006; Jönsson et al. 2006; Bayer et al. 2009).

Role of auxin in defining the initiation pattern of lateral organs sparks from sufficiency of auxin in triggering organ initiation when applied at the tip of naked meristem of *pin1* mutant, which is defective in polar auxin transport (Reinhardt et al. 2000). The presumed model suggests that auxin is transported to the site of lateral organ primordia inception by polar auxin efflux carrier PIN1. Growing lateral organ primordium acts as sink and this leads to depletion of auxin from surrounding cells, creating an inhibitory field which in turn controls the spacing between lateral organs to define a specific phyllotactic pattern (Reinhardt 2003; Fig. 2). While role of PIN1 was predicted earlier, only recently it has been shown that *pin1* hypomorphs result in switch of spiral pattern to opposite pattern (Prasad et al. 2011). Several computational models attempt to explain how a defined pattern is initiated. There is some consensus of assumptions found in various models made at the cellular and/or tissue level. The commonly used assumptions are:

(a) All decisive events that determine phyllotaxis occur in the outermost L1 layer except (Bayer et al. 2009) all models reviewed here limit themselves to the study of a single sheet of cells that form the shoot apical meristem.
(b) Movement of auxin through the apoplast is not considered.
(c) Auxin flows between cells by active transport and diffusion. Active transport is mediated by membrane proteins (PIN proteins) that show dynamic localization which is dictated by the specific polar auxin mechanism being modeled.
(d) Primordia are formed when auxin levels increase beyond a certain threshold, and once initiated, it is irreversible.
(e) Nascent and growing primordia act as auxin sinks (the empirical counterpart of the "inhibitory field"), modeled by the induction of another layer of "vascular tissue" beneath them that funnels auxin away from surrounding regions (de Reuille et al. 2006; Stoma et al. 2008), or an increased rate of degradation (van Mourik et al. 2012), both of which have equivalent mathematical formulations.

The distinct consequences of these sinks arise when applied over time. In some models, the converse is assumed, with developing primordia producing more auxin (Smith et al. 2006); in this case also, primordia act as sinks because of the concentration-based feedback involved in this model.

3 PIN Trafficking and Localization

Subcellular polarity of PINs determines the direction of auxin efflux out of that cell and thus coordinated PIN localizations along the chain of cells channelize directionality of auxin transport (Wisniewska et al. 2006; Dhonukshe et al. 2008, 2010). Subcellular analyses of PIN trafficking suggest that the PINs are not statically localized at the plasma membrane but undergo rapid endocytic cycling involving PIN internalization from the plasma membrane via clathrin-mediated endocytic pathway and PIN recycling back to the plasma membrane via ARF–GEF-regulated polar recycling (Geldner et al. 2001; Friml et al. 2002; Dhonukshe et al. 2007, 2008; Kleine-Vehn et al. 2008). The protein phosphatase 2A and Ser/Thr protein kinase PINOID are one of the major determinants of polar PIN localization (Christensen et al. 2000; Benjamins et al. 2001; Michniewicz et al. 2007; Dhonukshe et al. 2010). Apically localized PIN2 or basally localized PIN1 in the root meristem seems to be delivered originally in a nonpolar fashion after the de novo synthesis. Their apical or basal polarity is then established in the next step involving internalization from plasma membrane and phosphorylation-state-based polar recycling (Dhonukshe et al. 2008, 2010; Fig. 3). Additionally, molecules involved in intracellular trafficking of PIN and other proteins include endocytosis regulators, endosomal sorting/recycling regulators, and transcytosis regulators which are all associated in modulating polar localization of auxin transporters (Geldner et al. 2003; Kleine-Vehn et al. 2008).

3.1 Action of ARF–GEF GNOM in Polar Recycling of PINs

The *gnom* mutant phenocopies *pin1* mutant during early embryogenesis in Arabidopsis (Steinmann et al. 1999). GNOM encodes a BFA (brefeldin A)-sensitive guanine nucleotide exchange factor (GEF) for ARF GTPases that control vesicle budding (Steinmann et al. 1999; Geldner et al. 2003; Robert et al. 2008). GNOM protein resides in both the plasma membrane and endosomes (Steinmann et al. 1999; Geldner et al. 2003) and co-localizes with PIN1. In *gnom* mutant, PIN1 recycling is abnormal and as a result coordinated polar localization of PIN1 (and perhaps other PIN family members) is perturbed, leading towards drastic auxin transport-related

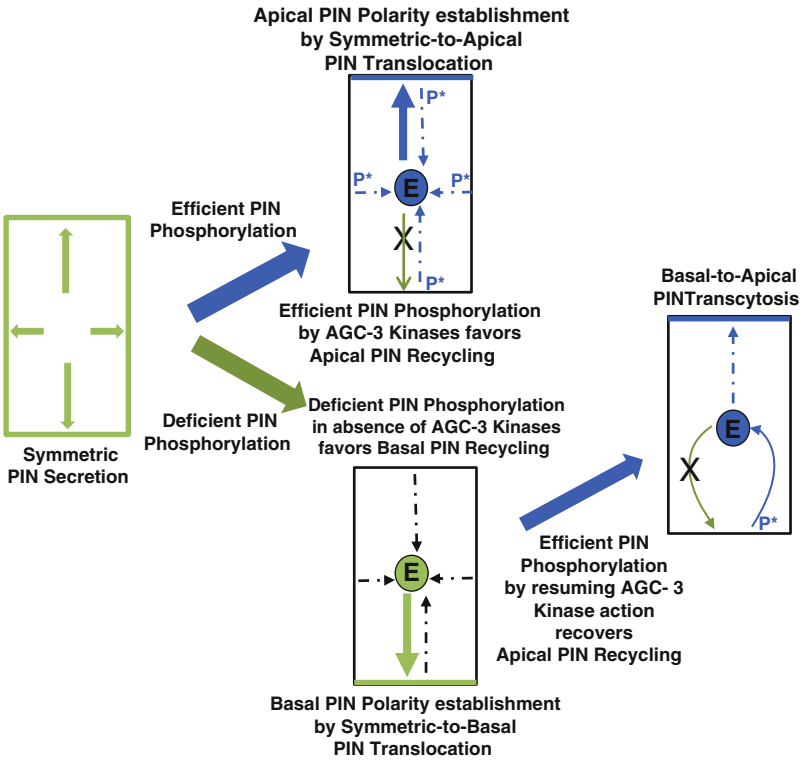

Fig. 3 Mechanism of establishment of PIN polarity by AGC-3 kinases. Non polar PIN gets translocated to apical upon phosphorylation by AGC-3 kianses and to basal in absence of phosphorylation. This figure is adapted from (Dhonukshe et al. 2012)

morphogenic defects. Transcytosis refers to the translocation of cargos from one polar domain to another. GNOM regulates basal PIN1 recycling and thus its basal localization. PINs have discrete polar plasma membrane localization on the apical, basal, or lateral side of cells, based on specific cell types (Kaplinsky and Barton 2004). GNOM-dependent transcytosis is crucial for early cell polarization. In the developmental progression of provascular cells, PIN1 can effectively shift from the apical side to the basal side in a wild-type line. In contrast, the *gnom* mutant fails to transport PIN1, localized on the apical side, to the basal side and shows abnormal embryo patterning. The BFA-treated wild-type seedlings also display *gnom*-like phenotypes (Geldner et al. 2001; Friml et al. 2003). In roots, PIN1, which is found in the stele, and PIN2, which is found in the cortex, are localized at the basal side of cells. However, PIN2 is localized at the apical side of epidermal cells. It was revealed that the BFA-sensitive GNOM ARF–GEF is obligatory for the basal but not apical localization of PINs. Loss-of-function mutations in *GNOM* or BFA treatment affected the basal PINs by recruiting them to the apical side. The shift in polarity of PINs due to the introduction of BFA was reversible in a protein

synthesis-independent manner. It was thus resolved that transcytosis through endosome-mediated trafficking plays a significant role in modulating PIN polarity (Kleine-Vehn et al. 2008).

3.2 Action of PID (PINOID) Kinase Providing Phosphorylation Bias for PIN Recruitment into Inverse Recycling Pathways

The *pid* mutant shows shoot phenotype similar to that of *pin1* mutants, displaying pin-like inflorescence, which indicates PID's role in auxin transport and/or signaling (Christensen et al. 2000; Benjamins et al. 2001). PID encodes a plant-specific serine/threonine protein kinase (Christensen et al. 2000). Interestingly, upon PID overexpression, PIN1 localization shifts from basal to apical cell side of root stele cells. The PID protein kinase directly phosphorylates PIN1, and the status of PID-mediated PIN1 phosphorylation governs its apical or basal distribution via its differential recruitment into apical or basal recycling pathway (Fig. 3). In addition to PID, WAG1 and WAG2, other two members of the AGC kinase protein family, instruct recruitment of PINs into the apical recycling pathway by phosphorylating the middle serine in three conserved TPRXS(N/S) motifs within the PIN central hydrophilic loop (Dhonukshe et al. 2010).

3.3 PP2A: A Protein Phosphatase Counteracting to the Action of PID Kinase

Given the significance of PIN phosphorylation status in determining PIN polar localization, PIN dephosphorylation is predicted to bear counteractive role. Recent study demonstrates that the PP2A phosphatase activity is essential for appropriate polar PIN localization and auxin transport-dependent plant development (Michniewicz et al. 2007). Mutations in PP2As (in Arabidopsis, there are three closely linked PP2A, including PP2A1, PP2A2, and PP2A3) cause various developmental abnormalities consistent with defective polar auxin transport. Genetic studies pointed out that PP2A (Ser/Thr phosphatases) and PID (Ser/Thr kinase) have counteracting roles in regulating auxin-dependent embryo and root development. A confined signaling pathway operates probably at the plasma membrane where PINs, PID, and PP2A all co-localize. Calcium also seems to play a role in PID/PP2A-mediated PIN phosphorylation/dephosphorylation. PID phosphorylation activity is negatively controlled by calcium and is concentration dependent. Auxin-related cell elongation and root alignment have been suggested to respond to changes in cytoplasmic calcium levels, and PINOID-mediated auxin signaling includes calcium-binding protein (Benjamins et al. 2003).

3.4 Role of SNX1 and VSP29 for PIN Trafficking and Localization

A retromer is a heteropentameric compound comprising of a sorting nexin dimer (through indefinite grouping of SNX1, SNX2, SNX5, or SNX6) and a trimer made up of VPS26, VPS29, and VPS35 in mammalian cells (Jürgens and Geldner 2007; Jaillais et al. 2006, 2007). The SNX dimers accomplish the recruitment of the pentameric retromer to endosomes, while the VPS22–VPS29–VPS35 triple subcomplex is thought to be binding cargos, which travel between endosomes and the *trans*-Golgi network (TGN). An *Arabidopsis thaliana* sorting nexin 1 (AtSNX1) was discovered in a novel endosomal compartment (Jaillais et al. 2006). AtSNX1 exists along with endosomal markers RABF1 and RABF2b in endosomes but not in Golgi, in the *trans*-Golgi network (TGN), and in endosomes having GNOM in them (Jaillais et al. 2006). Wortmannin, a PI3K (phosphatidylinositol 3-kinase) inhibitor deregulating the SNX localization in mammalian cells, also induced the generation of an enlarged AtSNX1-containing compartment in Arabidopsis. Wortmannin along with cycloheximide (protein synthesis inhibitor) triggered PIN2 accumulation in wortmannin-induced compartments. Based on these observations, it was established that polar PIN2 distribution is maintained by a novel AtSNX1-mediated endosomal pathway that is dissimilar to the GNOM-dependent PIN1-trafficking pathway, and it was suggested that two distinct populations of endosomes are involved in PIN1 and PIN2 trafficking, respectively.

3.5 Role of Rab5 for PIN Trafficking and Localization

Rab5 affects endocytosis via regulation of clathrin-coated vesicle formation at the plasma membrane, fusion of vesicles to endosomes, and fusion between endosomes (van der Bliek 2005). Rab5 localizes in the endosome and acts as a molecular switch by cycling between GDP-bound and GTP-bound states (Vitale et al. 1995). Arabidopsis possesses two direct homologues of Rab5 proteins. They are Ara7 and Rha1. The double mutant of ara7rha1 is gametophytic lethal (Dhonukshe et al. 2008). AtVps9a is the activator of Ara7 and Rha1. In Arabidopsis Rab5 homologues are identified in the endosomes. PINs are internalized largely by clathrin-mediated endocytosis pathway. The Rab5 interference affects PIN endocytosis. Interference with PIN endocytosis by manipulating the Arabidopsis Rab5 GTPase pathway prevents PIN polarization. Intriguingly, symmetric PIN1 leads to abnormal auxin distribution and one of the outcomes of such distribution causes enhanced local accumulation of auxin at cotyledon primordia eventually leading to root formation from places of cotyledon emergence (Dhonukshe et al. 2008), suggesting that maintenance of distinct polarity is prerequisite to specify cell fate and organ identity.

3.6 Role of ROP/RAC GTPases for PIN Trafficking and Localization

The plant-specific ROP/Rac subfamily of the extremely conserved Rho-family GTPases modulates signaling to set up the cell polarity in yeast and animal cells. Interestingly, it has also been shown to control cell polarity in several cell types of plants (Yang 2008). GFP–ROP2 was shown to be polarly confined to the plasma membrane in a similar manner to that of PIN2–GFP (Li et al. 2005). Gravity stimulation was found to induce vectorial re-localization of GFP–ROP2 in a way analogous to PIN2 re-localization. Furthermore, ROP2 overexpression intensifies PIN2–GFP polar localization and amplifies gravity responsiveness (Li et al. 2005). Further, auxin transported by PIN1 at the site of ROP2 activation induces ROP2 and ROP6 to regulate interdigitated growth of *Arabidopsis* leaf epidermal pavement cells (Xu et al. 2010).

3.7 Importance of Sterol Composition for PIN Localization

Sterols are integral components of the plasma and endomembranes. Sterols play a central role in the formation of membrane microdomains such as lipid rafts in the plasma membrane, which have a noteworthy effect on cell polarity. The chemical structure of plant sterol is similar to animal cholesterol. Polar delivery of cargos in plants also depends on the sterol composition of plasma membrane (Grebe et al. 2003; Willemsen et al. 2003; Kleine-Vehn et al. 2006). Recent work uncovers the major role of sterol in reiteration of PIN2 polarity after the division of polarized cells (Men et al. 2008). Sterols are also dynamic in endocytic trafficking among the plasma membrane and endosomal compartments, which is sensitive to the application of BFA.

4 Perspective

Recently, there has been a burst of literature on plant growth hormone auxin. We have begun to understand the mechanisms of polar localization and subcellular trafficking of auxin efflux carrier PINs. Precise localization of PINs, together with auxin influx carriers AUXs, shapes up the local accumulation of auxin during plant development. Recent experimental studies have allowed laying down conceptual models that can explain polar auxin transport and dynamics of PINs in generating the patterns. A major challenge ahead is to integrate the auxin-signaling pathways to the mechanistic aspects of polar auxin transport. How do auxin-signaling pathways instruct the subcellular trafficking of PINs and their polar localization and thus influence the polar auxin transport? Intriguingly, cell polarity is

instrumental to set the organ polarity. How the polar localization of PIN in individual cell and thus auxin transport from one cell to other gets communicated at the multicellular level to generate the polar organ remains largely unknown. However, gaining collaborations between in silico modeling studies and in vivo lab experiments are beginning to uncover the details of the morphogenetic auxin gradients in orchestrating various developmental processes.

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Signal Integration, Auxin Homeostasis, and Plant Development

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Abstract Auxin has been the most widely studied plant hormone in relation to plant development. The pattern of auxin distribution in plants plays a key role in organogenesis and tropic responses. Since auxin is synthesized by actively dividing cells, it is transported from the site of synthesis to the site of action to create auxin maxima. There has been a lot of effort in the past to dissect out the complex mechanism of auxin biosynthesis, signaling, and transport and to understand its role in controlling plant growth and development. Recently, there have been lots of reports of interaction between auxin and various hormones to regulate plant growth and development. This chapter summarizes the signaling integration cross talks and their role in affecting auxin homeostasis and plant growth and development.

1 Auxin Signaling

1.1 Auxin and Plant Development

Auxin was the first hormone to be studied in plants. In 1881, Charles Darwin published his results in *The Power of Movement in Plants* which stated that there is some kind of signal generated in the coleoptile tips which travels to the elongation zone and causes shaded side to grow more than the non-shaded portion. Based on this report, many experimentations were performed, but only in early 1926, Frits Went was able to demonstrate and obtain the diffusible growth-promoting factor from the tip of oat (*Avena sativa*) coleoptiles and it was termed as auxin. Indole-3-acetic acid (IAA) was determined as the principal natural auxin in the mid-1930s, and since then IAA has always been associated effectually with every aspect of plant growth and development. The multitude of processes affected by auxin has

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led to its consideration as a prime hormone. Auxin homeostasis is executed through the coordination of a set of three complex processes of auxin metabolism, translocation, and response.

1.2 Auxin Biosynthesis

Auxin biosynthesis is a complex process in plants. Auxin biosynthesis occurs both in aerial portions of the plant and in roots (Ljung et al. 2001, 2005). Auxin when required by plant is produced either by de novo synthesis involving multiple pathways or by facilitating the release from its conjugates (Zhao 2010). Previous genetic and biochemical analysis showed that two pathways operate for auxin biosynthesis: one is tryptophan (Trp) dependent and the other is tryptophan independent (Woodward and Bartel 2005). The Trp-dependent auxin biosynthesis pathway is categorized into four parts depending on their intermediates, which included the indole-3-acetaldoxime (IAOx) pathway, the indole-3-pyruvic acid (IPA) pathway, the tryptamine (TAM) pathway, and the indole-3-acetamide (IAM) pathway (Fig. 1). The IAOx pathway is catalyzed by cytochrome P450 monooxygenase (CYP79B2/B3) which converts Trp to IAOx. This pathway was identified in glucosinolate-producing plant species (Ruiz Rosquete et al. 2012). IAM pathway was well studied in bacterial system, and it is also conserved in plants. In this pathway, Trp is converted to IAM and with the help of indole-3-acetamide hydrolase, which is encoded by *AMI1* gene IAM, is then converted to IAA (Mano et al. 2010). IPA pathway is the main auxin biosynthesis pathway in Arabidopsis (Mashiguchi et al. 2011). In this pathway, Trp is converted to IPA with the help of Trp aminotransferase encoded by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) gene. Downstream to TAA1 and IPA acts the YUC proteins which are flavin monooxygenase-like proteins encoded by YUCCA (YUC) gene family. YUC family acts at a rate-limiting step in the IPA pathway and facilitates conversion of IPA to IAA (Mashiguchi et al. 2011). In TAM pathway tryptophan decarboxylase converts Trp to TAM; IAA is produced from indole-3-acetaldehyde (IAAld), a precursor produced from TAM (Mashiguchi et al. 2011). Any deficiency in these intermediates or Trp causes various developmental defects during organogenesis. In addition to Trp-dependent pathway in plants, a Trp-independent pathway also operates simultaneously, which was discovered after the study of Trp biosynthetic mutants, and it was found that they can also synthesize IAA without using Trp intermediates, but the detailed pathway is still to be elucidated.

Apart from auxin biosynthesis, auxin levels in plants are also maintained by conjugation of IAA. These conjugates are formed between IAA and sugars, amino acids, peptides, etc. These conjugations can be either reversible or irreversible. The IAA conjugates and indole-3-butyric acid (IBA) provide free IAA upon hydrolysis or β-oxidation. Other conjugates with glutamates (Glu) and aspartate (Asp) do not

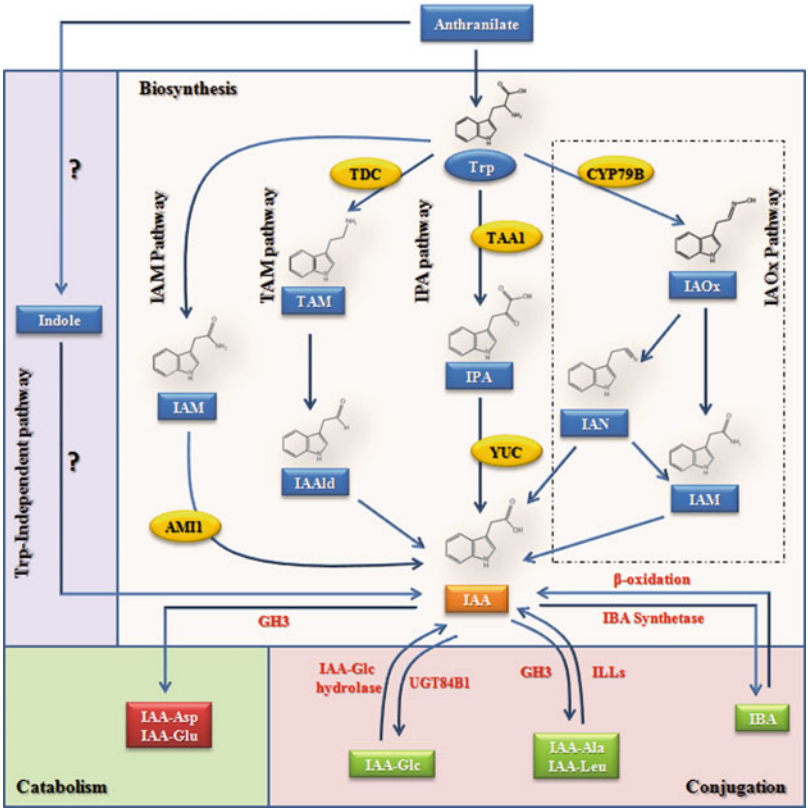

Fig. 1 Auxin biosynthesis, conjugation, and catabolism

hydrolyze and ultimately lead to catabolism (Tromas and Rechenmann 2010) (Fig. 1). Both auxin biosynthesis and its conjugation are important to maintain auxin levels in plants thus contributing to auxin homeostasis.

1.3 Auxin Transport

For proper plant development, a well-defined transport of auxin is required throughout the system. Various studies have shown that auxin transport is necessary for lateral root development, development of vasculature, embryonic axis formation, tropism, and phyllotaxy (Woodward and Bartel 2005). The site of auxin synthesis and its place of action in plant body are separated, and thus, to reach its destination auxin is translocated in a directional manner. Auxin translocates in plant through two distinct modes. One is the source to sink transport, which is faster (5–10 cm/h), nonpolar, and passive. In this type of transport, auxins are loaded in mature phloem

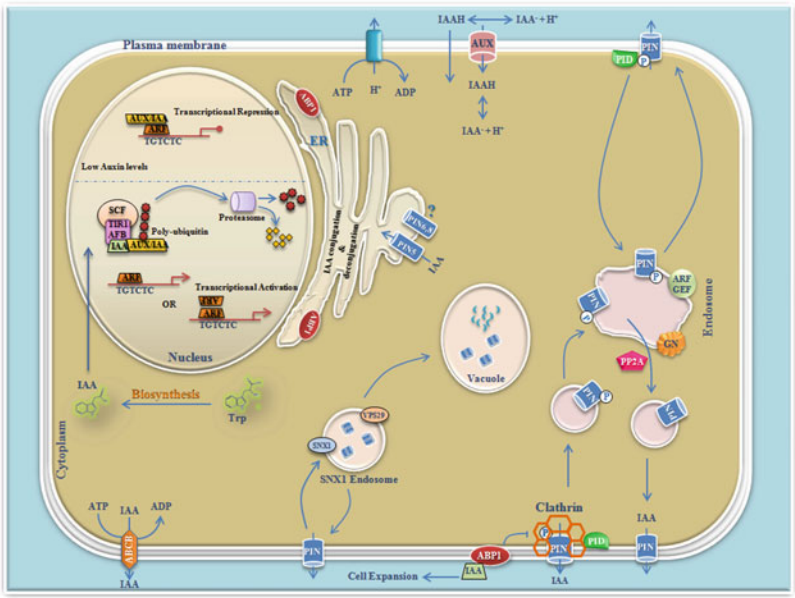

Fig. 2 Auxin signaling, transport, and trafficking mechanism of PIN

of biosynthetically highly active tissue and unloaded again in sink tissues, such as root (Marchant et al. 2002). The other is polar auxin transport (PAT), which is slower and more controlled. The pattern of plant is not only dependent upon the rates of auxin synthesis and its catabolism, but it is also dependent upon cells' capacity to retain or release auxin. Auxin distribution has an involvement of lots of proteins, but the most investigated family of proteins includes the PIN-FORMED (PIN) family consisting of eight members, PIN 1, 2, 3, 4, 5, 6, 7, and 8. These PINs are polarly localized plasma membrane proteins, which are found throughout the plant kingdom and mediate auxin efflux. Another family of proteins comprising of four members in Arabidopsis—AUXIN RESISTANT1 (AUX1) and LIKE AUX1 (LAX) 1, 2, and 3—facilitates auxin influx (Peer et al. 2011). PAT is facilitated mainly by these two family members. Other well-characterized members of auxin transporters include MULTIDRUG RESISTANCE/P-GLYCOPROTEIN (MDR/PGP) transporters, of which some facilitate auxin efflux and others mediate auxin influx, and members of "B" subclass of plant ATP-binding cassette (ABC) membrane transporters, which function as ATP-dependent amphipathic anion carriers during cellular auxin efflux (Fig. 2). Together PIN and ABCB transporters regulate cellular auxin homeostasis. Due to diversity of PIN protein promoters, PINs have specific tissue expression, which plays an important role in various developmental processes (Krecek et al. 2009). Aerial plant development is regulated by *At*PIN1 in Arabidopsis. PIN1 is mostly expressed in vascular tissues, in apical meristems of shoot, and in early embryos (Scarpella et al. 2006). The *At*PIN7 is found to be

expressed in the basal region of developing embryo and in the root tip and root primordia at the later stages. *At*PIN2, *At*PIN3, and *At*PIN4 are located in the root tip. These PINs mediate proper auxin distribution thus governing gravitropism (Krecek et al. 2009). *At*PIN5 and *At*PIN8 are characterized as short PINs. In these PINs, central hydrophilic loop is virtually absent. Among these *At*PIN5 is expressed weakly throughout, while *At*PIN8 is specifically expressed in the male gametophyte, i.e., the pollen (Krecek et al. 2009).

The localization of PIN proteins in subcellular compartments depends on their category of long PINs or short PINs. Long PINs decide the direction of intercellular auxin flow. Therefore, these are often localized on plasma membrane in a polarized manner (Wisniewska et al. 2006). The short PINs (*At*PIN5 and *At*PIN8) are localized on the endoplasmic reticulum (Fig. 2). This localization on endoplasmic reticulum helps to regulate the subcellular auxin levels. Localization of PIN proteins is asymmetric, complex, and regulated by other proteins. Changing the phosphorylation status of PIN proteins, which depends on the counterbalance activities of PINOID kinase (Friml et al. 2004) and protein phosphatase 2A (PP2A) (Michniewicz et al. 2007), changes the polarity of PIN proteins. Calcium and a 3-phosphoinositide-dependent protein kinase 1 (PDK1) act upstream of PINOID and PP2A; PDK1 phosphorylates and activates PINOID, whereas calcium negatively regulates PINOID phosphorylation activity (Fig. 2). Subcellular PIN localization is very dynamic. PIN proteins undergo endosomal recycling between plasma membrane and endosomal compartment. During the recycling, endocytosis of PIN proteins occurs in a clathrin-dependent manner (Dhonukshe et al. 2007), whereas ARF-GEFs and ARF-GTPase-activating protein such as GNOM and VASCULAR NETWORK DEFECTIVE 3 (VAN3) are involved in exocytotic step (Naramoto et al. 2010).

GNOM is a guanine nucleotide exchange factor for small G-protein of ARF class and regulates the targeting of PIN protein from the endosome to plasma membrane. On inhibiting GNOM function, apical localization of PIN protein is not strongly affected. This shows that GNOM plays a major role in basal polar targeting of PIN proteins (Kleine-Vehn et al. 2008). SORTING NEXIN1 (SNX1) and VACUOLAR PROTEIN SORTING 29 (VPS29) proteins are involved in the endosomal recycling of PIN proteins in *Arabidopsis*, which is distinct from GNOM-mediated pathway (Gao et al. 2008) (Fig. 2). Endogenous flavonoids negatively regulate auxin transport and alter gene expressions and subcellular localization of PIN1, PIN2, and PIN4. In addition, auxin itself provides feedback regulation of its own distribution by regulating the expression, stability, and localization of its own transporters. Auxin also inhibits PIN endocytosis and itself acts as self-polarizing signaling molecule by modulating PIN localization. Repolarization of PIN proteins is TRANSPORT INHIBITOR RESPONSE1 (TIR1)-dependent and is cell type specific. PAT is also modulated by other phytohormones such as cytokinin, GA, ethylene, and brassinosteroids, which eventually contribute to the auxin homeostasis in plants.

1.4 Auxin Signaling

Every event related to auxin biosynthesis, metabolism, and transport together collaborate to maintain an optimum auxin level so that a proper auxin signaling and its downstream effect can ensure optimal plant development. The physiological auxin response of specific cells can be controlled transcriptionally and non-transcriptionally. The non-transcriptional auxin signaling machinery is not yet well characterized, and AUXIN BINDING PROTEIN (ABP1) is considered an auxin receptor involved in non-transcriptional auxin signaling. ABP1 mediates rapid cell expansion by directly binding to auxin thus activating membrane potential and ion channels, which affect structural changes in cell wall and cytoskeleton. ABP1 also regulates the clathrin-mediated endocytosis of PIN proteins from the plasma membrane (Hayashi 2012; Robert et al. 2010). The transcriptional auxin signaling machinery was examined through the isolation of mutants with altered auxin response and also by examination of transcriptional changes after auxin administration. Initially, it was found that three families of transcripts were rapidly induced, which include the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA), GRETCHEN HAGEN3 (GH3), and SMALL AUXIN-UP RNA (SAUR) gene families (Chapman and Estelle 2009). The GH3 family encodes enzymes responsible for conjugation of auxin (Ludwig-Muller 2011). This family was first identified in soybean as an auxin-induced family (Hagen and Guilfoyle 1985). Intracellular auxin levels in cell are maintained by the feedback regulatory loop involving GH3 enzymes. SAURs are the largest family of early auxin-responsive genes. The SAUR proteins have been found to bind with Ca2+/CaM, but their physiological function is largely unexplored (Yang and Poovaiah 2000). SAUR63, a membrane-associated Arabidopsis SAUR protein, has been reported to regulate auxin-stimulated organ elongation positively (Chae et al. 2012).

Recently, SAUR19, SAUR20, SAUR21, SAUR22, SAUR23, and SAUR24 genes have also been reported to modulate auxin transport positively which leads to cell expansion in Arabidopsis (Spartz et al. 2012). Auxin response factors (ARFs) modulate expression of numerous auxin-responsive genes and AUX/IAA transcriptional repressor proteins. Auxin leads to transcription activation that most likely involves the participation of genes encoding AUX/IAA and ARF proteins. The AUX/IAA gene family in Arabidopsis consists of at least 29 genes encoding short-lived nuclear proteins, the transcription of which is rapidly upregulated by auxin (Remington et al. 2004). The AUX/IAA family consists of four conserved domains. Study of gain-of-function mutants of AUX/IAA gene family such as *short hypocotyl 2/indole-3-acetic acid inducible 3* (*shy2/iaa3*), *auxin resistant 2/indole-3-acetic acid inducible 7* (*axr2/iaa7*), *bodenlos/indole-3-acetic acid inducible 12* (*bdl/iaa12*), *solitary root/indole-3-acetic acid inducible 14* (*slr/iaa14*), *auxin resistant 3/indole-3-acetic acid inducible 17* (*axr3/iaa17*), and *massugu 2/indole-3-acetic acid inducible 19* (*msg2/iaa19*) showed that AUX/IAA repressor proteins undergo proteolytic cleavage. This is the crucial step during the expression of early auxin-responsive genes (Mockaitis and Estelle 2008). AUX/IAAs form oligodimers with

themselves and with the ARF family that consists of 23 genes in Arabidopsis. The ARF proteins contain the B3 DNA-binding domain, an activation/repressor domain, and two domains, which are highly similar to domains III and IV of AUX/IAA (Lau et al. 2008).

ARFs permanently bind to auxin-upregulated genes through auxin response element TGTCTC. Under low auxin concentration, they form dimers with AUX/IAA through domains III and IV, thereby shutting down transcription of auxin-responsive genes (Lau et al. 2008). Upon auxin accumulation, AUX/IAA is rapidly degraded, and this process allows ARF homodimerization and transcription (Leyser 2002) (Fig. 2). Degradation of AUX/IAA is executed via the ubiquitin-dependent proteolysis system. Major components of this pathway are E1 and E2 ubiquitin-activating and ubiquitin-conjugating enzymes and the E3 ligase which transfers the ubiquitin to target proteins. Auxin is perceived by TIR1 and AUXIN SIGNALING F BOX (AFB) protein receptors. Together they form integral component of SKP1/CULLIN1/F-BOX PROTEIN $(SCF)^{TIR1/AFB}$ complex (Kepinski and Leyser 2005). AUX/IAA through domain II binds to TIR1. Auxin enhances interaction between AUX/IAA and TIR1 by acting as molecular glue (Lau et al. 2008). The SCF ligase consists of four subunits Skp1, Cullin, F-box protein, and RING protein Rbx1. The Cullin1 (CUL1) protein acts as the scaffold for Skp1 and Rbx1, whereas F-box protein is attached to Skp1. CUL1 undergoes modification by a ubiquitin-related protein RUB/NEDD8 (Hayashi 2012). Conjugation of RUB/NEDD8 with CUL1 is facilitated by regulatory proteins such as RUB-specific E1 AUXIN RESISTANT1 (AXR1), E1 C-TERMINAL RELATED 1 (ECR1), and E2 enzyme RUB1 CONJUGATING ENZYME 1 (RCE1). Mutations within F-Box, Cullin, or Rub-activating enzyme in Arabidopsis are responsible for the auxin-insensitive phenotype. Specific interactions between AUX/IAA and ARFs define the gene expression pattern during different developmental processes of plant. The receptor-mediated cleavage regulates the degradation of transcription factors and other regulatory proteins.

2 Cross talk in Plant Hormone Signaling

2.1 Interaction of Brassinosteroids with Auxin

Brassinosteroid (BR), one of the most recently characterized phytohormone, plays a very crucial role in overall plant growth and development. In plants, a broad range of responses is regulated by BR, which includes cell expansion, cell proliferation, seed germination, vascular differentiation, apical dominance, flowering initiation, and flowering time regulation. BR signaling is also found to be involved extensively during plant immune response and stress tolerance. BRs have a marked effect on cell elongation and/or proliferation that is distinct from that of auxins, although it interacts with other plant hormones and also with environmental signals, such as light and temperature, in complex ways.

BR receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1), a LRR receptor-like kinase, is localized on plasma membrane. In Arabidopsis a small gene family members BRI1-LIKE1 (BRL1) and BRL3 are also found which encodes functional BR receptors but the BRL1 and BRL3 are specifically expressed in vascular cells, whereas BRI1 is expressed in all types of dividing cells (Kim and Wang 2010). In the absence of BR, a negative regulator BRI1 KINASE INHIBITOR 1 (BKI1) inhibits BRI1 and prevents the downstream signaling events by inhibiting its interaction with the positive regulators BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAKI) and BR SIGNALING KINASES (BSKs). Brassinolide (BL), the active BR, is perceived by the extracellular domain of BRI1 leading to an active BRI1 homo-oligomer formation. The signal is then transmitted through its cytoplasmic kinase domain and stimulates the dissociation of BKI1 from plasma membrane. Activated BRI1 then forms hetero-oligomer with BAK1 or its homologs BAK1-LIKE 1 (BKK1) and SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (SERK1), and by a series of transphosphorylation events form a completely active BR receptor complex (Shan et al. 2012). This activated BR receptor complex phosphorylates BSKs and in turn releases it to activate the BRI1 SUPPRESSOR 1 (BSU1) protein phosphatase.

Activated BSU1 dephosphorylates the Ser/Thr kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2) which, when phosphorylated, causes phosphorylation of transcription factors BRI1-EMS SUPPRESSOR 1/BRASSINAZOLE RESISTANT 1 (BES1/BZR1) during the absence of BR. Thus, BSU1 with the help of PROTEIN PHOSPHATASE 2A (PP2A) dephosphorylates BES1/BZR1 (Tang et al. 2011). The active unphosphorylated BES1/BZR1 can bind to DNA and either associate with themselves or other transcription factors such as BIM1, MYB30, AtIWS1, and ELF6/REF6 to control various BR responses via regulating BR-responsive gene expression (Shan et al. 2012).

Earlier studies on BR revealed that for most of the developmental processes, BR and auxin triggered similar response, providing indirect hints for a BR-auxin signaling overlap. BRs elicit very clear responses in auxin bioassays (Grove et al. 1979; Yopp et al. 1979; Katsumi 1985). The dependency of BR responses on a functional auxin signaling has been confirmed by the differential BR resistance of auxin response mutants (Nemhauser et al. 2004). Similarly, a functional BR signaling is also required for proper auxin responses as suggested by resistance of a weak allele of BR perception mutant *bri1* to temperature-induced hypocotyl elongation (Nemhauser et al. 2004). Such temperature-induced hypocotyl elongation has previously been shown to be dependent on altered auxin levels. This auxin and BR interdependency is reflected at the transcriptional level also (Clouse et al. 1992; Zurek et al. 1994; Nemhauser et al. 2004; Kim et al. 2006). Several genes were reported to be both auxin and BR regulated (Goda et al. 2004). BR and auxin share the same response element "TGTCTC" to regulate common transcriptional responses of BR and auxin (Goda et al. 2004; Nemhauser et al. 2004). Genes such as TCH4 (Xu et al. 1995), SAUR6B (Zurek et al. 1994), IAA2, IAA3, IAA13, IAA19, IAA22, and SAUR-AC1 (Goda et al. 2002; Mussig et al. 2002) are regulated by both BRs and auxin. Mainly SAUR, GH3, and IAA gene families were reported to be regulated by auxin and BR in Arabidopsis (Goda et al. 2004;

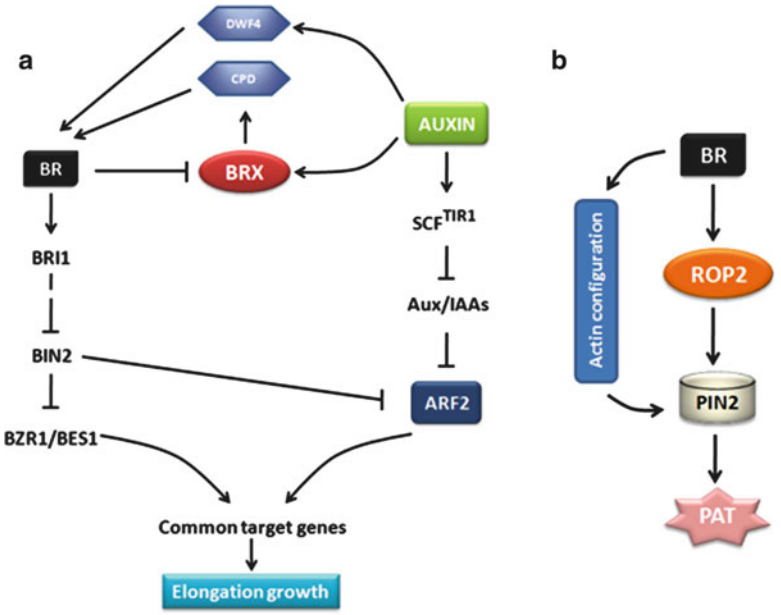

Fig. 3 A simplified model for auxin–BR crosstalk in plants. (**a**) Additive or synergistic effect of auxin and BR signaling on target gene expression. (**b**) Effect of BR on PIN2-mediated polar auxin transport

Song et al. 2009). Although previous studies have shown that exogenous BR application could not alter auxin levels (Nakamura et al. 2003), Song et al. (2009) have shown that transcriptional level of auxin biosynthesis-related gene YUCCA3 was promoted under BL treatment, indicating that BR may regulate auxin biosynthesis.

In BR-deficient mutant *det2* and BR perception mutant *bri1*, the transcription of several AUX/IAA genes was significantly reduced. These AUX/IAA genes are mainly involved in root development. Therefore, AUX/IAA can be the converging point for BR-auxin interaction during root development (Kim et al. 2006). BR-regulated BIN2 kinase can modulate auxin signaling by phosphorylating ARF2, which leads to the loss of DNA-binding repressive activities of ARF2. The BIN2 regulation of auxin-induced genes via inactivation of ARF2 shares the node of interaction between auxin and BRs (Vert et al. 2008). BREVIS RADIX (BRX), a gene required for optimal embryonic and postembryonic root growth, also improves our understanding of auxin and BR signaling interaction (Mouchel et al. 2004, 2006). Proper auxin signaling maintains BRX expression. BRX is also involved in a feedback loop which maintains threshold BR levels by regulating expression of BR biosynthesis-related gene CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD) (Mouchel et al. 2006). This threshold BR level is critical for optimal auxin action. Proper brassinosteroid levels, thus, might be rate limiting for the auxin-induced transcriptional responses required for optimal root growth (Fig. 3a).

In addition, BRs stimulate PAT and modifies the distribution of endogenous auxin. In plants treated with brassinolide (BL) or mutants defective in BR biosynthesis or signaling, the transcription of PIN genes, which facilitate functional auxin transport in plants, was differentially regulated (Li et al. 2005). BL also enhanced plant tropistic responses by promoting the accumulation of the PIN2 protein from the root tip to the elongation zone and stimulating the expression and dispersed localization of ROP2 during tropistic responses (Li et al. 2005). BRI1 expression pattern is reported to be related to root meristem size and is associated with regulation of PIN2 and PIN4 genes (Hacham et al. 2011, 2012). BR modulates auxin homeostasis to induce reset of hypocotyl directional growth and gravitropism in Arabidopsis (Gupta et al. 2012). Actin cytoskeleton plays a central role in auxin-BR signaling interaction. BR can modulate actin filament reconfiguration which leads to altered polar localization of PIN2 (Fig. 3b). Thus, BR balances auxin gradient and enhances auxin responses such as root gravitropism by actin cytoskeleton reconfiguration (Lanza et al. 2012).

Auxin has been shown to be one of the triggers for BR biosynthesis. When auxin was administered to the DWF4pro:GUS plants, the expression of DWF4 was increased. Similarly, DWF4 expression was found to be induced by auxin in various BR biosynthesis and signaling mutant background, suggesting that auxin has a direct control on BR biosynthesis (Chung et al. 2011). Exogenous treatment with auxin increases BR biosynthesis in Arabidopsis roots, which suggests that part of the auxin signal is transmitted through BR biosynthesis and subsequent signaling pathways (Chung et al. 2011).

These findings suggest that auxin and BR signaling pathways interact extensively and a proper BR biosynthesis and signaling is necessary for an optimal auxin response and vice versa. Both these signaling pathways converge on a set of common target genes partially to control many plant developmental processes. BR-auxin interactions are crucial for overall plant growth and developments such as root and hypocotyl tropic responses, meristem size, and control.

2.2 Interaction of Ethylene with Auxin

Ethylene being a gaseous hormone is considered special among all the hormones in plants. For most of the phytohormone signal transduction pathways, receptors are positive regulators, but in case of ethylene, all the five ER-integrated receptor proteins including ETR1, ETR2, ERS1, ERS2, and EIN4 act as negative regulators in Arabidopsis (Guo and Ecker 2004). Ethylene follows a linear signaling pathway that initiates by its binding and consequently inhibition of the activity of receptor protein ETR1 via 26S proteasome (Kevany et al. 2007). ETR1 inactivation causes inhibition of the CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) kinase, a negative regulator of signaling which when activated causes inhibition of ETHYLENE INSENSITIVE 2 (EIN2). EIN2 is a positive regulator which activates the EIN3 and EIN3-like (EIL) family of transcription factors, which in turn promote transcription

of genes containing an ethylene-responsive element (ERE) in their promoter region. EIN2 activity is modulated by two F-box proteins named EIN2 TARGETING PROTEIN 1(ETP1) and ETP2 (Qiao et al. 2009). Similarly, EIN3 and EIL1 level is regulated by F-box proteins, EIN3-BINDING F-BOX 1 (EBF1) and EBF2 (Guo and Ecker 2003; Potuschak et al. 2003). Ethylene through these hierarchical events of signal transduction by SCF complexes regulates many developmental processes in plant by controlling gene expression either positively or negatively (Zhao and Guo 2011).

The interaction between auxin and ethylene has been well studied and it was found that these hormones interact differently for different development processes. Synergistic interaction is followed to control developmental processes, such as root elongation and root hair formation, whereas antagonistic interaction is followed in processes, such as lateral root formation and hypocotyl elongation (Muday et al. 2012). Some ethylene-insensitive mutants were also found to be defective in auxin transporters such as *aux1* and *ethylene insensitive root 1/pinformed 2* (*eir1/pin2*), giving the first glimpse of auxin and ethylene signaling convergence in root growth regulation (Pickett et al. 1990; Luschnig et al. 1998). Several studies suggested that auxin and ethylene have similar effect on root elongation. Mutants with enhanced ethylene biosynthesis and signaling have reduced root elongation, while ethylene-insensitive mutants showed reduced growth inhibition (Stepanova et al. 2007; Ruzicka et al. 2007). Similarly, auxin overproducer mutants and exogenous application of auxin show reduction in root elongation, while auxin-insensitive mutants *tir1*, *axr2*, *axr3*, and *slr1* show loss in auxin-induced root elongation (Muday et al. 2012).

Study of two mutants *wei2* (*weak ethylene insensitive* 2) and *wei7* (*weak ethylene insensitive* 7) showed that ethylene via inducing expression of *WEI2* and *WEI7* promotes auxin biosynthesis in root meristem (Stepanova et al. 2005). Ethylene also promotes the expression of *TAA1* (*TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1*) and its homolog *TAR2* (*TAA1-RELATED2*) enhancing the local auxin production in root meristematic region thus inhibiting root elongation in an auxin-dependent manner (Stepanova et al. 2005). Auxin-inducible reporters DR5:GUS when treated with ethylene show increased auxin levels in root elongation zone (Ruzicka et al. 2007).

Ethylene and auxin synergistically regulate root hair formation and elongation. In loss of ethylene signaling mutant *ein2-1*, root hair initiation was significantly reduced by application of auxin influx inhibitors as compared to control. Root hair initiation is also significantly reduced in *aux1ein2* double mutant suggesting that ethylene may affect the cellular auxin level in controlling root hair initiation (Rahman et al. 2002). But during lateral root formation, the situation is just opposite. Auxin stimulates lateral root formation by stimulating pericycle cells within elongating primary roots to enter de novo organogenesis leading to the establishment of new lateral root meristems (Ivanchenko et al. 2008). When treated with ethylene, the primary root grows but lateral root initiation is reduced. Also, the ethylene *ctr1* and *eto1* mutants have reduced number of lateral roots, whereas dominant negative *etr1* and ethylene-insensitive *ein2* have elevated number of lateral roots (Negi et al. 2008). While emergence of new lateral root primordia is

inhibited with high concentrations of ACC, the emergence of existing lateral root primordia is not hindered. Ethylene-stimulated auxin biosynthesis mutants *wei2* and *wei7* show reduced response toward the high concentration of ethylene-mediated lateral root inhibition. The mutants with perturbed auxin responses, such as *slr1* and *arf7 arf19*, were also found to be insensitive to the low-ethylene promotion of lateral root initiation. This suggests that the effect of ethylene on lateral root initiation is mediated by both auxin synthesis and signaling (Ivanchenko et al. 2008).

Enhanced ethylene levels increase both acropetal and basipetal auxin transport. Ethylene promotes auxin transport from root meristematic zone to elongation zone in an AUX1- and PIN1-dependent manner (Ruzicka et al. 2007). In ethylene-insensitive *etr1* and *ein2* mutants, auxin transport is reduced. Therefore, ethylene regulates both acropetal and basipetal auxin transport. The increased auxin transport depends on AUX1 since *aux1*-7 mutant is insensitive to ethylene and thus for the inhibition of lateral root formation (Negi et al. 2008). It is reflective that effect of ACC is more pronounced on lateral root formation than on inhibition of primary root length and both these process can be uncoupled (Muday et al. 2012).

Ethylene also modulates gravitropism in plants. It reduces the early gravitropic response in both root and shoot. Treatment with ethylene or its biosynthetic precursor, ACC, reduces root gravitropic curvature. Ethylene modulates root gravitropism by enhancing basipetal auxin transport. ACC treatment leads to increased DR5:GFP expression on the upper side of the gravistimulated root, therefore minimizing the auxin gradient leading to reduced gravitropic curvature. Mutations in the auxin influx protein AUX1 and in the auxin efflux carrier, EIR1/PIN2/WAV6/AGR1, caused insensitivity to ethylene. Also, treatment with the auxin transport inhibitor, NPA, inhibited both root curvature and ethylene effects; these insensitivities are probably because auxin transport was affected (Masson et al. 2002). Thus, ethylene insensitivity in the roots of *aux1* and *agr1/eir1/pin2/wav6* mutant plants may relate to the role of ethylene in regulating auxin transport and further pointing to the importance of the interactions between these two plant hormone signaling pathways. The *alh1* [1-aminocyclopropane-1-carboxylic acid (ACC)-related long hypocotyl 1] Arabidopsis mutant, which is less sensitive to ethylene and auxin, displays a faster root response to gravity. *alh1* is altered in the cross talk between ethylene and auxin, probably at the level of auxin transport (Vandenbussche et al. 2003). The auxin signaling mutants *axr1*, *tir1-1*, *axr2*, and *axr3* have reduced sensitivity to ethylene and also have altered root gravitropic curvature. The *tt4* mutant defective in flavonoid synthesis is insensitive to ACC-mediated inhibition of gravitropic response. Flavonoids act as negative regulator of auxin transport, ACC, and auxin-induced flavonoid biosynthesis (Lewis et al. 2011).

These findings suggests that auxin and ethylene act in accordance to regulate root elongation, lateral root development, root hair initiation and elongation and gravitropic response, etc. It can be concluded that both ethylene and auxin signaling pathways interact extensively and along with other complex signaling processes can regulate overall growth and development of plant.

2.3 Interaction of Gibberellins with Auxin

Gibberellins (GAs) were the second group of phytohormones to be characterized. GA is known to regulate many developmental processes of plants such as stem elongation, promotion of seed germination, pollen development, and transition to flowering.

Various positive and negative regulators of GA signaling have been identified using genetic approaches. DELLA proteins, suppressor of GA signaling, are among the most extensively studied and characterized component of GA signaling. DELLA nuclear proteins, in general, act as suppressor of plant growth and are the member of GRAS family of transcriptional regulators. In rice and Arabidopsis, discovery of a soluble GA receptor GA INSENSITIVE DWARF1 (GID1) has led to better understanding of GA cascade (Ueguchi-Tanaka et al. 2005; Nakajima et al. 2006; Griffiths et al. 2006). Three major components of GA signaling are the following: a receptor (GID1), a DELLA protein, and an F-box protein SLEEPY1 (SLY1/GID2). When GA binds to GID1, it stimulates the interaction of GID1 with the DELLA domain of the DELLA proteins (Griffiths et al. 2006) leading to the destabilization of DELLA proteins. The complex thus formed with the help of specific F-box proteins (SLY1/GID2) stimulates DELLA proteins' binding to an SCF E3 ubiquitin ligase leading to polyubiquitination and promotes its degradation through the 26S proteasome pathway (Sasaki et al. 2003; Dill et al. 2004; Griffiths et al. 2006). This $SCF^{SLY1/GID2}$-mediated GA signaling is parallel to SCF^{TIR1}-mediated auxin signaling in mode of regulation.

In literature, several reports suggest a cross talk between GA and auxin. GA is mostly reported to be positively interacting with auxin in regulating cell expansion and tissue differentiation. GA application enhances auxin transport causing increased apical dominance (Jacobs and Case 1965; Bjorklund et al. 2007). Arabidopsis mutants with reduced GA responses are reported to have reduced apical dominance (Silverstone et al. 1997). GA pathway has been linked to regulate PAT indirectly during root meristem growth. At early stages of meristem development, elevated GA levels repress the expression of cytokinin-responsive ARR1 gene. At later stages of meristem development, a decrease of gibberellin biosynthesis stabilizes the DELLA protein REPRESSOR-of-ga1-3 (RGA), which causes transcriptional activation of ARR1. ARR1 along with ARR12 enhances the expression of auxin-inducible gene SHORT HYPOCOTYL 2 (SHY2) leading to repression of PIN (Moubayidin et al. 2010). Therefore, both GA and DELLA proteins balance cell division and differentiation during root meristem growth by controlling auxin transport (Moubayidin et al. 2010). GA deficiency or aberrations in signaling leads to reduced auxin transport in the inflorescences of Arabidopsis mutants which correlate with a reduction in PIN abundance. This reduced level of PIN protein can be recovered by exogenous GA application. GA deficiency also promotes vacuolar degradation of PIN2 and such GA-deficient mutants because of perturbed auxin transport have defects in cotyledon differentiation and root gravitropic responses (Willige et al. 2011) (Fig. 4).

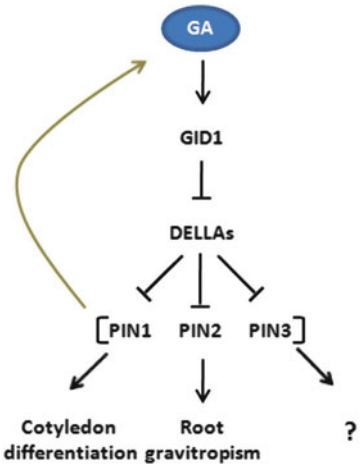

Fig. 4 GA regulation of PIN protein abundance and PIN-mediated development and growth responses (Model Adapted from Willige et al. 2011). GA-mediated DELLA repressor degradation is required for normal PIN protein accumulation at the plasma membrane (Fu and Harberd 2003; Frigerio et al. 2006)

Similarly, auxin can also modulate plant growth by modulating GA levels. Frigerio et al. (2006) have reported that auxin was able to enhance GA biosynthesis via inducing the expression of GA biosynthesis genes such as GA20ox and GA2ox in Arabidopsis. Root cells with lesser auxin levels due to impaired auxin transport also have reduced GA biosynthesis (Frigerio et al. 2006). Suboptimal GA levels lead to stabilization of DELLA protein hence inhibiting root elongation in general. Auxin can also regulate GA signaling via regulating DELLA protein degradation. Both auxin signaling and transport are involved in the degradation of DELLA. In auxin signaling mutant *axr1* GA-induced degradation of RGA is compromised (Fu and Harberd 2003). A perturbed auxin transport also facilitates stabilization of RGA. Proper degradation of DELLA proteins in cells is a prerequisite for proper root elongation. Stabilized DELLA proteins can retard root growth in plants having excised shoot apices and thus deficient in auxin (Fu and Harberd 2003).

Both GA regulation of PAT and auxin stimulation of GA biosynthesis and signaling via deactivation of DELLA proteins orchestrate a balanced growth and development in plants. The cooperation of GA and auxin is fundamental in maintaining cell wall plasticity and cytoplasmic protein synthesis to regulate cell elongation. GA pathway interacts with auxin transport and controls various developmental processes such as embryogenesis, root elongation, and gravitropic responses.

2.4 Interaction of Abscisic Acid with Auxin

In plants, abscisic acid (ABA) is known to regulate many developmental processes that include initiation and maintenance of seed dormancy, root growth inhibition, stomatal closure regulation, and many biotic and abiotic stress responses.

Combining several genetic, biochemical, and cellular studies, many cell surface-localized and intracellular ABA receptors have been identified in plants (Cutler et al. 2010). Cytosol- and nuclear-localized PYR/PYL/RCAR (PYRABACTIN RESISTANCE/PYRABACTIN RESISTANCE LIKE/REGULATORY COMPONENTS OF ABA RECEPTOR) proteins, which are the members of ligand-binding protein superfamily START, function as direct receptors of ABA. PYR/PYL/RCAR proteins after binding to ABA positively regulate ABA signaling via inhibition of negative regulators, group A PP2Cs (PROTEIN PHOSPHATASE 2C) (Nishimura et al. 2010). Group A PP2Cs when activated negatively regulate ABA signaling by inhibiting SnRK2 (SNF1-RELATED PROTEIN KINASE 2) kinases. SnRK2 kinases are positive regulators of ABA signaling which by directly phosphorylating bZIP transcription factors (i.e., ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR, ABFs/AREBs) regulate ABA-induced gene expression leading to ABA responses in plants. Both the PP2Cs and SnRKs are likely to have many more substrates and/or interaction partners than are currently identified. CHLH proteins, a subunit of Mg-cheletase enzyme, which are localized on plastid membranes, are also reported to be involved in ABA perception. The role for these CHLH proteins in ABA perception and signaling was further strengthened by analyzing ABA sensitivity of CHLH knockdown and overexpression lines with respect to ABA-induced gene expression, seed germination, dormancy, and stomatal regulation. The exact mechanism by which CHLH proteins function in ABA signaling is still very less understood. Another class of ABA receptors which are localized on plasma membrane includes GPCR-type G proteins (GTG1 and GTG2) which bind to ABA either positively or negatively and participate in ABA signaling. The mechanism of GTG1/2-mediated ABA signal transduction is not well characterized. There is tremendous redundancy and feedback at every level of the ABA signaling network (Cutler et al. 2010).

There are few reports of ABA and auxin signaling interactions during root growth and tropic responses (Brady et al. 2003; Rock and Sun 2005). Cross talk between ABA and auxin during lateral root development has been observed in lines overexpressing maize VIVIPAROUS1 (VP1, an ortholog of Arabidopsis ABI3) gene in the *abi3* mutant background. In the presence of ABA, these lines could not form lateral roots even after auxin application (Suzuki et al. 2001). Brady et al. (2003) have shown that ABI3 itself is auxin inducible in lateral root primordia suggesting the involvement of ABA signaling in auxin-induced lateral root formation. Under drought conditions both auxin and ABA play a key role in root growth. MYB96, a R2R3-type MYB transcription factor, has been reported to regulate drought stress response and lateral root development by integrating ABA and auxin signals (Seo et al. 2009). ABA signals via MYB96 regulate a subset of GH3 genes which modulates endogenous auxin levels during lateral root development (Seo et al. 2009). These observations suggest that MYB96 acts as connecting link for ABA-auxin signal integration during lateral root development under drought stress. ABA also inhibits root growth through altering the distribution of auxin in root tips. ABI4 was reported to alter auxin distribution by negatively regulating PIN1 expression during lateral root growth (Shkolnik-Inbar and Bar-Zvi 2010).

Auxin also interacts with ABA during seed germination. An auxin transport-defective *aux1* mutant was firstly isolated during a genetic screen for mutants deficient in ABA responses. This *aux1-301* mutant shows accelerated seed to seedling transition in the presence of ABA providing clues for interconnections between ABA and auxin signaling during seed germination and post germination growth (Belin et al. 2009). AUX1 was found to be critically essential for ABA-dependent repression of embryonic axis elongation. Root cap- and elongation zone-localized AUX1 and PIN2 were involved in auxin transport to peripheral elongation zone during embryonic axis elongation. ABA can independently repress the AXR2/IAA7 transcript level in cells of those regions thus enhancing auxin responses (Belin et al. 2009). More recently, an auxin response factor *arf2-101* mutant was isolated during a genetic screen to find the new genes in ABA response. *arf2-101* was found to be hypersensitive to ABA during seed germination as well as primary root growth (Wang et al. 2011). These reports altogether suggest that AXR2/IAA7 negatively regulates both auxin and ABA signaling pathways. ARF2 acts as a central integrator for auxin and ABA signaling cross talk during seed germination and early seedling development in which ABA-dependent repression of growth is potentiated by auxin.

ABA control of seed germination and post germination growth mainly involves modulation of auxin signaling and transport. ABA can also regulate auxin signaling via maintaining auxin levels in controlling lateral root development during stress conditions. Therefore, both auxin and ABA signals alone or integrated are essential for proper seed germination and development.

2.5 *Interaction of Cytokinin with Auxin*

Cytokinins (CKs) are adenine-derived molecules which are of two types, isoprenoid and aromatic CKs. The first CK, kinetin, was isolated as a breakdown product from autoclaved herring sperm DNA as a cell division-promoting factor (Miller et al. 1955). CKs are involved in a number of processes as embryogenesis, de novo organogenesis, root and shoot development, shoot branching, lateral root formation, vascular morphogenesis, nodule organogenesis, leaf expansion, seed germination, senescence, apical dominance, sink-source relationship, nutrient acquisition, increased crop productivity, interaction with pathogens, circadian rhythms, abiotic stress, and more (Muller 2011; Su et al. 2011; Hwang et al. 2012). Recently, apart from gravitropism, Kushwah et al. (2011) reported the role of CK in a novel root directional response termed as CK-induced root growth response in addition to coiling and waving.

CK signaling involves a multistep phosphorelay pathway using the following order of phosphorylation (1) Arabidopsis histidine kinases (AHK2, AHK3, AHK4) that act as CK receptors, (2) Arabidopsis histidine phosphotransfer proteins (AHP1–AHP5), and (3) Arabidopsis response regulators (ARRs), type-A (ARR3–ARR9, ARR15–ARR17) and type-B (ARR1, ARR2, ARR10–ARR14, ARR18–ARR21). After phosphorylation, AHPs move from cytoplasm into the

nucleus where they phosphorylate ARRs. Phosphorylated type-B ARRs act as positive regulators of CK signaling pathway and induce transcription of type-A ARRs and other CK early responsive genes (Hwang and Sheen 2001). Type-A ARRs are negative regulators of CK signaling and are regulated by a mechanism involving stabilization by CK and/or proteasomal pathway (Ren et al. 2009).

Skoog and Miller (1957) in their classical paper have shown that in tobacco pith tissue cultures, high and low cytokinin–auxin ratio supported shoot and root formation, respectively, whereas at equal concentrations of both, the tissue tended to form callus. CK and auxin mutually regulate their signaling and/or their metabolism. If we compare CK and auxin signaling pathway, then type-B ARRs are similar to that of ARFs and type-A ARRs are similar to AUX/IAA proteins, both involving proteasomal pathway as a common mean of regulation (Ren et al. 2009). There are several developmental processes controlled by either antagonistic or synergistic action of CK and auxin. The CK and auxin cross talk depends upon the plant species and organ being studied, which hampered the elaboration of a general model.

The root architecture comprises of primary root and lateral roots. The root system plasticity according to constantly changing environmental conditions is important to optimize nutrient acquisition and therefore represents an important agronomical trait. CK and auxin act antagonistically for specifying the first root stem cell niche during Arabidopsis early embryogenesis (Muller and Sheen 2008). Auxin maxima in hypophysis-derived basal cell directly activate transcription of *ARR7* and *ARR15* (negative regulators of CK signaling). *ARR7* and *ARR15* function is essential for stereotypical cell division pattern and thus for normal embryonic root stem cell niche formation (Fig. 5a).

During embryogenesis CK is required for the procambial cell divisions and for the acquisition of the correct identity of newly formed vascular cells. CK works through AHKs, primarily AHK4, to initiate phosphorelay via the AHPs to type-B ARRs (ARR1, ARR10, and ARR12) to induce transcription of genes regulating root vascular differentiation. This pathway is regulated by AHP6 and possibly by some type-A ARRs, which act as a negative regulator of CK signaling pathway. Phloem-transported CKs direct auxin flow into the xylem axis by modulating the distribution of PIN3 and PIN7. A high auxin level at the xylem axis activates *AHP6* expression (Fig. 5b).

Root meristem comprises of meristem zone, transition zone, and elongation-differentiation zone. The root meristem size established by a fine balance between the antagonistic interaction of CK (induce cell differentiation) and auxin (induce cell division). The nature of this antagonistic interaction has recently been unveiled by landmark discovery of Dello Ioio et al. (2008). They proposed the model that CK activates the *SHY2* gene (member of the Aux/IAA gene family) via AHK3/ARR1 branch of CK signaling cascade and thus reduced auxin response. This leads to *PIN1*, *PIN3*, *PIN7*, and *IPT5* downregulation and differentiation. Moreover, SHY2 protein on one hand negatively regulates auxin transport and on the other hand negatively controls CK biosynthesis. High auxin concentration mediates SHY2 protein degradation via proteasomal pathway sustaining PIN activity and cell division (Fig. 5c). CK effect on root meristem size is ethylene independent, while

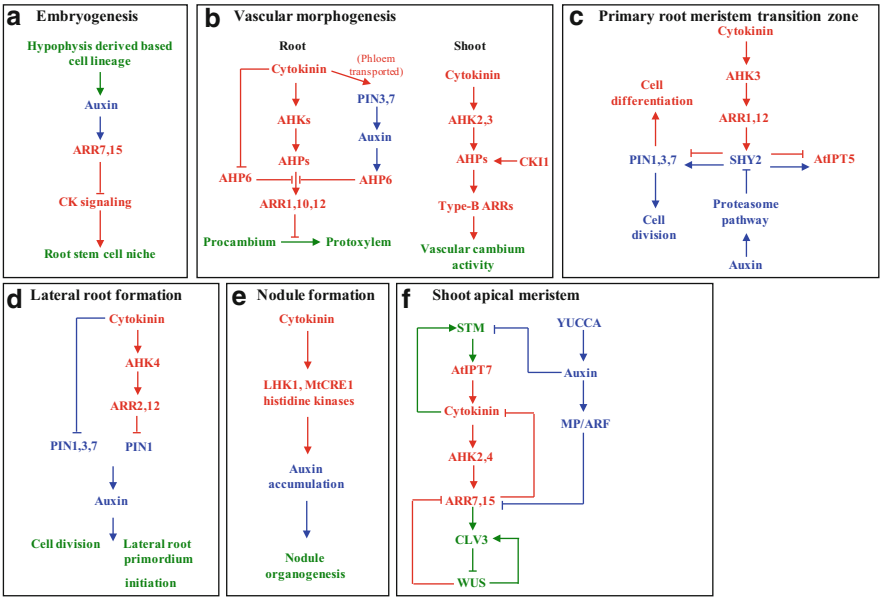

Fig. 5 Cytokinin (CK) and auxin interaction (Dello Ioio et al. 2008; Hwang et al. 2012) in (**a**), embryogenesis; (**b**), vascular morphogenesis; (**c**), primary root meristem transition zone; (**d**), lateral root formation; (**e**), nodule formation; and (**f**), shoot apical meristem. In shoot meristem, STM (SHOOTSTEMLESS) increases CK biosynthesis via inducing AtIPT7 and CK increases STM expression. CK increases ARR7/15 expression, which in turn represses CK signaling. ARR7/15 are required for CLV3 (encodes peptide ligand) expression. CLV3 decreases WUS expression, which in turn limits the ARR7/15 expression. WUS also increases CLV3 expression. Auxin produced by YUCCA genes decreases ARR7/15 expression through the STM-mediated pathway and MP-mediated auxin signaling. *Red* indicates CK components, *blue* indicates auxin components, and *green* indicates other components and processes

on overall root growth and cell elongation, it is ethylene dependent (Ruzicka et al. 2009). In agreement with Dello Ioio et al. (2008), the classical work of Pernisova et al. (2009) and Ruzicka et al. (2009) have also shown that CK might regulate the auxin efflux via modulation of the *PIN* transcription.

CK mediates lateral root inhibition (ethylene independent) via perturbation of PIN gene expression but does not disrupt auxin perception and the auxin-mediated cell division (Laplaze et al. 2007). CK treatment leads to delayed and disorganized cell division in lateral root primordia. CK involves quick and strong downregulation of plasma membrane PIN1 abundance by its alternative sorting to lytic vacuoles for degradation (Marhavy et al. 2011). CK-mediated regulation of the lateral root formation involves AHK4/ARR2 and ARR12 branch of CK signaling cascade (Fig. 5d).

CK and auxin act synergistically to promote nodule organogenesis in symbiotic interaction with rhizobium bacteria. CK is important not only for the initiation of nodule organogenesis but also for its later development. Plants with reduced CK

level and loss-of-function mutant of CK receptor (*MtCRE1* of *Medicago truncatula* and *LHK1* of *Lotus japonicus*) show a strong decrease or even the absence of nodulation. Gain-of-function mutant of LHK1 in *L. japonicus* produced root nodules even in the absence of rhizobia. Bacterial nod factors induce auxin accumulation by inhibiting PAT in dividing pericycle and cortical cells, and the latter is crucial for nodule initiation. Treatment with auxin transport inhibitors also leads to pseudonodule formation and nodulation gene expression. In *M. truncatula* MtCRE1 histidine kinase regulates *MtPINs* (auxin efflux carriers) expression, resulting in CK-dependent auxin accumulation in developing nodule primordia (Fig. 5e).

Shoot apical meristem (SAM) arises during embryogenesis and produces aerial parts of the plant body. The active cell division and differentiation occurs in SAM; thus, a fine balance between both these processes is necessary to control SAM size and structure. In agreement with the pioneer work of Skoog and Miller (1957), CK positively controls SAM size and activity, since it is reduced by CK deficiency. Auxin also plays crucial role in the SAM maintenance. Auxin and CK signaling converge on *ARR7* and *ARR15* (type-A ARRs) during SAM development (Fig. 5f). Auxin antagonizes CK for organ initiation both in the embryonic SAM and in the inflorescence meristem. High levels of CK are required for the stem cell maintenance in the meristem, but not for organ initiation. CK–auxin interaction is also involved in the predominant shoot apex growth, which inhibits the outgrowth of axillary bud as well as also in shoot branching. In inflorescence stem procambium cells, the CK signaling from constitutively active CKI1 and CK-activated AHK2 and AHK3 is integrated into the phosphorelay cascades to maintain the vascular cambium activity (Fig. 5b).

In summary there are numerous developmental processes controlled either by antagonistic or synergistic action of CK and auxin. This interaction involves cross talk of these signaling pathways as well as regulation of each other's level. CK–auxin cross talk is important for responses to environmental signals throughout the plant life cycle. The combination of relevant queries and development of powerful techniques will shed further light on CK–auxin interaction in future.

2.6 Interaction of Jasmonic Acid with Auxin

Jasmonates (JA) are a recently discovered class of phytohormone which play a pivotal role in growth and development, stress alleviation, etc. Chemically, JAs are oxylipins synthesized through lipoxygenase pathway in chloroplast and peroxisomes (Wasternack 2007). Their role in reproductive development, defense against biotic attack, and abiotic stress mitigation is well studied (Wasternack 2007). It is also found that plants release some JA derivatives as volatile organic compounds (VOCs) to communicate with each other in mutual danger situations (Baldwin et al. 2006).

The cross talk between auxin and JA is quite extensive. Some of the hormones have similar signal perception and transduction pathways and auxin and JA are a

classic example for this analogy. JA signaling pathway shows a strikingly high similarity to SCF^{TIR1}-mediated auxin signal transduction. An SCF^{COI1} complex and JASMONATE ZIM-DOMAIN (JAZ) proteins are identified as the most important components of JA signaling (Thines et al. 2007; Chini et al. 2007; Yan et al. 2009). When JA-Ile binds to SCF^{COI1} complex, it recruits JAZ protein for polyubiquitination and subsequent degradation (Chini et al. 2007). This will relieve the transcription factors from JAZ protein-mediated inhibition and they activate the JA-responsive genes in response to the particular signal. In the absence of JA, JAZ proteins repress the JA-responsive gene expression by binding to bHLH transcription factors like MYC2/JASMONATE INSENSITIVE1 and inhibit their activity (Chini et al. 2007). These bHLH transcription factors are the effectors of JA signaling which activate the transcription of the JA-responsive genes in response to stress, wounding, etc. (Dombrecht et al. 2007).

This SCF^{COI1}-mediated JAZ degradation through 26S proteasomal pathway is identical to SCR^{TIR1}-mediated degradation of AUX/IAA proteins in auxin signaling (Gray et al. 2001). CORONATINE INSENSITIVE 1 (COI1) shows approximately 33 % sequence similarity TIR1 in amino acid sequence and both have leucine-rich repeats and an F-box motif (Yan et al. 2009). Consistent with this, COI1 showed an overall architecture similar to TIR1 in crystal structure studies. They share a similar N-terminal tri-helical F-box motif and C-terminal solenoid domain of 18 tandem leucine-rich repeats. Although both COI1 and TIR1 show overall similarity in structural contour, they evolved distinct hormone-binding sites which differentiate the signaling pathway of both molecules (Sheard et al. 2010). Likewise, inositol phosphate works as a cofactor in both auxin and JA perception (Tan et al. 2007; Sheard et al. 2010). These observations confirm the overall analogy in signal perception, transduction, and downstream gene activation of auxin and JA.

Auxin and JA also interact at the level of signaling components. JASMONATE-ZIM-DOMAIN PROTEIN 1 (JAZ1/TIFY10A) is responsive to both auxin and JA. The auxin-dependent induction of JAZ1/TIFY10A is independent of JA signaling and is controlled by the IAA-ARF signaling pathway. JAZ proteins are the repressors of JA signaling and this is one of interaction site of the complex interplay between auxin and JA signaling (Grunewald et al. 2009). An Arabidopsis mutant defective in JA response is found to be allelic to *axr1* which shows the extent of interaction between these two molecules at the level of signaling components (Tiryaki and Staswick 2002).

Auxin and JA modulate the homeostasis of each other through the GH3 family proteins. It is a family of acyl-acid-amido synthetases which conjugate auxin, JA, and SA to amino acids which results in activation or inactivation of hormonal activity (Wang et al. 2008). GH3.9 is a group II GH3 family member which can conjugate IAA with amino acids and influence primary root growth negatively. Mutation in GH3.9 caused an increase in primary root length and auxin sensitivity. This mutation also altered IAA- and MeJA-mediated root growth inhibition

signifying the role of GH3.9 as a potential cross-talking point at the level of auxin homeostasis (Khan and Stone 2007a, b). It is also found that auxin-mediated adventitious root initiation is achieved by reduction of JA level through GH3.3, GH3.5, and GH3.6 proteins (Gutierrez et al. 2012). Additionally, it is reported that JA can regulate biosynthesis of auxin. MYC2, the key transcription factor for JA-regulated gene expression, negatively regulates tryptophan and tryptophan-derived secondary metabolism during JA signaling, suggesting a role of MYC2 in auxin biosynthesis (Dombrecht et al. 2007).

Auxin can also influence JA levels eventually, controlling flower maturation and fertility. Auxin response transcription factors ARF6 and ARF8 are important in the development of fertile flower, and mutation of these genes can cause severe defects like undehisced anther and immature gynoecium. JA measurement and JA feeding experiments revealed that reduced endogenous JA level is responsible for the defect in anther but not for the defect in gynoecium (Nagpal et al. 2005). It is found that ARF6 and ARF8 increase JA biosynthesis through the activation of JA biosynthetic gene DEFECTIVE IN ANTHER DEHISCENCE1 (DAD1) (Tabata et al. 2010). These studies altogether suggest that auxin and JA can influence the homeostasis of each other which result in the fine tuning of many developmental events.

Auxin is a very important phytohormone in root development and vascular development. PAT and auxin gradient is important for proper root development and differentiation (Overvoorde et al. 2010). The role of JA in root development is emerging and it is shown that many of the auxin effects on root development are achieved by modulating JA homeostasis and signaling. The role of jasmonate in root growth inhibition is well known. Methyl jasmonate (MeJA) can inhibit the primary root growth very significantly (Staswick et al. 1992, 2002).

Auxin controls the adventitious root initiation by modulating JA homeostasis through ARFs and GH3s (Gutierrez et al. 2012). JA also regulates root stem cell activity through MYC2. MYC2 binds to the promoters of PLETHORA 1 (PLT1) and PLT2 and inhibits their expression (Chen et al. 2011). PLETHORA transcription factors are important in maintaining stem cell niche and auxin gradient (Galinha et al. 2007; Dhonukshe et al. 2012).

JA has a positive effect on lateral root induction and growth which is mediated by enhanced auxin biosynthesis and localized accumulation. JA decreases the expression of PIN1 and PIN2 and affects their endocytosis. It also induces ANTHRANILATE SYNTHASE α1 (ASA1)-dependent auxin synthesis. This will in turn result in increased auxin concentration in root basal meristem which promotes lateral root formation (Sun et al. 2009, 2011) (Fig. 6). COI1, a part of JA receptor complex, is also found to be involved in JA-induced pericycle cell activation and lateral root initiation and is auxin dependent (Raya-Gonzalez et al. 2012). In addition to its role in auxin signaling, AXR1 is also responsive to MeJA. *axr1-1* mutant is highly susceptible to pathogen and showed resistant to JA-mediated root growth inhibition (Tiryaki and Staswick 2002). This can be one of the potential interaction sites between JA and auxin signaling.

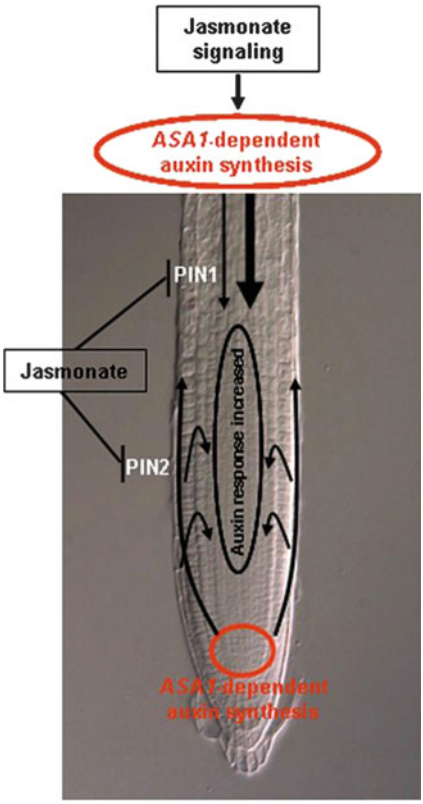

Fig. 6 Model showing jasmonate modulation of auxin accumulation in the root basal meristem (Adapted from Sun et al. 2009). JA stimulates the ASA1-dependent auxin synthesis in shoot and root tissues, which are transported to the root basal meristem through phloem-based and/or polar transport pathways. On the other hand, jasmonate reduces the protein levels of auxin efflux carriers (PIN1 and PIN2), which leads to decreased auxin transport capacities to the root basal meristem. The net effect of jasmonate is to increase local auxin accumulation in the root basal meristem and promote LR formation

JA also has effect on gravitropic responses. Hormone content measurement by GC-MS/MS revealed that a JA gradient is also formed opposite to the auxin gradient and the total content of JA is also increased during gravitropic response. Flooding of JA delays the gravitropic bending because it will perturb the JA gradient. Consistent with this, a JA-deficient rice mutant *hebiba* bends very slowly on gravitropic stimulus (Gutjahr et al. 2005). Similarly, tryptophan conjugates of JA (JA-Trp) will cause agravitropic root growth in Arabidopsis in dose-dependent manner. This response is independent of JA co-receptor COI1 and dependent on auxin receptor TIR1, suggesting a role of JA in gravitropic responses along with auxin (Staswick 2009).

In short auxin and JA have an extensive network of cross talking which is evident from analogous signal perception and transduction pathway, interaction at the level of signaling molecules, and modulation of metabolism of each other. It is found that JA have a critical role in root development, gravitropism, etc. which is primarily dependent on PAT and localized auxin gradient. Molecular and physiological studies instituted this extensive cross talk and further studies are needed to dissect the broad interaction between these phytohormones at molecular level.

2.7 Interaction of Salicylic Acid with Auxin

Salicylic acid (SA) is a phenolic phytohormone involved in many faces of plant development and defense against pathogens. SA is involved in systemic acquired resistance (SAR) response and work as an endogenous and exogenous signal which prepares plants against pathogen attack through the activation of pathogenesis-related (PR) proteins (Vlot et al. 2009).

It is found that upon pathogen attack, the endogenous level of SA is increased to elicit the defense responses. SA induces defense-related genes by both NONEXPRESSER OF PR GENES 1 (NPR1) dependent and independent mechanisms (Loake and Grant 2007). The NPR1-dependent pathway is well studied among the two; NPR1, with the help of TGACG MOTIF-BINDING FACTOR (TGA) class transcription factors, induces a large number of SA-responsive genes (Wang et al. 2006). However, the exact nature of SA perception is not known yet. Very recently, it is found that NPR1, NPR3, and NPR4 can perceive SA (Wu et al. 2012; Fu et al. 2012). SA causes a conformational change in NPR1 which releases the C-terminal transactivation domain from the inhibition of N-terminal BTB/POZ domain, thus inducing the SA-mediated gene expression through TGA transcription factors (Wu et al. 2012). NPR3 and NPR4 also bind to SA in different affinities in which NPR4 shows the higher affinity. NPR3/4 also works as adapters of the Cullin 3 ubiquitin E3 ligase for NPR1 degradation in a SA-dependent manner. From this observations it can be concluded that NPR3/4 works as regulators of NPR1-dependent SA-mediated gene expression and the role of ubiquitin-mediated protein degradation pathway in SA signaling pathway is emerging (Fu et al. 2012). The fine details of this NPR3/4-dependent SA control mechanism are still elusive.

The role of auxin in plant defense is identified many years ago, but the molecular mechanisms underlying this are very poorly understood. Recent molecular and physiological studies revealed that auxin is involved in the defense responses by interacting with JA and SA. In most of the cases, auxins and SA show almost an antagonistic interaction in modulating defense responses (Kazan and Manners 2009).

Many phytopathogens produce auxin or use host's auxin synthesis machinery for successful invasion and establishment on host (Fu and Wang 2011). Elevated auxin level is critical for many pathogens for a successful attack on host. Microarray analysis revealed that SA-mediated defense against pathogen is achieved by downregulating auxin synthesis and signaling while increasing auxin conjugation. SA can downregulate a large portion of auxin-related genes including TIR1, SAURs, and PINs, which suggests that a reduced auxin level is important in fighting against pathogens (Wang et al. 2007). At molecular level, studies on Arabidopsis MAP KINASE KINASE 7 (*At*MKK7) revealed the antagonism of SA and auxins in defense responses. *At*MKK7 overexpression resulted in an elevated level of SA which leads to constitutive expression of PR proteins conferring enhanced resistance against *Pseudomonas syringae* and *Hyaloperonospora parasitica* (Zhang et al. 2007a). Interestingly, the AtMKK7 overexpression reduced PAT and free IAA levels suggesting a negative role of auxin in defense (Zhang et al. 2008; Dai et al. 2006).

There are reports also where auxins increase the resistance against pathogens through SCF-mediated ubiquitination pathway (Llorente et al. 2008). A synergism between auxin and SA in defense response is observed at the homeostasis level of both hormones. GH3.5 acts as a bifunctional modulator of auxin and SA during pathogen attack. Over-expression of GH3.5 resulted in an increased SA and IAA accumulation and induction of PR1 proteins against avirulent pathogens and showed a disrupted auxin response, while the knockdown mutants showed high susceptibility and compromised SAR response. Microarray analysis of *gh3.5-1D* mutant revealed that the SA signaling pathway is augmented by GH3.5 through SA-responsive genes, while auxin pathway is activated through enhanced IAA biosynthesis and inhibition of auxin-repressor genes (Zhang et al. 2007b). However, direct molecular evidences are lacking to establish the synergism between SA and auxin in disease resistance.

The role of auxin in plant defense responses is a prime area of interest in auxin research. In short, auxin promotes biotic perturbation and SA provides resistance by downregulating auxin biosynthesis and transport and upregulating auxin conjugation. However, there are reports also in which auxin offers resistance against pathogens. Further molecular and physiological works are required for establishing the role of auxins in plant defense and its interaction between SA.

2.8 Interaction of Strigolactones and Auxin

Strigolactones are newly discovered phytohormone (Gomez-Roldan et al. 2008; Umehara et al. 2008). Strigolactones were first isolated from plant root exudates, where they trigger germination of root parasitic plants as well as stimulate hyphal branching of the symbiotic arbuscular mycorrhizal fungi (Xie et al. 2010). Strigolactones also participate in different plant growth and developmental processes like lateral shoot branching and root development (Beveridge 2006; Ongaro et al. 2008; Leyser 2008; Kapulnik et al. 2011). Chemically, strigolactones are terpenoid lactones, which contain a tricyclic lactone (ABC part) and a butenolide group (D ring), which connect to each other via an enol ether bridge. Strigolactones are synthesized mostly in roots and transported via xylem to shoot (Cardoso et al. 2011). Strigolactones are derived from carotenoids by sequential oxidation and oxidative cleavage (Xie et al. 2010). Initial steps of strigolactone biosynthesis occur in plastid. In Arabidopsis two plastid-targeted carotenoid cleavage dioxygenases (CCDs), MAX3 and MAX4, are involved in these steps. A cytochrome P450 family member, MAX1, acts downstream to MAX3 and MAX4, involved in non-plastidic steps of strigolactone biosynthesis pathway (Turnbull et al. 2002; Sorefan et al. 2003; Booker et al. 2004, 2005; Auldridge et al. 2006). A Leu-rich F-box protein, MAX2 (MORE AXILLARY GROWTH 2), is involved in strigolactone signal transduction pathway, by either acting as strigolactone receptor or associating with a receptor complex (Stirnberg et al. 2002, 2007; Johnson et al. 2006; Ishikawa et al. 2005).

Studies on Arabidopsis pea and rice showed that strigolactones negatively regulate shoot branching by interacting with auxin and cytokinin (Ferguson and Beveridge 2009). Strigolactones are synthesized mostly in roots and transported acropetally to the stem which directly suppress auxin-mediated axillary bud outgrowth (Durbak et al. 2012). Auxin control of shoot branching is explained by canalization-based model (Sachs 1981). According to this model, buds act as auxin source and the stem acts as an auxin sink. For the activation of buds, auxin must export out from buds to the main stem. The initial flow of auxin from a bud to stem occurs through files of cells which have higher auxin transport activity. These cell files act as auxin transport canals. The auxin transport from first activated bud to main stem reduces the sink strength of stem and inhibits auxin export by other buds to the sink tissue. Strigolactones dampen auxin transport canalization by limiting PIN accumulation on the plasma membrane through MAX2-dependent pathway in *Arabidopsis* (Crawford et al. 2010). When strigolactones are low, the polar auxin transport becomes high and the competition between buds for sink gets reduced which results in more bud activation. In contrast, more endogenous strigolactone concentration inhibits polar auxin transport resulting in high sink auxin concentration, which in turn inhibits outgrowth of other buds. In *Arabidopsis* pea and rice, strigolactone biosynthetic MAX3 and MAX4 genes are upregulated by auxin, suggesting that a feedback loop exists between auxin and strigolactones (Hayward et al. 2009; Brewer et al. 2009). Strigolactones inhibit auxin transport and auxin induces the expression of strigolactone biosynthetic genes resulting in higher production of strigolactones, which in turn potentially reduced polar auxin transport (Durbak et al. 2012). Further, Agusti et al. (2011) found that strigolactones and their interaction with auxin signaling strongly regulate secondary growth. By using genetic and pharmacological data, they found that strigolactones act downstream of auxin in a common signaling cascade and positively regulate cambium activity (Agusti et al. 2011).

In addition to its role in fungal hyphal branching, germination of root parasitic plants and shoot branching, strigolactones also work as a modulator of root development. In Arabidopsis, strigolactones regulate primary root elongation, lateral root formation, and root hair elongation by cross talk with ethylene and auxin signaling (Koltai 2011; Ruyter-Spira et al. 2011). Exogenous application of synthetic strigolactones GR24 regulates primary root and lateral root development in Arabidopsis. These effects of strigolactones are mediated by modulating local auxin levels in the root tip and developing lateral root primordial (Ruyter-Spira et al. 2011). In Arabidopsis strigolactone-insensitive mutant *max2* showed same sensitivity as wild type toward exogenous IAA application in regulating root hair length, whereas auxin receptor mutant *tir1-1* was less sensitive than wild type at low concentration of strigolactones (Kapulnik et al. 2011). The results suggest that strigolactone signaling is not necessary for the auxin response, but auxin signaling enhances the root hair elongation response of strigolactones (Kapulnik et al. 2011). Ethylene directly interacts with strigolactone, while auxin and strigolactone cross talk is mediated through ethylene signaling by modulating PAT (Koltai 2011).

Auxin and strigolactones modulate many of the common developmental events. It is found that auxin inhibits shoot branching through strigolactones. Auxin induces strigolactone synthesis which in turn dampens PAT, creating an auxin accumulation in buds which keeps buds undifferentiated. Strigolactone perturb auxin level in root which is important in root elongation and lateral root emergence, while in regulating root hair elongation, auxin and strigolactone interaction is mediated through ethylene signaling.

3 Conclusions

It is very important to understand the accurate mechanism of signaling interaction to understand how plants sense different parameters of various exogenous and endogenous cues and turn them into different cellular events. Plant hormones affect a number of common responses in plants. These common responses depend on specific hormone combination rather than depending on the specific action of a single hormone individually. Molecular mechanisms of hormone signaling integration have now started to get unraveled. The signals and signaling mechanisms by which the major pathways of growth and development work in specific cell types are now known. The extent of integration of these pathways with each other and other signaling pathways within cells is now being explored and understood.

The phytohormone auxin is essential for plant development and controls almost all aspects of the plant life cycle. It is now becoming clear how auxin metabolic pathways together with auxin carrier-dependent regulation of cellular auxin homeostasis get integrated to dynamically modulate the plant architecture. Several reports as mentioned in this chapter are making it increasingly clear as how other plant hormones or growth regulators also influence the rate of auxin metabolism and transport. These reports together suggest that on one hand hormonal cross talk might provide the coordinative link between auxin metabolism and transport, while on the other hand, auxin signaling feeds back into other hormonal pathways. Hence, the global phytohormonal steady-state levels seem to balance the cellular auxin homeostasis. Such a mechanism conceptually provides a robust means to stabilize auxin levels and is yet flexible enough to induce substantial changes in auxin homeostasis in response to exogenous and endogenous cues. Local manipulation of auxin levels appeared to be an elegant way to improve plant developmental characteristics of agricultural interest. The cell type-specific manipulation of auxin levels has been used to induce parthenocarpy for the production of seedless fruits and to improve the quality as well as the yield of the cotton fiber production. Auxin minima formation in specific cells has a role in fruit development and seed dispersal and can be exploited for regulating these features. Cell type- or tissue-specific modification of auxin levels seems to be a successful strategy for agricultural applications. Thus, the regulation of cell-specific auxin production and manipulation of intracellular auxin carrier by various phytohormones could eventually be used to shape plant development via the cell type-specific regulation of free IAA levels.

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Regulation of Polar Auxin Transport by Protein Phosphorylation

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Abstract Protein phosphorylation is one of the most important posttranslational mechanisms regulating polar auxin transport (PAT). The best known example is the phosphorylation carried out by the protein kinase PINOID (PID), which determines PIN polar targeting at the plasma membrane. PID belongs to the family of plant AGC kinases, which play prominent roles in the regulation of PAT and in the cross talk between PAT and the light-signaling pathways. Moreover, kinases belonging to other families, such as protein kinase CK2 or MAP kinases, are now emerging as important regulators of PIN recycling and/or other aspects of PAT, and the phosphoinositide biosynthetic pathway appears to play a significant role on PIN trafficking. On the other hand, identification of protein phosphatases that may reverse the effects of protein kinases is far behind, although rapid progress is being made, providing new insights about the role of protein phosphorylation on PAT regulation.

1 Introduction

The phytohormone auxin regulates plant cell division and expansion and has a central role in the patterning of plant vasculature and the control of phyllotaxis. The most abundant of the naturally occurring auxins is the indole-3-acetic acid (IAA). An important finding in recent years is that IAA is nonuniformly distributed within the plant tissues and that establishment of auxin maximum or minimum is a key step for organogenesis. The asymmetric distribution of IAA relies principally on auxin cell-to-cell transport, also called polar auxin transport (PAT) (Vanneste and Friml 2009). In the 1970s, the chemiosmotic hypothesis of PAT postulated that the apoplastic, protonated IAA can passively diffuse into the cell and that cytosolic pH

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favors the deprotonated form of auxin IAA, which requires active transport to exit the cell (Rubery and Sheldrake 1974; Raven 1975). Several auxin carriers, belonging to different protein families, have been identified to date. The AUX1/LAX family are auxin-influx transporters that facilitate the entry of the protonated apoplastic IAA. Regarding the auxin-efflux transporters, two families of proteins performing this function have been identified, (1) the PIN-formed proteins (PIN proteins) and (2) members of the multidrug-resistant/P-glycoprotein (MDR/PGP) subfamily of ATP-binding cassette (ABC) proteins (Noh et al. 2001; Friml et al. 2003; Blakeslee et al. 2004; Bouchard et al. 2006a; Geisler and Murphy 2006; Petrasek et al. 2006). PAT as the basis of pattern generator in plant development can be traced back to early land plants and is also present in algae and mosses (Cooke et al. 2002).

Eight members of the PIN family have been identified so far, five of them localizing at the plasma membrane (PIN1, 2, 3, 4, and 7) (Petrasek et al. 2006) and three at the endoplasmic reticulum (PIN5, 6, and 8) (Mravec et al. 2009). More recently, a family of PIN-like proteins (PILs) localizing at the endoplasmic reticulum membrane have been reported, the function of which is to facilitate the intracellular auxin accumulation (Barbez et al. 2012). The plasma membrane-resident PIN proteins show a polar distribution, which varies depending on cell type (Feraru and Friml 2008). Regulation of the highly specific polar targeting of the PIN proteins is an intriguing question, in which signals derived from cell membrane composition (in particular sterols) (Willemsen et al. 2003), posttranslational protein modification, and cell-type specificity might interplay. In addition, PIN expression and localization are controlled by auxin (Paciorek et al. 2005; Tanaka et al. 2006), thus establishing a feedback regulatory loop for auxin distribution through the plant organs.

The newly synthesized PIN proteins are delivered to the plasma membrane (PM) in a nonpolar manner and are then transported to their final destination by the complex network of vesicle trafficking (Geldner et al. 2001, 2003; Jaillais et al. 2006; Dhonukshe et al. 2008). Vesicle trafficking is also responsible for the flexible changes of PIN localization in response to stimulus such as gravity or light (Abas et al. 2006; Kleine-Vehn et al. 2010; Wan et al. 2012). Although still not completely understood, one of the mechanisms that modulate PIN polar localization is phosphorylation. The protein kinase PINOID (PID), which belongs to the family of AGC protein kinases, is the first described regulator of PIN polar localization (Christensen et al. 2000; Benjamins et al. 2001; Friml et al. 2004). In general terms, PID-dependent PIN phosphorylation results in PIN apicalization, whereas hypophosphorylated PIN proteins show a basal localization (Dhonukshe et al. 2010; Huang et al. 2010; Li et al. 2011). PID-mediated regulation of PIN polarity occurs in concert with ENHANCER OF PINOID (ENP)/MACCHI-BOU4 (MAB4)/NAKED PINS IN YUC MUTANTS 1 (NPY1), which belongs to a family of regulatory proteins called NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3)-like proteins (Treml et al. 2005; Furutani et al. 2007, 2011).

Recently, it was reported that the ABCB/PGP auxin-efflux carriers are also substrates of PID. PID seems to have a dual, counteractive effect on ABCB/PGP

Table 1 Summary of protein kinases and phosphatases functionally involved in polar auxin transport

	Target processes
AGC kinases/NPH3-containing proteins	• PIN and PGP polar targeting • PIN degradation[a] • PAT-phototropism cross talking
Protein kinase CK2	• PIN recycling[a] • AGC/NPH3 regulation[a]
MAP kinases	• PAT-pathogenesis cross talking
Enzymes involved in phosphoinositides metabolism	• Regulation of phosphoinositide levels involved in – Membrane composition – Enzyme activity – Protein anchoring to plasma membrane – Vesicle trafficking
Phosphatases: PP1, PP2A, PP2C, PP6	• PIN polar targeting • InsP3 levels • PIN expression[a] • NPH3 regulation • PKS4 regulation

[a]Hypothesized function, not fully demonstrated.

activity, depending on another ABCB interactions (Henrichs et al. 2012). Moreover, additional kinases are likely to be involved in the regulation of PAT, although the identification of their substrates remains elusive. In this chapter we review the different kinases and phosphatases for which experimental evidence sustains a significant role in the regulation of PAT (Table 1).

2 The AGC Family of Protein Kinases and Their Regulators

The AGC family of Ser/Thr kinases plays crucial roles in fundamental processes in animals and yeast, and there is strong evidence that members of this family are key regulators of auxin-signaling pathways. AGC kinases were named in animals after the cAMP-dependent protein kinase A, cGMP-dependent protein kinase G, and phospholipid-dependent protein kinase C. The *Arabidopsis* AGC family of kinases contains 39 members, which have been grouped in six subfamilies (Bogre et al. 2003). The best characterized plant members belong to the plant-specific AGC VIII subfamily and are the most closely related to mammalian protein kinase A. One characteristic of the AGC VIII members is the substitution of the conserved DFG amino acid motif for DFD in the subdomain VII of the catalytic domain. Another characteristic, specific to plant AGC VIII kinases, is the presence of an amino acid insertion between the conserved subdomains VII and VIII of the catalytic domain, which ranges from 36 to 90 residues in the *Arabidopsis* family members. Plant AGC VIII kinases have been subdivided in two subgroups (AGC VIIIa and b) (Bogre et al. 2003) or in four subgroups (AGC1–4) (Galvan-Ampudia and Offringa 2007), depending on the criteria used for the alignment. For the sake of simplicity,

in this review we will use the classification of Bogre et al. (2003). The AGC VIIIa subgroup includes PID and its three closest homologues, PID2, WAVING 1 (WAG1), and WAG2 (Santner and Watson 2006), all of them well-known regulators of PAT. The AGC VIIIb subgroup includes two blue-light-activated protein kinases, PHOTOTROPIN 1 (PHOT1) and PHOT2, involved in the regulation of the phototropic response and of other light responses such as chloroplast movement and stomata opening. On the other hand, a separate group of AGC kinases also involved in PAT is the PDK1 subfamily, which is formed by two highly similar *Arabidopsis* members of the 3-phosphoinositide-dependent protein kinase (PDK1). In animals, PDK1 is a central integrator of signaling events from receptors that stimulate the production of phosphoinositides and regulate processes such as the balance between growth, cell division, and apoptosis (Alessi 2001).

2.1 The AGC VIIIa Subfamily

The Ser/Thr kinase PINOID (PID) is the most studied member of this group of kinases. *Arabidopsis pid* loss-of-function mutant shows pleiotropic defects in the development of floral organs, cotyledons, and leaves (Christensen et al. 2000; Benjamins et al. 2001), whereas *35S::PID* transgenic plants exhibit dwarf stature, decreased apical dominance, root agravitropic growth, and defects in lateral root formation (Christensen et al. 2000; Benjamins et al. 2001). Simultaneous disruption of the four PID homologues (PID, PID2, WAG1, and WAG2) abolishes the formation of cotyledons. Numerous studies have demonstrated that PID is directly involved in the regulation of the polar delivery of PIN proteins (Friml et al. 2004). PID phosphorylates Ser 231, 252, and 290, located in the conserved motif TPRXS (S/N) that is repeated three times within the central hydrophilic loop (PINHL) of PIN proteins (Michniewicz et al. 2007). There is evidence that PID-dependent PIN phosphorylation takes place at the plasma membrane, but in contrast to their PIN substrates, PID/WAG1/WAG2 has a symmetrical, nonpolar localization (Dhonukshe et al. 2010). PID is predominantly expressed in the epidermis and the lateral root cap (Dhonukshe et al. 2010), and its PM-localization is reduced in the *cpi* sterol biosynthesis mutant (Men et al. 2008). However, PIN apical targeting might depend on other factors than PID-dependent phosphorylation, since *pid* loss-of-function mutants do not show a significant perturbation of the polar localization of PIN1 and PIN2 in root cells, even though they exhibit enhanced PIN2 accumulation in endomembrane compartments (Sukumar et al. 2009). WAG1 and WAG2, the closest homologues of PID, act redundantly with PID on PIN phosphorylation and polar targeting (Geldner et al. 2003; Dhonukshe et al. 2010).

The current model for PIN polar targeting, supported by experimental results, claims that this process takes place in two steps: (1) the newly synthesized PIN proteins are delivered to the plasma membrane in a nonpolar manner; and (2) PIN proteins are subsequently recruited by the apical or basal recycling pathway, depending on their phosphorylation status. PID/WAG1/WAG2 kinases are the

main regulators of the apical recycling pathway, controlling the apical targeting of new proteins or the apical shift of basal proteins. GNOM, a member of the large family of ARF guanine nucleotide exchange factors (ARF-GEF), is the main regulator of the basal recycling pathway (Steinmann et al. 1999; Geldner et al. 2003). GNOM is predominantly localized at the recycling endosomes and its activity is inhibited by the drug brefeldin A (BFA) (Geldner et al. 2003; Kleine-Vehn et al. 2008a). Studies performed with BFA and with a BFA-insensitive GNOM version have demonstrated that the basal targeting of PIN1 and PIN2 is GNOM dependent, whereas PIN apical localization is GNOM independent (Dhonukshe et al. 2010). These data suggest that PID and GNOM act antagonistically in the regulation of apical/basal PIN targeting. Moreover, PID-GNOM antagonism might be a particular example of a common mechanism, in which an AGC kinase and an ARF-GEF protein mediate the establishment of PIN polarity in different environmental conditions and/or developmental programs, such as in phototropic and gravitropic responses (Kleine-Vehn et al. 2010; Ding et al. 2011), lateral root formation (Kleine-Vehn et al. 2008b), leaf epidermal cell indentations (Li et al. 2011), and fruit valve margin formation (Sorefan et al. 2009).

Additional but still unidentified kinases do likely contribute to the polar localization of PIN proteins. For instance, PID-independent PIN1 phosphorylation at Ser337/Thr340 is sufficient to trigger PIN1 apical localization (Zhang et al. 2010). Moreover, a new phosphorylation site in *Arabidopsis* PIN3 has been recently identified; this site is adjacent to a TPRXS(S/N) consensus motif located in the M3 region of the hydrophilic loop (PIN3HL) (Ganguly et al. 2012). Mutations of Ser residues at the M3 region affect PIN3 subcellular trafficking and cause defects in auxin distribution, root growth, and root gravitropism. However, the mutated domain is still phosphorylated by PID, raising the question of whether additional PID-dependent phosphorylation sites exist at the PIN3HL region or the mutated Ser residues are phosphorylated by other still unidentified kinases. Quite interestingly, Ganguly et al. (2012) propose that the M3 phosphorylation sites at PIN3-HL operate distinctively depending on the cell type, suggesting the existence of cell-type-specific trafficking factors that can decipher the M3 phosphorylation code differently.

The ABCB1/PGP1 auxin-efflux carrier is also phosphorylated by PID. Interestingly, the impact of the PID-dependent phosphorylation on the ABCB1 activity is different depending on whether or not ABCB1 is interacting with TWISTED DWARF1 (TWD1). TWD1 is a central regulator of the ABCB-mediated auxin transport (Bouchard et al. 2006b). The results show that PID-dependent ABCB1 phosphorylation enhances auxin efflux but blocks ABCB1 activity in the presence of TWD1. PID phosphorylates ABCB1 in a serine residue of its linker domain, but surprisingly, the phosphorylated site shows no obvious sequence homology to the TPRXS(N/S) motif of the PIN proteins (Henrichs et al. 2012).

Other protein kinases belonging to the AGC VIIIa family are also involved in the regulation of PAT, but its activity does not affect PIN polarity. This is the case of D6 protein kinases (D6PKs), with four members in Arabidopsis sharing a high degree of sequence homology (Zourelidou et al. 2009). Arabidopsis T-DNA

insertional lines in those genes show auxin-related phenotypes, such as fused cotyledons, agravitropic root growth, and reduced lateral root formation; these phenotypes were accompanied by defects in auxin maxima and auxin transport. D6PKs localize at the basal side of the plasma membrane of stele cells, thus co-localizing with basal PIN proteins. It has been demonstrated that the PIN proteins are in vitro and in vivo substrates of D6PKs, although PIN targeting is not modified in *d6pks* mutants. This suggests that D6PKs do not regulate PIN endocytosis and/or recycling. Although it has been demonstrated that *pin1* phenotype is enhanced in loss-of-function *d6pk* double and triple mutants, the exact nature of the role of D6PKs kinases in auxin transport is still unknown (Zourelidou et al. 2009).

2.2 The AGC VIIIb Subfamily

The best characterized plant members of this subfamily are the PHOTOTROPINS (PHOT) (Bogre et al. 2003), blue-light-activated protein kinases that mediate phototropism and other light responses. *Arabidopsis* genome contains two phototropin-encoding genes sharing 60 % homology: *PHOT1* (originally named NPH1, for *NON-PHOTOTROPIC HYPOCOTYL 1*) and *PHOT2* (originally named *NPL1*, for NPH1-like). Two major domains can be distinguished in their amino acid chain: the amino-terminal domain, which contains two light-, oxygen-, or voltage (LOV)-responding domains, and the carboxyl-terminal protein kinase domain. PHOT2 has a slightly shorter amino-terminal region relative to PHOT1. The LOV1 and LOV2 domains are PER/ARNT/SIM (PAS)-like domains and bind one molecule of flavin mononucleotide (FMN), which provides the UVA/blue-light response (Celaya and Liscum 2005). Blue light promotes phototropin autophosphorylation at multiple sites in the N-terminal region, and the kinase activity is necessary for normal phototropism (Briggs et al. 2001; Sakai et al. 2001; Christie et al. 2002).

The existence of a cross talk between phototropism and auxin was discovered by a series of experiments in the early twentieth century that culminated with the Cholodny and Went hypothesis. This theory postulates that phototropism is caused by an asymmetric distribution of auxin in the hypocotyl, which causes the differential growth associated with the light-driven curvature. Molecular studies discovered that mutations in the auxin carriers PIN1 and PIN3 have clear phenotypic effects on hypocotyl phototropism (Galweiler et al. 1998; Friml et al. 2002) and that proper re-localization of PIN1 in hypocotyl cells is essential for the light-driven shoot bending (Blakeslee et al. 2004). Moreover, two members of the *Arabidopsis* PGP/ABCB/MDR family regulate photomorphogenesis by mediating PAT (Lin and Wang 2005), and PGP1 and PGP19 interact with PIN1 and PIN2 in a tissue-dependent manner (Blakeslee et al. 2007). The membrane composition, in particular sterol composition, plays a critical role in the PIN/PGP interaction and stabilizes PIN1 in particular membrane microdomains (Titapiwatanakun et al. 2009). Changes of auxin fluxes driven by the auxin transporters lead to downstream effects, producing changes of gene expression, in which the ARF7/NPH4 factor

appears to be involved (Harper et al. 2000). However, the way in which the light-driven modification of the phosphorylation status of phototropins is communicated to produce the downstream effects is still poorly understood. A recently identified substrate of PHOT1 is the auxin transporter ABCB19 (Christie et al. 2011). PHOT1-dependent ABCB19 phosphorylation inhibits its auxin-efflux activity, producing an increase of auxin levels in and above the hypocotyl apex; auxin is then channeled by PIN3 to the elongation zone of the shoot, preceding shoot bending. One possible consequence of PHOT1-mediated phosphorylation could be to disrupt the interaction of ABCB19 with its positive regulator, TWD1. On the other hand, the PHYTOCHROME KINASE SUBSTRATE 4 (PKS4) is a known transducer of PHOT1 signaling (Lariguet et al. 2006). It was recently shown that PKS4 is transiently phosphorylated by PHOT1 in a blue-light-dependent manner and that this is an early event following phototropin autoactivation. The accumulation of the PKS4 phosphorylated form is modulated by a phosphatase of the PP2A type, which reverses the action of PHOT1. Moreover, phytochromes repress the accumulation of the PKS4 phosphorylated form, and thus PKS4 appears as an integration point between phototropin and phytochrome signaling. PKS4 phosphorylation occurs upstream of auxin redistribution. However, the authors conclude that PKS4 phosphorylation is not essential for phototropism, but it is part of a negative feedback mechanism (Demarsy et al. 2012).

2.3 The NPH3/BTB Proteins as Signal Transducers of the AGC VIII Kinases

The family of proteins containing the plant-specific NPH3 (from NON-PHOTOTROPIC HYPOCOTYL) domain are signal transducers of light and auxin responses and, in general, of processes triggered by plant AGC kinases. The founding members of the family are NPH3 and RPT2. NPH3 was discovered in a screening for phototropin-interacting proteins, and RPT2 (for ROOT PHOTOTROPISM 2) was found after additional screenings for mutants affected in phototropism. Both proteins belong to a family of 32 members in Arabidopsis, characterized by the presence of the NPH3 domain. In addition, 30 out of the 32 members also contain a BTB (broad complex/tramtrack/bric-a-brac) domain, which is evolutionarily conserved and involved in interacting with other proteins to form homo- or heterodimers. NPH3 and RPT2 likely have different functions in phototropism. Both of them interact with PHOT1, but RPT2 interacts with the LOV domains (Inada et al. 2004), whereas NPH3 interacts with the C-terminal region (Motchoulski and Liscum 1999). Pedmale and Liscum (2007) reported that NPH3 is in a phosphorylated state under dark conditions and that blue-light illumination causes NPH3 dephosphorylation via a PHOT1-dependent mechanism. NPH3-phosphorylation status is controlled not only by blue light but also by auxin (Chen et al. 2010), suggesting that NPH3 might be involved in the auxin feedback response. However, mutations in the NPH3 putative

phosphorylation sites have no effect on the phototropic response, and thus it is difficult to ascertain the functional significance of this phosphorylation, if any, on NPH3 function. On the other hand, NPH3 regulates the dynamic equilibrium of PIN2 localization in the phototropic response (Wan et al. 2012). PIN2 localizes in the pre-vacuolar compartment in darkness, and under blue light it is recycled to the plasma membrane in a NPH3/PHOT-dependent manner. The authors suggest that NPH3 might also regulate PIN2 targeting to the vacuole for its subsequent destruction. Moreover, NPH3 was recently shown to function as a substrate-specific adapter in a CULLIN3-based E3 ubiquitin ligase (Roberts et al. 2011). As such, NPH3 can promote mono-, multi-, and poly-ubiquitination of PHOT1, and thus modify both the subcellular localization and the amount of PHOT1 at the plasma membrane.

NPY1 is another member of the NPH3/BTB family involved in auxin-regulated plant development. The loss-of-function *npy1* (for *NAKED PINS IN YUC MUTANTS 1*) mutant was identified as an enhancer of the phenotype of the *yuc1yuc4* double mutant (Cheng et al. 2007). *YUCCA* (*YUC*) genes encode flavin monooxygenases involved in auxin biosynthesis. Loss of function of NPY1 results in pin-like inflorescences in a *yuc1yuc4* background, but not in a wild-type background. *NPY1* is allelic to *ENHANCER OF PINOID* (*ENP*) and *MACCHI-BOU 4* (*MAB4*), and loss-of-function mutants fail to develop cotyledons in the *pid* background. The *NPY* subgroup includes five members in *Arabidopsis*, named *NPY1 to 5*. The five *NPY* genes show unique and overlapping expression patterns in embryogenesis and in the inflorescence apex (Cheng et al. 2008). The single mutants display either slight defects in cotyledons (*npy1*) or no defects at all, whereas double and triple mutants have pin-like inflorescences. The NPY proteins play also a role in root morphogenesis and root gravitropism. The phenotypic changes are visible only in quadruple or quintuple mutants (with defects in gravitropism and root elongation) but not in single mutants, revealing synergistic effects between the members of the family.

A mechanistic similarity between the phototropic response and the auxin-regulated plant development has been pointed out (Cheng et al. 2007). In both cases, a central module of two core proteins (one of them an AGC kinase and the other a NPH3-containing protein) triggers the appropriate response, which also requires the downstream activation of an auxin-response factor (ARF). In the case of the blue-light-induced phototropism, the PHOT1 receptor (an AGC kinase), which is located at the plasma membrane, interacts with the transducer NPH3 to activate the pathway. In the case of the auxin-driven responses, the combination of PID (the AGC kinase) with a protein of the NPY family will most likely form the core module. However, no protein–protein interaction has been yet demonstrated between PID and any of the members of the NPY subgroup, although NPY1 and PID show partial co-localization in Arabidopsis protoplasts (Furutani et al. 2011). It is worth noting that NPY1 lacks the coiled-coil domain present in other members of the NPH3 family, a domain that is important for the NPH3–PHOT1 interaction. Moreover, regulation of the pathway by phosphorylation/dephosphorylation has been demonstrated only for the pair PHOT1/NPH3.

2.4 The AGC PDK1 Subfamily

The 3-phosphoinositide-dependent protein kinases (PDKs) have attracted considerable attention because of their central position in coupling lipid signals derived from the membrane with downstream signaling events (Mora et al. 2004). In Arabidopsis, the PDK1 subfamily of AGC kinases is composed of two members, PDK1 and PDK2 (Bogre et al. 2003). Arabidopsis PDK1 binds to a wide range of signaling lipids (Deak et al. 1999), although the functional impact of this binding is not known.

Numerous studies support the idea that PDK1-dependent phosphorylation is an evolutionary conserved mechanism for regulation of the AGC kinases (Mora et al. 2004). In mammals, PDK1 interacts with downstream AGC kinases by their C-terminal hydrophobic domain (called PDK1-interacting fragment, or PIF). This interaction drives the phosphorylation of a Ser residue located at the activation loop, a highly conserved region upstream of the PIF domain, triggering the autocatalytic activation of the interacting AGC kinase. In vitro studies showed that Arabidopsis AGC kinases do not require the presence of the PIF domain to bind to PDK1 (Zegzouti et al. 2006b). Most of the Arabidopsis AGC kinases not only bind to but are also phosphorylated by PDK1 in vitro. Exceptions to this mode of regulation are WAG1 and WAG2, which have an amino acid change at the second position of the activation loop (within the consensus PDK1 target site) and the KCBP-interacting protein kinase (KIPK). KIPK contains both the PIF domain and the PDK1 consensus signature sequence, but also it has an extra-large N-terminal extension. Consequently, neither the PIF domain nor the activation loop alone, nor the combination of both domains, is sufficient to confer PDK1 substrate identity (Zegzouti et al. 2006a, b). In particular, *Arabidopsis* PID kinase binds PDK1 in vivo and in vitro, and this binding stimulates autophosphorylation and in vitro transphosphorylation of the maltose-binding protein, the classical in vitro substrate of PID. In addition, PID is able to bind phospholipids, and PID activity is stimulated by Ca^{2+} (Zegzouti et al. 2006a). These results raise the possibility that PID is recruited to the PM where it is subsequently activated by PDK1, thus initiating a signaling cascade resulting in the regulation of the apical/basal position of auxin-efflux transporters. This model suggests that PID might play a role in plant auxin transport mechanistically similar to that of the PKB/PKC kinases in mammalian glucose transport (Watson et al. 2004).

3 Phosphatidylinositol Metabolism and Polar Auxin Transport

Phosphorylation/dephosphorylation reactions in phosphoinositide metabolic pathways regulate the accumulation of different metabolites. Many of these metabolites are important second messengers that regulate plant growth and development as well as

cellular responses to environmental changes or stress (see Xue et al. 2009, for a review). Here we focus on the enzymes and compounds of this pathway that are relevant for PAT.

The link between phosphoinositides and PAT was primarily established by the fact that the AGC kinases PDK1 and PINOID can bind to phosphatidic acid (PA), phosphatidylinositol (PtdIns) 3-phosphate, PtdIns(3,4)P_2, and PtdIns(4,5)P_2, through a pleckstrin homology (PH) domain (Zegzouti et al. 2006a). Phosphatase PP2A, which reverses PID effect during PAT, can also bind PA (Testerink et al. 2004).

Phosphatidic acid (PA) production is induced by auxin and NO and is an early signaling event during adventitious root formation in cucumber explants (Lanteri et al. 2008). PA also regulates cytoskeleton and vesicle trafficking (Li and Xue 2007). Moreover, accumulation of PtdIns(4,5)P_2 and Ins(1,4,5)P_3 (due to a mutation in the phosphoinositide phosphatase gene, *SAC9*) results in shorter primary roots and fewer lateral roots (Williams et al. 2005). Ins(1,4,5)P_3-mediated Ca^{2+} signaling regulates the gravitropic response and modulates PIN expression and PIN polarity (Yun et al. 2006; Zhang et al. 2011). PtdIns(4,5)P_2 is synthesized from PI-4-phosphate by the PI monophosphate 5-kinase 2 (PIP5K); in animal cells, PIP5K plays critical roles in vesicle trafficking. In *Arabidopsis*, *pip5k* knockout mutant shows reduced lateral root formation, delayed root gravitropism, and reduced levels of auxin. Even more, Arabidopsis *pip5k* mutant shows a decelerated vesicle trafficking of PIN2 and PIN3 that can be rescued by incubation with PtdIns(4,5)P_2 (Mei et al. 2012).

Additionally, PIN2 internalization during gravistimulation is sensitive to wortmannin, a drug that inhibits phosphatidylinositol 3-kinase (PI3K) and PI4K (although the latter with lower affinity) and involves sorting nexin 1 (SNX1)-containing endosomes (Jaillais et al. 2006). SNX1 co-localizes with PtdIns3P-enriched membrane subdomains (Kleine-Vehn et al. 2008a) and is a putative downstream effector of PI3K (Vanoosthuyse et al. 2003). Inhibition of PI3K suppresses endocytosis (Emans et al. 2002) and inhibits protein sorting to vacuoles in Arabidopsis (Matsuoka et al. 1995). PtdIns3P, the product of PI3K, is present at very low levels in plant cells and has been shown to regulate root hair cell elongation (Lee et al. 2008). PtdIns3P can activate kinases and their downstream signaling processes, suggesting a role similar to that of PtdIns(3,4,5)P_3 in animal cells (Deak et al. 1999).

All these findings reveal the intricate relationship between the phosphatidylinositol signaling pathway and the auxin responses, and provide new insights into the control of PAT.

4 Protein Kinase CK2

The protein kinase CK2 is a ubiquitous Ser/Thr kinase, essential for cell survival in all eukaryotes (Ahmed et al. 2002). CK2 is a tetrameric enzyme, composed by two catalytic (α) and two regulatory (β) subunits, even though monomeric forms

composed only by α catalytic subunit occur also in vivo. Plant genomes contain several genes encoding the catalytic or the regulatory subunit, the expression of which gives rise to different active isoforms. For example, *Arabidopsis* genome contains four genes for the CK2α subunit and four genes for the CK2β subunit (Salinas et al. 2006), and the different isoforms can make a diversity of interactions among them. The wide variety of the resulting holoenzymes will likely have specific but also overlapping functions.

In plants, CK2 gene expression and activity are higher in proliferative tissues and are cell cycle regulated (Espunya et al. 1999; Espunya and Martinez 2003; Moreno-Romero et al. 2008). It has been demonstrated that inhibition of CK2 activity with 4,5,6,7-tetrabromobenzotriazole (TBB) leads to cell cycle arrest, specifically at G1/S and M phases (Espunya et al. 1999; Moreno-Romero et al. 2008). A CK2 dominant-negative mutant has been generated in Arabidopsis by conditional expression (dexamethasone inducible) of a CK2α-inactive subunit. CK2-defective plants exhibited phenotypic traits characteristic of auxin-deficient mutants, such as a dramatic decrease of lateral root formation, inhibition of root growth, or overproliferation of root hairs (Marques-Bueno et al. 2011a). Moreover, gravitropism was enhanced (mutant plants responded quicker to the gravitropic stimulus), whereas phototropism was inhibited (Marques-Bueno et al. 2011a, b). In addition, CK2-defective plants were hypersensitive to exogenous auxin and to N-1-naphthylphthalamic (NPA), an inhibitor of PAT.

The activity of *DR5rev::GFP* (a gene reporter of auxin levels) in CK2 mutant plants showed reduction of auxin levels in roots, particularly in columella cells, and increase of auxin levels in cotyledons. Moreover, PIN1, PIN2, PIN4, and PIN7, as well as protein kinase PID, were strongly upregulated in CK2 mutant plants. Global transcript profiles showed significant changes in 121 genes of a total of 330 auxin-related genes present in the arrays; this result agrees with the observed auxin-related phenotypes. Furthermore, the use of *PIN::PIN-GFP* constructs revealed massive internalization of some of the PIN proteins in CK2-defective roots. Particularly, PIN4 and PIN7, which normally localize in an apolar manner at the plasma membrane of columella cells, were found massively internalized in endosome-like vesicles (Marques-Bueno et al. 2011a). All these data support the idea that protein kinase CK2 is a regulator of PAT in *Arabidopsis*, by controlling PIN localization and/or expression. Thus, the well-known role of CK2 in the regulation of plant cell division might be mediated by the hormone auxin.

A functional similarity between the asymmetrical localization of PINs during auxin transport and the asymmetrical distribution of the GLUT4 glucose transporter in response to insulin signaling has been highlighted, suggesting that both processes might be mechanistically analogous (Muday and Murphy 2002). During insulin signaling, two AGC kinases, PKB and PKC, are recruited to the membrane and activated by PDK1 kinase, generating a secondary signaling cascade that results in the PM-asymmetrical localization of GLUT4 (Watson et al. 2004). In mammals, CK2-mediated phosphorylation affects the PI3K/PDK1/PKB pathway at multiple levels (Dominguez et al. 2009) and involves phosphorylation of PKB (Di Maira et al. 2005, 2009). PID kinase, a key regulator of auxin signaling and transport, belongs to

the AGC family of protein kinases and interacts with and is phosphorylated by the Arabidopsis homologue of PDK1 (Zegzouti et al. 2006a). The link between membrane-localized phospholipid ligands and downstream auxin-mediated signaling events might now include protein kinase CK2 (Marques-Bueno et al. 2011b; Scherer et al. 2012). CK2 is known to locate in different compartments within the cell, which in mammals includes the plasma membrane (Bosc et al. 2000; Olsten et al. 2004).

It is worth noting that the AGC kinase PID and several members of the NPH3 protein family contain numerous acidic-based motifs in their sequence, which meet the requirement of CK2 phosphorylation consensus site. CK2 might be one of the protein kinases regulating the activity of the AGC/NPH3 core module that has a central role in auxin-signaling pathways (Marques-Bueno et al. 2011b). Unfortunately, phosphorylation of the above proteins by CK2 has not yet been demonstrated.

5 MAP Kinases

The mitogen-activated protein kinase (MAPK) cascade consists of three protein kinases sequentially activated through phosphorylation by the upstream component: a MAP kinase kinase kinase (MAPKKK or MEKK), a MAP kinase kinase (MAPKK or MKK), and a MAP kinase. There are more than 80 putative MAPKKKs, 10 MAPKKs, and at least 20 MPKs in the *Arabidopsis thaliana* genome. It has been extensively reported that MAP kinases have a key role in plant defense against pathogens (for a review see Pitzschke et al. 2009). More recently, some components of the MAPK pathway have been implicated in auxin signaling, and there is evidence that they might serve as cross-talk points between auxin signaling and defense responses.

Mockaitis and Howell (2000) reported the increase of an unidentified MAPK activity upon auxin treatment (Mizoguchi et al. 1994). On the other hand, Kovtun et al. (2000) showed that an oxidative stress-activated MAPK cascade could negatively regulate early auxin responses. Genetic screens for auxin-response mutants in Arabidopsis identified *ibr5* (*iba response5*), showing reduced sensitivity to auxin (Monroe-Augustus et al. 2003). The *ibr5* locus encodes a putative MAP kinase phosphatase, suggesting that IBR5 might act as a positive regulator of auxin signaling. Even more, IBR5 interacts specifically with MAPK12, which is a negative regulator of auxin signaling. Thus, inactivation of MAPK12 by the IBR5 phosphatase leads to upregulation of auxin-responsive genes and the appearance of auxin-hypersensitive phenotypes (Lee et al. 2009).

The implication of MAP kinases in auxin transport was demonstrated by the isolation of an Arabidopsis mutant that showed *bushy* and *dwarf* phenotypes (*bud1* mutant) and developed significant less numbers of lateral roots (Dai et al. 2006). The mutation was due to a T-DNA insertion at 513 bp upstream of the start codon of the MAP kinase kinase 7 (MAPKK7) encoding gene, resulting in gene overexpression. Sensitivity to exogenous IAA and IAA content was similar in

bud1 and wild-type Arabidopsis, but *bud1* showed enhanced shoot gravitropism and defects in IAA transport. Interestingly, *bud1* plants accumulated elevated levels of salicylic acid and displayed constitutive expression of pathogenesis-related genes. Consequently, they also exhibited enhanced resistance to bacterial and oomycete pathogens. Recent experimental evidences indicate that SA inhibits pathogen growth partly through the suppression of auxin signaling. Some pathogens have evolved to counteract this plant defense mechanism by inducing auxin synthesis in the host plant. According to the above data, MKK7 may serve as a cross-talk point between auxin signaling and defense responses. This relationship between auxin signaling and plant defense is likely to be evolutionarily conserved, since soybean MAPK4 (GmMPK4) is a negative regulator of plant defense responses and a positive regulator of growth and development (Liu et al. 2011). GmMPK4-silenced plants show high levels of SA and constitutive expression of defense-related genes, whereas auxin-related genes are repressed. The SA-biosynthetic pathway that appears activated in these plants is the phenylpropanoid pathway.

6 Phosphatases

The identification of particular phosphatases involved in the regulation of PAT is far behind that of protein kinases. One of the first reports was the isolation of the *ROOT CURLING ON NPA1 (RCN1)* mutant, with altered responses to N-1-naphthylphthalamic acid (NPA) (Garbers et al. 1996). *rcn1* is a loss of function mutant of a regulatory subunit of PP2A. Later studies with three closely related regulatory A subunits of the PP2A complex in Arabidopsis (PP2AA1/RCN1, PP2AA2, and PP2AA3) demonstrated that *pp2a* mutants exhibited phenotypes affecting root and embryo development that were similar to PID gain-of-function phenotypes (Michniewicz et al. 2007). Moreover, it was demonstrated that PINOID and PP2A act antagonistically on the phosphorylation of the hydrophilic loop of PIN proteins. PP2A is broadly distributed in the cell, but where its action takes place is still unclear (Michniewicz et al. 2007).

It was recently reported that a phytochrome-associated Ser/Thr protein phosphatase, FyPP1, plays a role in regulating the interdigitated expansion pattern of leaf epidermis cells by influencing PIN1 localization (Li et al. 2011). Mutations simultaneously disrupting the function of *Arabidopsis FyPP1* and of its homologous gene *FyPP3* cause severe defects in a wide range of developmental processes, resulting in shorter roots, fewer lateral roots, defective columella cells, root meristem collapse, abnormal cotyledons, and altered leaf venation (Dai et al. 2012). FyPP1 and FyPP3 encode the catalytic subunits of protein phosphatase 6 (PP6), and they interact with SAL (for SAPS DOMAIN-LIKE) proteins and with regulatory subunits of PP2A (PP2AAs) to form the PP6 heterotrimeric holoenzyme complex. Dai et al. (2012) propose that it is the PP6 heterotrimeric holoenzyme that

specifically interacts with and dephosphorylates the conserved Ser residues of TPRxS(N/S) motifs in the PIN proteins, playing antagonistic functions with PID kinase.

Screenings for *suppressors of PIN1 overexpression* (*supo*) identified an inositol polyphosphate 1-phosphatase mutant (*supo1*) (Zhang et al. 2011). *SUPO1*/*SAL1* encodes a bifunctional enzyme, which functions in InsP3 catabolism (Quintero et al. 1996) and in posttranslational gene silencing (Gy et al. 2007). *supo1* mutant has enhanced $InsP_3$ and Ca^{2+} levels and shows suppression of the PIN1 gain-of-function phenotypes. It is suggested that regulation of $InsP_3$ levels by SUPO1/SAL modulates PAT by affecting PIN polar targeting by a PID-related mechanism. These results establish a clear link between phosphoinositide metabolism and the molecular machinery directly involved in auxin transport.

The two related phosphatases POL and PLL1 are members of the type 2C phosphatases and play crucial roles in maintaining shoot stem cells both embryonically and post-embryonically (Song et al. 2008; Yu et al. 2003). POL/PLL1 promote expression of WOX5, a homologue of WUSCHEL (WUS), which is necessary for stem cell maintenance. *polpll1* double mutant shows reduced accumulation of PIN1 during embryogenesis, although no obvious asymmetric localization was observed. Auxin distribution, measured with the *DR5::GFP* reporter, and auxin responses, were normal in the *polpll1* mutant. Thus, although these two phosphatases seem to regulate the asymmetrical divisions necessary for stem cell maintenance and specification and their mutation slightly perturbs PIN1 expression, they do not seem to be directly involved in the regulation of PAT.

As far as it is known, phosphatases seem to have a special relevance in the phototropin-mediated signaling pathways. It is known that blue light stimulates dephosphorylation of NPH3 by a type 1 protein phosphatase. NPH3 dephosphorylation is PHOT1 dependent, and it is a key step for the phototropic response (Pedmale and Liscum 2007). On the other hand, the PKS4 phosphorylation status is controlled by a PP2A phosphatase (Demarsy et al. 2012). Interestingly, PSK4 is first phosphorylated by PHOT1 in response to light, but the PP2A-mediated dephosphorylation step appears to be a key regulatory feedback mechanism to prevent excessive phototropism.

7 Conclusions

The initial cloning and characterization of diverse families of auxin-carrier proteins and, later on, the discovery of their asymmetrical localization at the plasma membrane have been landmarks in the understanding of the regulation of auxin fluxes directionality. Since then, an overwhelming amount of work from different laboratories has shed light on the molecular basis controlling the polar targeting of the auxin carriers. Currently, it is widely accepted that PID-mediated PIN phosphorylation constitutes a basic mechanism that regulates PIN delivery to the apical domain of the plasma membrane. However, the picture is growing in complexity.

Not only PIN proteins and other auxin-efflux carriers are likely phosphorylated by other kinases than PID, but in some cases, PID phosphorylation is not sufficient to switch PIN localization from the basal to the apical cell side, although it enhances PIN accumulation in endosomes. Also, there has been a progressive discovery of the upstream PID regulators, which turn out to be protein kinases as well. In addition, the emergence of molecular mechanisms that link PAT and the phototropin-mediated responses (the phototropins being also kinases of the same family as PID), or PAT and plant defense (with MAP kinases as important players), is contributing to entangle the web of protein kinases involved in PAT regulation. More recently, protein kinase CK2, a ubiquitous and pleiotropic kinase, has been added to this web, and although its substrates have not yet been identified, it is foreseen that CK2 could regulate the central core formed by the AGC/NPH3 pair of proteins.

As PIN recycling is highly dependent on vesicle trafficking, the regulation of the different subroutes of this traffic also has consequences on PAT. Key steps such as endocytosis or the delivery of cargo proteins to particular endocytic compartments, or to the plasma membrane, influence auxin fluxes. A great amount of protein kinases and phosphatases are involved in the biosynthetic pathways that regulate the accumulation of different phosphoinositols and phosphoinositides. The different compounds synthesized by this route are likely to play a role in either the anchorage of proteins to the membranes, the direct stimulation of enzymatic activity, or as structural components of particular microdomains in the plasma membranes, which, in turn, might be important for the specificity of the polar targeting. The different subpopulations of endocytic particles are not equal in their lipid composition, and this fact, together with the posttranslational modification of the cargo proteins (PIN phosphorylation or PIN ubiquitination, for instance), acts as signals instructing differential recruitment to alternative trafficking pathways.

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Control of Auxin Transport by Reactive Oxygen and Nitrogen Species

María Fernández-Marcos, Luis Sanz, Daniel R. Lewis, Gloria K. Muday, and Oscar Lorenzo

Abstract Auxin transport is a central process in plant growth and development and as a result is highly regulated. The amount and direction of auxin transport is defined by a set of auxin influx and efflux carriers with precise localization that lead to long-distance polar auxin transport. These auxin transport proteins are regulated by transcriptional and posttranslational mechanisms and through protein-targeting machinery that directs them to the appropriate plasma membrane location. A variety of signals initiate regulatory changes in the abundance, activity, or localization of these proteins, with plant hormones, light, and other environmental signaling implicated in this process. Recent evidence indicates that changing levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) may also fine-tune the activity or synthesis of these proteins. This insight has been obtained by using mutants or treatments that alter the levels of ROS or RNS and demonstration of changing auxin transport and abundance of transport proteins. The molecular mechanisms by which ROS and RNS lead to changes in auxin transport are not yet clear but likely include changes in protein synthesis and abundance. This chapter briefly introduces the key proteins and antioxidant molecules that control the levels of ROS and RNS and focuses on the evidence linking these changes to altered auxin transport.

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1 Introduction

Important roles of reactive oxygen species (ROS) and reactive nitrogen species (RNS) have recently been described in many plant developmental processes (Swanson and Gilroy 2010; Mittler et al. 2011) including guard cell physiology, flower development, root hair elongation, and most relevantly cellular differentiation in the root apex and modulation of auxin transport via action on PIN auxin efflux proteins (Bashandy et al. 2010; Tsukagoshi et al. 2010). ROS and RNS make excellent signaling molecules as their toxicity requires they be kept at low levels in cells, which allows subtle changes in their synthesis to lead to large magnitude differences in their levels, like other important signaling molecules, such as calcium and protons. As a result, there is a precise balance between synthesis and scavenging of these molecules that allows their level to be carefully modulated.

ROS include H_2O_2, $O_2^{\bullet -}$, and $^1O_2$, which are by-products of aerobic cellular metabolism. Their accumulation is determined by the balance between production and detoxification by antioxidants. In both plants and animals, ROS can be generated through respiratory burst/NADPH oxidases (Suzuki et al. 2011; Marino et al. 2012), while ROS levels are reduced through antioxidant protein networks including thioredoxins, glutathione/glutaredoxins, and peroxidases (Mittler et al. 2011) or by chemical antioxidants, such as flavonoids or ascorbate (Hernandez et al. 2009; Pollastri and Tattini 2011). Plants with mutations in the genes encoding proteins that synthesize ROS or antioxidants have linked ROS to plant development (Mittler et al. 2011).

The most abundant RNS in plants is nitric oxide (NO), which is a gaseous free radical displaying a broad spectrum of regulatory functions involved in physiological processes (Delledonne 2005). In animals, nitric oxide synthase (NOS) defines NO levels, but no obvious plant NOS homolog has yet been identified. Some studies have shown the importance of a NOS-like pathway in mediating NO responses in plants, while other studies suggest that the enzyme nitrate reductase (NR) is more important (Besson-Bard et al. 2008a, b). The diverse enzymatic and nonenzymatic reactions in plant NO synthesis are illustrated in Fig. 1. NO levels and bioactivity are also controlled by scavengers, which include class 1 hemoglobin (Hb1) (Fig. 1; Dordas et al. 2004; Perazzolli et al. 2004; Igamberdiev and Hill 2004) in a reaction that is dependent on the presence of both NAD(P)H and AHb1 (Igamberdiev et al. 2005). Strong hypoxic induction of the *AHb1* gene (At2g16060) occurs in Arabidopsis under conditions that also induce enzymes of nitrogen metabolism (Klok et al. 2002), suggesting a mechanism for a rapid and transient elevation of NO levels, followed by scavenging to prevent cellular damage.

Both RNS and ROS directly modulate the activity of proteins through posttranslational modification (PTM). PTMs mediated by RNS, such as cysteine S-nitrosylation or tyrosine nitration (Moreau et al. 2010; Arc et al. 2011), can result in an alteration of diverse protein functions. Similarly, ROS leads to oxidation of specific cysteine residues, which control protein activity. The best described ROS-dependent regulation of a mammalian protein is tyrosine phosphatase 1B (PTP1B), where the activity

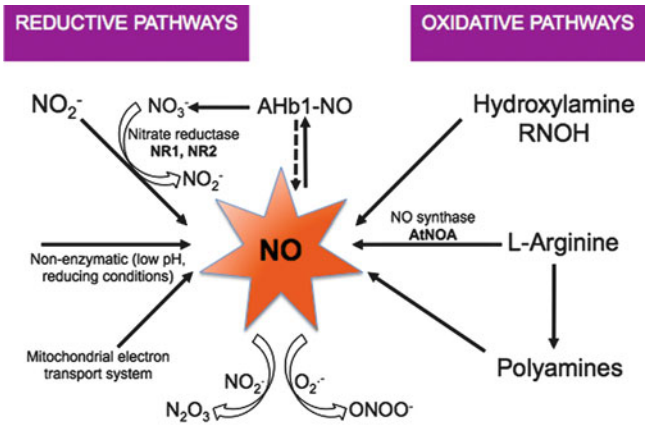

Fig. 1 Formation of reactive nitrogen species (RNS) with emphasis on the diverse enzymatic or nonenzymatic reactions potentially involved in NO synthesis in plants. *AtNOA Arabidopsis thaliana* nitric oxide associated, *RNOH* substituted derivatives of hydroxylamine. *NO* nitric oxide, NO_3^- nitrate, NO_2^- nitrite, N_2O_3 nitrogen trioxide, $NO_2^\bullet$ nitrogen dioxide radical, $O_2^{\bullet-}$ superoxide, $ONOO^-$ peroxynitrite. Adapted from Moreau et al. (2010)

of the protein has been shown to be regulated by a specific single cysteine oxidation which in turn regulates the insulin signaling pathway (Tonks 2006). Recent data suggest that protein nitration and S-nitrosylation could be more than a biological marker of nitrosative stress and could participate in protein turnover or signal transduction in plants (Corpas et al. 2007; Ischiropoulos 2009; Stamler et al. 1992; Jaffrey and Snyder 2001). The specificity of these modifications indicates that they may act as regulatory switches in signal transduction pathways (Hess et al. 2005), analogous to protein phosphorylation (Spickett et al. 2006). Recent evidence, using mutants or chemical treatments that raise or lower ROS and RNS, suggests that auxin transport proteins (or proteins that control their synthesis or targeting) may be targets of ROS and/or RNS regulation by oxidation, nitration, or S-nitrosylation.

2 ROS Regulation of Auxin Transport

ROS have been reported to modulate polar auxin transport by influencing auxin efflux and influx-dependent transport. Recent genetic analysis indicates that polar auxin transport is impaired in plants with altered ROS accumulation. Plants with defects in genes encoding thioredoxin reductase (*ntra* and *ntrb*) and an enzyme of glutathione biosynthesis (*cad2*) exhibit altered ROS homeostasis, due to the absence of these antioxidant proteins (Bashandy et al. 2010). The *ntra ntrb cad2* triple mutant has impaired auxin transport and developmental phenotypes consistent with altered auxin transport including PIN-shaped inflorescences and reduced lateral root formation (Bashandy et al. 2010). In addition, *atgrxs17* and *ntra ntrb*

cad2 plants show auxin-related morphological phenotypes and reduced expression of the auxin-responsive reporter, $DR5_{pro}$:*GUS* indicating that they have deficiencies related to auxin action or auxin accumulation (Bashandy et al. 2010; Cheng et al. 2011). Consistent with this latter possibility, root growth defects of this triple mutant are rescued by auxin treatment, suggesting a defect in transport, rather than signaling (Bashandy et al. 2010). Decreased transcript levels for auxin efflux and influx carriers in response to elevated ROS suggest that ROS may modulate auxin polar transport at the level of synthesis of transport proteins (Blomster et al. 2011; Tognetti et al. 2012). Intriguingly, the auxin transport inhibitor TIBA and O_3 treatment (used as a tool to produce an apoplastic ROS burst) exhibited similar transcriptional effects on auxin-responsive genes (Blomster et al. 2011). Similarities between the responses to oxidative stress and auxin transport inhibitors suggest ROS may act on plant morphology by inhibiting auxin transport either at the level of synthesis or turnover of auxin transport proteins.

Elevated ROS may also alter auxin transport by affecting the stability of auxin efflux carriers. The fluorescence of GFP fusions to PIN1, PIN2, PIN3, and PIN7 are decreased in the root tips of primary roots, but not adventitious roots, treated with BSO (Koprivova et al. 2010; Bashandy et al. 2010). One set of authors suggest that since BSO did not abolish transcription of PIN1, and the effect of BSO was complemented by dithiothreitol, the authors conclude that as yet an uncharacteristic post-transcriptional redox mechanism regulates the accumulation of PIN proteins, and thus auxin transport, in the root tips (Koprivova et al. 2010). Taken together, these data suggest the intriguing result that BSO treatment decreases PIN protein abundance with both transcriptional and post-translational role implicated.

Mutations in genes encoding proteins that are involved in the synthesis of chemical antioxidants, such as flavonols, suggest an additional link between ROS and auxin transport (Pollastri and Tattini 2011). Flavonols have been shown to regulate auxin transport and dependent physiological processes, including root elongation, gravitropism, and branching (Brown et al. 2001; Buer and Muday 2004; Peer et al. 2004; Buer and Djordjevic 2009; Lewis et al. 2011). Auxin transport is elevated in inflorescences, hypocotyls, and roots of plants with the *tt4-2* mutation, which make no flavonoids (Murphy et al. 2000; Brown et al. 2001). A comparison of the root gravitropic responses of wild-type and several *tt4* alleles identified a delay in root gravitropism when flavonoid synthesis is abolished, which is reversed by chemical complementation by naringenin (Buer and Muday 2004; Buer et al. 2006; Lewis et al. 2011). Flavonoids promote gravitropism presumably by regulating auxin movement in the root tip that modulates differential growth (Buer and Muday 2004). Finally, factors that regulate flavonoid biosynthesis also affect auxin transport, such as light levels (Jensen et al. 1998; Rashotte et al. 2003), wounding and pathogen attacks (Mathesius et al. 1998; Berleth and Sachs 2001), ethylene levels (Lewis et al. 2011), and gravity stimulation (Buer and Muday 2004; Buer et al. 2006).

What has not yet been demonstrated is whether the role of flavonols is to alter ROS in the root and thereby regulate auxin transport through ROS signaling pathways or through more direct mechanisms (Pollastri and Tattini 2011). The levels of ROS species are elevated in plants with defects in flavonol synthesis,

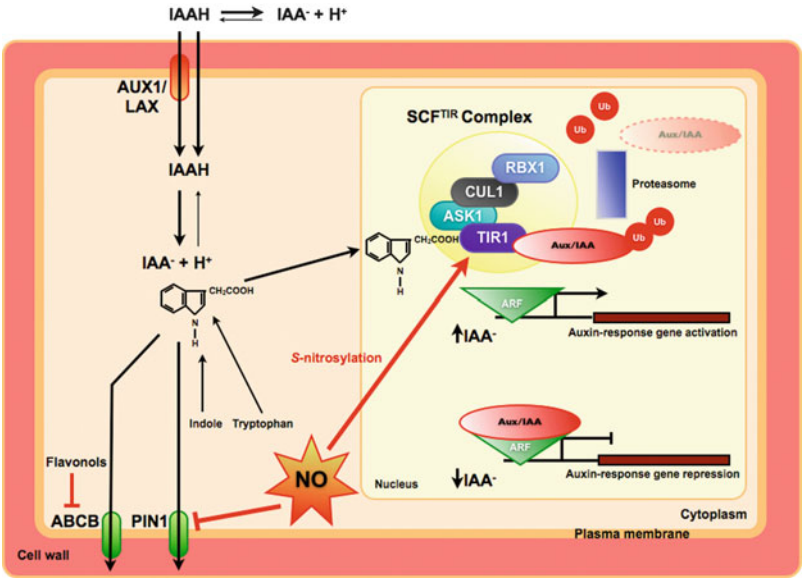

Fig. 2 Role of nitric oxide and flavonols in auxin transport and direct effect of NO in the auxin signaling pathway. *AUX1/LAX* auxin uptake carrier, *PIN1/ABCB* auxin efflux carriers

consistent with flavonols acting as antioxidants in vivo (Lewis and Muday, unpublished observation), but other mechanisms of flavonol regulation of auxin transport have been described. Quercetin has been shown to block auxin transport when ABCB proteins are expressed in heterologous systems (Fig. 2; Geisler et al. 2005; Bouchard et al. 2006). In addition, *ABCB4* was shown to be epistatic to *TT4* by double mutant analysis, indicating that flavonols act through ABCB4 to control basipetal auxin transport and gravitropism (Lewis et al. 2007). The inhibition of auxin transport includes disruptions of a complex between an ABCB protein and an immunophilin protein that is needed for maximal auxin transport (Bailly et al. 2008). Whether this protein complex is sensitive to oxidation state in the cell has not yet been reported. An additional intriguing possibility is that ROS and/or RNS control the activity of flavonols by converting them to a semiquinone state, which may have different inhibitory properties. In this scenario, the oxidized flavonol could then be reduced, restoring its capacity to inhibit protein complex formation. Resolving the role of flavonols in regulation of auxin transport via modulation of ROS levels awaits further experimentation.

3 RNS Regulation of Auxin Transport

Auxin transport has a central role in auxin-regulated growth processes. Despite the effort to understand the mechanism of NO regulation of polar auxin transport, our knowledge is still limited. Hu et al. (2005) showed that gravistimulation of soybean

primary roots induces asymmetric accumulation of NO, and this NO generation is stimulated by auxin since NPA treatments inhibit NO accumulation and gravitropic bending, suggesting that lateral auxin transport is essential for asymmetric NO generation.

Interestingly, high levels of endogenous NO in the *cue1*/*nox1* background produce a drastic reduction in auxin movement from the root shoot junction to the root tip (acropetal or rootward auxin transport), through use of [^{3}H]IAA radiotracer assays as described previously (Fig. 2; Lewis and Muday 2009; Fernández-Marcos et al. 2011). Additionally, high levels of endogenous or applied NO reduce the fluorescence of a PIN1:GFP fusion which participates in rootward auxin transport, without altering significantly PIN1 transcript levels (Fernández-Marcos et al. 2011). In contrast, the fluorescence of a GFP-fusion reporter for PIN2, which mediates basipetal or shootward IAA transport, was not altered significantly suggesting a specific effect of NO on rootward auxin transport in primary roots mediated by changes in PIN1 protein levels (Fernández-Marcos et al. 2011). Likewise, acropetal auxin transport is enhanced in mutants with lower levels of NO such as *atnoa1*, supporting the hypothesis that altered NO levels cause altered auxin transport capacity (Fernández-Marcos et al. 2011; unpublished data).

In a recent report, Bai et al. (2012) propose that treatment with 3-O-C10-HL (*N*-acyl-homoserine lactones, AHLs) promotes auxin-dependent adventitious root formation, possibly through H_2O_2- and NO-dependent cGMP signaling in mung bean (*Vigna radiata*) seedlings. This treatment is able to stimulate the generation of H_2O_2, NO, and the synthesis of cGMP to activate adventitious root formation. Treatment with 3-O-C10-HL enhances hypocotyl auxin basipetal transport and this effect can be reversed by scavenging H_2O_2 or NO, suggesting that these molecules act within a single pathway to promote hypocotyl basipetal auxin transport and adventitious root formation.

4 Other ROS and RNS Connections to Auxin

ROS and RNS can also affect the dynamics of the actin cytoskeleton and may alter actin-dependent targeting of auxin transport proteins. At the level of subcellular dynamics and polar targeting, there is increasing evidence that auxin regulates polar auxin transport by inhibiting PIN endocytosis (Dhonukshe et al. 2008; Lin et al. 2012; Nagawa et al. 2012), which is actin dependent (Geldner et al. 2001). Whether ROS have an effect on auxin distribution as a result of their regulation of the cytoskeleton, vesicle trafficking, and membrane dynamics remains to be elucidated. However, it is already known that NO affects the functioning of the actin cytoskeleton. In response to NO levels, actin cables change their orientation from longitudinal to oblique and cellular cross-wall domains become actin depleted/depolymerized (Kasprowicz et al. 2009). Additionally, actin-dependent vesicle trafficking is also affected. This was demonstrated through the analysis of recycled

wall material transported to newly formed cell plates (Kasprowicz et al. 2009). Thus, the dynamic actin cytoskeleton could be considered as a downstream effector of NO signaling *in planta* (Kasprowicz et al. 2009).

4.1 Auxin Promotes ROS Accumulation

Auxin induces changes in redox status leading to a more oxidizing cellular environment (Takahama 1996; Joo et al. 2001; Jiang and Feldman 2003; Li et al. 2009; Wang et al. 2010; De Tullio et al. 2010). This change in redox status is mainly due to the generation of several ROS, such as hydrogen peroxide (H_2O_2) (Brightman et al. 1988; Joo et al. 2001) and superoxide ions ($O_2^{\bullet -}$) (Schopfer 2001). These ROS may be generated by oxidation of IAA (Kawano 2003) or, indirectly, as a consequence of auxin affecting the activities or synthesis of redox-associated systems (Takahama 1996; Kisu et al. 1997; Jiang and Feldman 2003; Pignocchi et al. 2003).

Redox processes are important for regulating root growth. This regulation may act through mechanisms dependent (Duan et al. 2010) or independent (Tsukagoshi et al. 2010) on the auxin signaling pathway. ROS accumulation in the quiescent center (QC) is an interesting example of how auxin induces changes in redox status. The redox status of the QC, where auxin is strongly accumulated, is different from that in adjacent rapidly dividing cells. The QC has a more oxidizing environment (Kerk and Feldman 1995; Sanchez-Fernandez et al. 1997; Kerk et al. 2000; Jiang and Feldman 2003; Liso et al. 2004) and a large group of transcripts associated with regulating redox status are localized to this tissue (Jiang et al. 2010).

Auxin also promotes ROS accumulation during gravitropic bending (Joo et al. 2001). Gravistimulation elicits a transient increase in intracellular ROS. The action of asymmetrically applied H_2O_2 in causing root curvature does not depend upon auxin redistribution, suggesting that ROS play a role as a downstream component in the auxin response pathway. Increased ROS concentrations may in turn trigger nitric oxide (NO) generation by nitrate reductase (NR) (Wang et al. 2010) and NO synthase (NOS)-like enzymes (Neill et al. 2008; Li et al. 2009). This probably occurs through the rapid phosphorylation of MAP kinase 6 (MAPK6) (Kovtun et al. 2000; Wang et al. 2010) and/or the action of the protein kinase OX1 and involves Ca^{2+} (Rentel et al. 2004). Removal of NO with an NO scavenger or inhibition of NO synthesis via NO synthase inhibitors or an inhibitor of nitrate reductase reduces gravitropic bending, indicating that NO synthesis is an important component of the gravitropic response (Hu et al. 2005).

Additional experiments have revealed possible mechanisms of auxin-induced ROS synthesis. The activation of PtdIns 3-kinase and NADPH oxidase is required for auxin-induced production of ROS, regulating plant cell expansion through the activation of Ca^{2+} channels (Joo et al. 2001; Foreman et al. 2003). Recent results suggest that NADPH oxidase may also be regulated by the Feronia (FER) receptor-like kinase (Duan et al. 2010) and RAC/ROP GTPases (Tao et al. 2002; Xu et al.

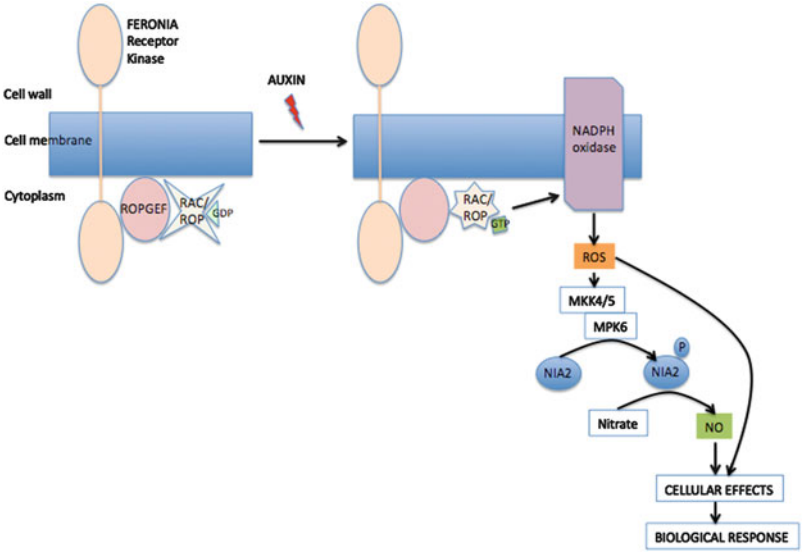

Fig. 3 A model for the putative pathway of ROS and NO biosynthesis and signal transduction mediated by auxin-activated FER–RAC/ROP complex (modified from Duan et al. 2010 and Wang et al. 2010)

2010). Specifically, FERONIA acts as a surface regulator of the RAC/ROP signaling pathway which in turn regulates NADPH oxidase-dependent ROS production (Wu et al. 2011) (Fig. 3).

4.2 ROS Represses Auxin-Inducible Promoters

Several lines of evidence suggest that ROS may modulate auxin sensitivity by repressing auxin-inducible gene expression (Navarro et al. 2006; Wang et al. 2007; Ludwikow and Sadowski 2008; Bashandy et al. 2010; Iglesias et al. 2010; Blomster et al. 2011; Cheng et al. 2011). Indeed, auxin-resistant mutants *axr1* and *axr3* are less sensitive to ROS than wild-type plants (Koprivova et al. 2010). This process seems to involve changes in MAPK activity. Specifically, ROS can activate an Arabidopsis MAPK, ANP1, which initiates a phosphorylation cascade involving two stress MAPKs, AtMPK3 and AtMPK6 (Kovtun et al. 2000). The activated MAPK cascade plays a dual role in regulation of gene expression activating stress-response genes that protect plants from diverse environmental stresses and repressing auxin-inducible promoters (Kovtun et al. 2000). Thus, the ANP-mediated MAPK cascade represents a molecular link between oxidative stress and the plant growth hormone auxin (Kovtun et al. 2000). In this scenario, NO may also collaborate with ROS to repress auxin-inducible promoters. Increased NO accumulation in *cue1*/*nox1* mutant, where endogenous NO levels are enhanced, depletes auxin-dependent reporter expression in the apical auxin maximum (Fernández-Marcos et al. 2011).

4.3 RNS Regulation of Auxin Signaling

The synergistic effects of auxin and NO have been well characterized in the regulation of a variety of physiological processes of plants. One of the best described NO functions in plants is their involvement in the auxin-regulated signaling cascades determining root growth and morphology. During the last decade it has been reported that NO is involved in the promotion of adventitious roots (Pagnussat et al. 2002), in primary root growth and lateral root formation (Correa-Aragunde et al. 2004; Fernández-Marcos et al. 2011), in root hair development (Lombardo et al. 2006), and in gravitropic responses (Hu et al. 2005).

The role of NO in root development and the cross talk with hormones such as auxin is an emerging area of study. We found that high levels of NO, released by NO donors or using NO overaccumulating mutants (*cue1*/*nox1*), produced a decrease in the primary root length by reducing root meristem size and cell division rates (Fernández-Marcos et al. 2011). As auxin gradients are important factors in the regulation of these processes, the spatial pattern of the auxin response reporter $DR5_{pro}$:*GUS*/*GFP* after NO treatment and in the *cue1*/*nox1* background was analyzed, showing an alteration in the root apical auxin maximum. NO also reduces elongation of root cells (Fernández-Marcos et al. 2012). It has been reported that attenuation of auxin transport and signaling delayed gibberellin (GA)-induced RGA (a DELLA protein) degradation (Fu and Harberd 2003), and as a consequence the NO-inhibition of elongation in the elongation-differentiation zone (EDZ) could be due to the promotion of DELLA activity and, consequently, PIN1 degradation in the presence of high levels of NO (Fernández-Marcos et al. 2012).

Consistent with robust changes in signaling and transcription, numerous NO-regulated genes have been identified. These genes are involved in different functional and biological processes (Huang et al. 2002; Polverari et al. 2003; Parani et al. 2004; Palmieri et al. 2008). However, the direct molecular targets of NO remain poorly documented in plants. Only a few intracellular S-nitrosylated proteins have been identified in plants (Astier et al. 2011; Lindermayr et al. 2005; Tanou et al. 2009). A recent and promising example is the NO-mediated modulation of auxin signaling through posttranslational modification of the TIR1 auxin receptor. S-nitrosylation of TIR1 promotes its interaction with Aux/IAA repressors, thereby facilitating their degradation (Fig. 2; Terrile et al. 2012).

5 Conclusions

In summary, ROS/RNS are important regulators of auxin-dependent growth and development through their effects on several distinct aspects of auxin biology. Recent reports indicate that auxin transport is perturbed in mutants affected in ROS/RNS homeostasis and/or under treatments to produce a burst of ROS/RNS. These results collectively suggest the existence of a regulatory loop between ROS

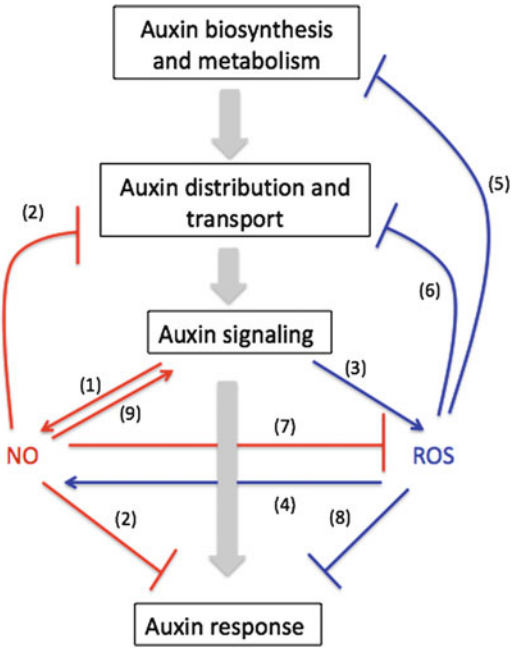

Fig. 4 The schematic diagram shows how ROS and NO impact auxin signaling and response by affecting its biosynthesis and distribution. (3) Joo et al. (2001); (5) Kawano (2003); (1) Hu et al. (2005); (4) Wang et al. (2010); (7) Astier et al. (2011); (6) Blomster et al. (2011); (8) Cheng et al. (2011); (2) Fernández-Marcos et al. (2011); (9) Terrile et al. (2012)

and auxin transport with profound implications for a broad array of signaling processes (Fig. 4).

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Hormonal Control of Polar Auxin Transport

Jiaqiang Sun, Linlin Qi, and Chuanyou Li

Abstract Polar auxin transport (PAT) is required for the formation and maintenance of local auxin gradients, which is of crucial importance for many aspects of auxin-mediated plant development. Different plant hormones regulate plant growth and development mostly by modulating PAT, particularly, pointing to the PINFORMED (PIN) proteins. In this book chapter, we review recent advances on hormonal regulation of PAT.

1 Polar Auxin Transport

Polar auxin transport (PAT) has been found throughout the higher plant species. It is characterized by its strictly controlled directionality, as designated by the name, polar auxin transport. Briefly, the major polar flow of auxin can be traced from apical tissues towards the base of the plant and further to the root tip. Once auxin reaches the tip of the root, part of it is redirected back upwards (basipetally) through the root epidermis into the root elongation zone (Rashotte et al. 2000) where it can be recycled back into the vasculature stream (Blilou et al. 2005). A number of physiological and genetic studies have clearly demonstrated that PAT, which is required for the formation and maintenance of local auxin distribution patterns so-called local auxin gradients, is of crucial importance for many auxin-mediated developmental processes.

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2 Subcellular Trafficking of PINs

The polar, subcellular localization of PIN auxin efflux carriers determines the direction of intercellular auxin flow, thus defining the spatial aspect of auxin response. The current model of polar targeting of PIN proteins towards different plasma membrane domains encompasses apolar secretion of newly synthesized PINs followed by endocytosis and recycling back to the plasma membrane (PM) in a polarized manner (Dhonukshe et al. 2008). The reversible phosphorylation of PIN proteins by the serine/threonine protein kinase PINOID (PID) and protein phosphatase 2A (PP2A) is important for the decision about apical or basal delivery of PIN proteins (Friml et al. 2004; Michniewicz et al. 2007).

The clathrin-dependent endocytosis is operational in plants and constitutes the predominant pathway for the internalization of numerous PM-resident proteins including PIN auxin efflux carriers (Dhonukshe et al. 2007; Kitakura et al. 2011). The *Arabidopsis* protein GNOM is a brefeldin A (BFA)-sensitive ARF-GEF that is required for the proper polar localization of PIN1 (Geldner et al. 2003). The apical and basal PIN targeting pathways are interconnected but molecularly distinct by means of ARF-GEF vesicle-trafficking regulators. The *Arabidopsis* ortholog of the yeast and mammalian vacuolar protein sorting 29 (VPS29), a member of the retromer complex, is required for endosome homeostasis, PIN protein cycling, and dynamic PIN1 repolarization (Jaillais et al. 2007). The SORTING NEXIN 1 (AtSNX1) retromer complex acts to retrieve PIN2 proteins from a late/pre-vacuolar compartment back to the recycling pathways (Jaillais et al. 2006; Kleine-Vehn et al. 2008).

3 Hormonal Control of PAT

The PAT-dependent auxin gradients mediate many aspects of plant growth and development. A large body of evidence shows that different plant hormones regulate plant growth and development partially by modulating PAT, in particular, pointing to the PIN proteins.

3.1 Auxin-Mediated Regulation of PAT

Auxin itself positively feeds back on PIN expression in a time- and concentration-dependent manner. Even when the protein synthesis was inhibited by cycloheximide, auxin effects on the expression of PIN still occur, demonstrating that the auxin-dependent PIN upregulation does not require *de novo* synthesis of any factors. Higher auxin concentrations, besides modulating *PIN* expression, posttranscriptionally downregulate the abundance of specific PIN proteins (Vieten et al. 2005). Moreover, local auxin application or accumulation during *de novo* organ formation

leads to rearrangements in the subcellular polar localization of PIN proteins (Sauer et al. 2006). This auxin effect on PIN polarity is cell specific, does not depend on PIN transcription, and involves the Aux/IAA-ARF (indole-3-acetic acid-auxin response factor) signaling pathway (Sauer et al. 2006).

Auxin can also inhibit PIN endocytosis and promote its PM localization, which is specific to biologically active auxins and requires activity of the Calossin-like protein BIG (Paciorek et al. 2005). By inhibiting the internalization step of PIN constitutive cycling, auxin increases levels of PINs at the PM, providing a mechanism for the feedback regulation of PAT (Paciorek et al. 2005). Pan et al. reported that the $SCF^{TIR1/AFB}$-dependent processes are involved in auxin regulation of endocytosis, recycling, and PM accumulation of the auxin efflux transporter PIN2 in *Arabidopsis thaliana* (Pan et al. 2009). Another recent report (Robert et al. 2010) showed that auxin signaling mediated by the auxin receptor AUXIN-BINDING PROTEIN 1 (ABP1) inhibits the clathrin-mediated internalization of PIN proteins. ABP1 acts as a positive factor in clathrin recruitment to the PM, thereby promoting endocytosis (Robert et al. 2010). Auxin binding to ABP1 interferes with this action and leads to the inhibition of clathrin-mediated endocytosis (Robert et al. 2010). Thus, ABP1 mediates a non-transcriptional auxin signaling that regulates the evolutionarily conserved process of clathrin-mediated endocytosis and suggests that this signaling may be essential for the developmentally important feedback of auxin on its own transport (Robert et al. 2010).

3.2 Cytokinin-Mediated Regulation of PAT

It was reported that exogenous cytokinin (CK) exhibits a negative effect on the transcription of several PIN efflux carriers (Laplaze et al. 2007). Significantly, the B-type response regulators (ARRs) can directly activate the expression of *SHY2/IAA3*, a negative regulator of auxin responses, leading to downregulation of *PIN* transcription in the root meristem (Dello Ioio et al. 2008).

A new study has recently shown that cytokinin regulates endocytic recycling of PIN1 by redirecting it for lytic degradation in vacuoles (Marhavý et al. 2011). Stimulation of the lytic PIN1 degradation is a specific mechanism to rapidly modulate the auxin distribution in cytokinin-mediated developmental processes. In another recent study, multiple *arr* mutants, which have a reduced root apical meristem (RAM) phenotype, were found to have reduced PIN protein levels, but not decreased *PIN* transcript levels (Zhang et al. 2011). In contrast, disruption of type-A *Arabidopsis* ARRs, which are negative regulators of cytokinin signaling, alters the levels of PIN proteins and results in increased sensitivity to N-1-naphthylphthalamic acid (NPA), an inhibitor of PAT (Zhang et al. 2011). Cytokinin, acting through the type-A ARRs, alters the level of several PIN efflux carriers and thus regulates the distribution of auxin within the root tip.

3.3 Gibberellin-Mediated Regulation of PAT

A previous study in *Populus* wood showed that gibberellin (GA) stimulates PAT and has a common transcriptome with auxin (Björklund et al. 2007). By feeding isotope-labeled IAA, the experiment showed that GA indeed increases auxin levels in the stem by stimulating PAT (Björklund et al. 2007). A recent study in *Arabidopsis* showed that gibberellin regulates PIN abundance and is required for auxin transport-dependent growth and development. PAT is reduced in the inflorescences of *Arabidopsis* mutants defective in GA biosynthesis and signaling. This reduced PAT correlates with a reduction in the PIN abundance in GA-deficient plants and that PIN protein levels recovered to the wild-type levels following GA treatment. The regulation of PIN protein levels cannot be explained by a transcriptional regulation of the *PIN* genes but that GA deficiency promotes, at least in the case of PIN2, the targeting of PIN proteins for vacuolar degradation (Willige et al. 2011). Thus, GA-dependent modulation of PIN turnover may be causative for the differential growth responses.

3.4 Brassinosteroid-Mediated Regulation of PAT

Brassinosteroids (BRs) are important plant growth regulators in multiple developmental processes. Previous reports have shown that BR treatment enhances auxin-related responses (Bao et al. 2004; Nakamura et al. 2003). Recent study showed that BRs stimulate PAT capacities and modify the distribution of endogenous auxin (Li et al. 2005). In plants treated with BR or defective in BR biosynthesis or signaling, the transcription of *PIN* was differentially regulated (Li et al. 2005). In addition, BRs enhance plant tropistic responses by promoting the accumulation of the PIN2 protein from the root tip to the elongation zone and stimulating the expression and dispersed localization of ROP2 during tropistic responses (Li et al. 2005).

3.5 Strigolactone-Mediated Regulation of PAT

During the last century, two key hypotheses have been proposed to explain apical dominance in plants: auxin promotes the production of a second messenger that moves up into buds to repress their outgrowth and auxin saturation in the stem inhibits auxin transport from buds, thereby inhibiting bud outgrowth (Crawford et al. 2010). Strigolactones (SLs), or their derivatives, were recently demonstrated to act as endogenous shoot branching inhibitors: auxin moving down the main stem inhibits branch activity by preventing the establishment of auxin transport out of axillary branches and that SLs act by dampening PAT, thus enhancing competition between branches (Crawford et al. 2010).

3.6 Jasmonate-Mediated Regulation of PAT

Previously, we have shown that jasmonate (JA) upregulates auxin biosynthesis and represses polar auxin transport during *Arabidopsis* lateral root formation and gravitropic growth (Sun et al. 2009). JA displays two distinct aspects of CORONATINE INSENSITIVE 1 (COI1)- and AUXIN RESISTANT 1 (AXR1)-dependent effects on PIN2 subcellular distribution: at lower concentration, JA inhibits PIN2 endocytosis, whereas, at higher concentration, JA reduces PIN2 accumulation in the PM. Mutations of *ASA1* (*ANTHRANILATE SYNTHASE a1*) and the *TIR1/AFBs* auxin receptor genes impair the inhibitory effect of JA on PIN2 endocytosis, suggesting that a lower concentration of JA inhibits PIN2 endocytosis through interaction with the auxin pathway. In contrast, mutations of *ASA1* and the *TIR1/AFBs* auxin receptor genes enhance, rather than impair, the reduction effect of JA on the PM accumulation of PIN2, suggesting that this action of JA is independent of the auxin pathway. In addition to the JA effects on PIN2 endocytosis and PM residence, JA also alters lateral auxin redistribution in response to gravistimulation and therefore impairs the root gravitropic response (Sun et al. 2011). Our studies highlight the importance of JA–auxin interaction in the coordination of plant growth and the adaptation response.

3.7 ABA-Mediated Regulation of PAT

Lateral root (LR) formation is promoted by auxin and inhibited by cytokinin and abscisic acid (ABA). A recent study showed that mutation of *ABSCISIC ACID INSENSITIVE4* (*ABI4*), which encodes an ABA-regulated AP2 domain transcription factor, results in an increased number of LRs. Expression of the auxin-efflux carrier protein PIN1 is reduced in the *ABI4* overexpressors, enhanced in the *abi4* mutants, and is less sensitive to inhibition by CK and ABA in the *abi4* mutants than in the wild-type plants. Transport levels of exogenously applied auxin were elevated in the *abi4* mutants and reduced in the *ABI4* overexpressors. Therefore, ABI4 mediates ABA and CK inhibition of LR formation via reduction of PAT (Shkolnik-Inbar and Bar-Zvi 2010).

3.8 Ethylene-Mediated Regulation of PAT

Root growth is controlled by the coordinated action of several phytohormones, including auxin and ethylene. Recent studies showed that the effect of ethylene on root growth is largely mediated by the regulation of the auxin biosynthesis and transport-dependent local auxin distribution (Ruzicka et al. 2007; Stepanova et al. 2007; Swarup et al. 2007). Ethylene stimulates auxin biosynthesis and basipetal

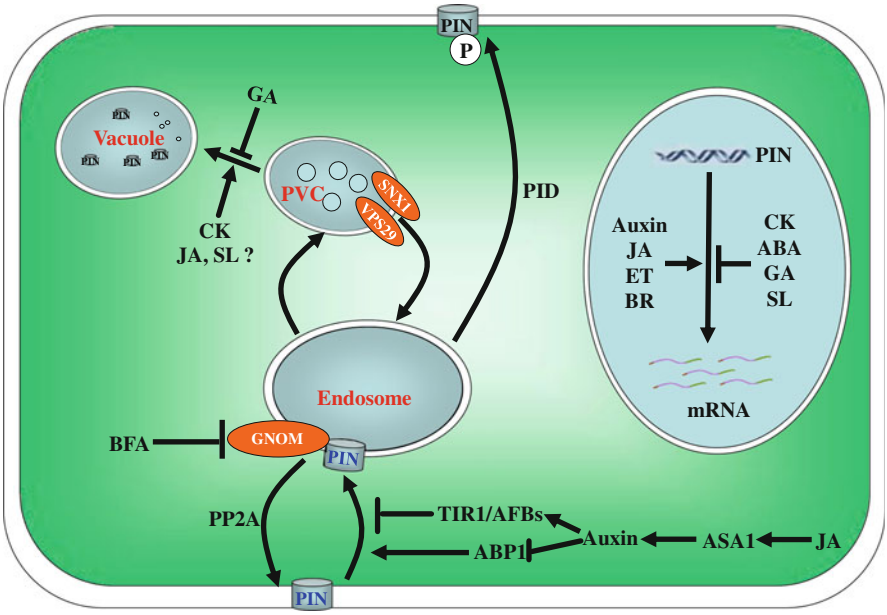

Fig. 1 Hormonal regulation of PIN proteins. Generally, hormones regulate PIN proteins at both transcriptional and posttranscriptional levels. Transcriptionally, auxin, JA, ET, and BR have been demonstrated to have positive effects on the expression of *PINs*, while CK, ABA, GA, and SL have negative effects. At the posttranscriptional level, PIN proteins cycle continuously between endosomal compartments and the plasma membrane. The endocytosis occurs in a clathrin-dependent manner, while the exocytotic step requires the activity of GNOM, an ADP-ribosylation factor GTPase guanine nucleotide exchange factor (ARF-GEF). The reversible phosphorylation of PIN proteins by the PINOID kinase (PID) and protein phosphatase 2A (PP2A) is important for the decision about apical or basal targeting of PIN proteins. Auxin inhibits PIN endocytosis through TIR1/AFBs auxin signaling pathways or affecting the ABP function in endocytosis. Lower concentrations of JA also inhibit endocytosis of PIN proteins through upregulation of ASA1-dependent auxin biosynthesis. CK and GA have contrasting effects on the PIN abundance. CK regulates endocytic recycling of PIN1 by redirecting it for lytic degradation in vacuoles. GA deficiency promotes, at least in the case of PIN2, the targeting of PIN proteins for vacuolar degradation. JA and SL reduce the PIN protein abundance at the plasma membrane posttranscriptionally through the unknown mechanisms. *JA* jasmonates, *ET* ethylene, *BRs* brassinosteroids, *CK* cytokinin, *ABA* abscisic acid, *GA* gibberellins, *SLs* strigolactones, *PVC* pre-vacuolar compartments

auxin transport towards the elongation zone, where it activates a local auxin response leading to inhibition of cell elongation. Consistently, in mutants affected in auxin biosynthesis (*wei2* and *wei7*) or basipetal auxin transport (*pin2* and *aux1*), ethylene cannot activate the auxin response nor regulate the root growth. In addition, ethylene modulates the transcription of several components of the auxin transport machinery (Ruzicka et al. 2007). Thus, ethylene achieves a local activation of the auxin signaling pathway and regulates root growth by both stimulating the auxin biosynthesis and modulating the auxin transport machinery.

4 Conclusions

The principal auxin transporters PIN can be regulated at multilevels, i.e., transcription, polarity, endocytosis, exocytosis, recycling, transcytosis, and degradation. The complexity and dynamic turnover of PIN provide multiple interaction nodes between auxin and other hormones, furnishing plants with great developmental flexibility and environmental plasticity. As discussed here, present studies have demonstrated that hormones can regulate PIN proteins at both transcriptional and posttranscriptional levels (Fig. 1). However, most of these studies are relatively limited to the alteration of PIN transcription or plasma membrane abundance, and the detailed cellular and molecular mechanisms underlying how hormones regulate PIN proteins remain largely unclear.

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Auxin Transport and Signaling in Leaf Vascular Patterning

Enrico Scarpella and Thomas Berleth

Abstract Reticulate tissue systems pervade most multicellular organisms, and the principles controlling the formation of these cellular networks have long been object of interest of biologists and mathematicians. In particular, the beautiful and varied networks of veins in plant leaves have intrigued mankind since antiquity. Vascular cells are aligned with one another within continuous veins that reproducibly supply all areas of the leaf, but the precise path of vein formation is highly variable. Recent advances suggest a self-organizing control mechanism in which an apical-basal continuous flow of signal could establish a basic coordinate system for body-axis and vascular-strand formation, and account for both the reproducible and the variable features of leaf vein patterns.

1 Introduction

Most multicellular organisms form tissue networks for transport function. In many animals, the patterns of these networks are stereotyped, suggesting tight positional controls (Lu and Werb 2008). By contrast, the patterns of vein networks of plant leaves are both reproducible and variable: reproducible because all parts of the leaf are supplied by veins; variable because the exact sites of vein formation are unpredictable (Sachs 1989) (Fig. 1). These observations argue against a tight specification of leaf vein patterns and suggest a self-organizing control mechanism that functionally integrates vein formation with leaf development (Berleth et al. 2000). Evidence

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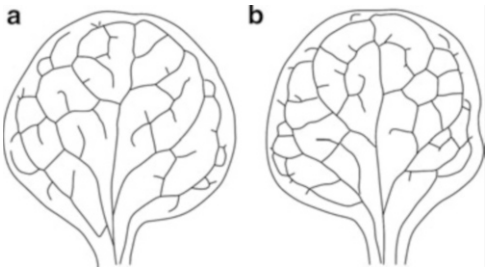

Fig. 1 Reproducibility and variability of leaf vein patterns. Authentic vein patterns of Arabidopsis leaves illustrate reproducibility and variability. Arabidopsis leaves have pinnate vein patterns in which veins of successively higher order branch off veins of lower order starting from a single first-order vein, also called midvein. Note that second-order veins reproducibly form closed loops and that third-order veins are more frequently connected to other veins at both ends, while fourth-order veins often end freely in the lamina. Though these features are reproducible, the precise locations of the veins are unpredictable. Thus, the patterning mechanism has to ensure continuity and certain network features without defining positions of veins (Drawn from: Donner et al. (2009) Regulation of preprocambial cell state acquisition by auxin signaling in Arabidopsis leaves. Development 136:3235–3246 with kind permission from The Company of Biologists)

for and objections against such mechanism are the focus of this chapter; other aspects of vascular development have been comprehensively reviewed in (Aloni 1987, 2001; Baucher et al. 2007; Bollhoner et al. 2012; Cano-Delgado et al. 2010; Carlsbecker and Helariutta 2005; Dejardin et al. 2010; Dengler and Kang 2001; Dengler 2001, 2006; Dettmer et al. 2009; Du and Groover 2010; Elo et al. 2009; Fukuda 2004; Fukuda et al. 2007; Hirakawa et al. 2010, 2011; Jung and Park 2007; Lehesranta et al. 2010; Moreno-Risueno et al. 2012; Nelson and Dengler 1997; Oda and Fukuda 2012; Ohashi-Ito and Fukuda 2010; Risopatron et al. 2010; Rolland-Lagan 2008; Samuels et al. 2006; Sieburth and Deyholos 2006; Spicer and Groover 2010; Turner and Sieburth 2002; Turner et al. 2007; van Bel et al. 2002; Ye 2002; Ye et al. 2002; Zhang et al. 2011; Zhong et al. 2010; Zhou et al. 2011).

2 Induction of Vascular Strand Formation in Mature Organs

Evidence for a mechanism that integrates vascular strand formation with organismal signals was first derived from experiments in which auxin had locally been applied to plant tissues (Sachs 1981) (Fig. 2a). In fact, not only does auxin promote differentiation of vascular cells—a property not unique to auxin (e.g., Aloni 1987; Cano-Delgado et al. 2010; Fukuda 2004; Vera-Sirera et al. 2010)—but it coordinates the differentiation of these cells along continuous lines to form vascular strands, a complex tissue response characterized by a number of unique properties (Berleth et al. 2000; Sachs 1981). First, the auxin-induced vascular-differentiation response is local, as it is initiated at the site of auxin application; second, it is polar, as it is

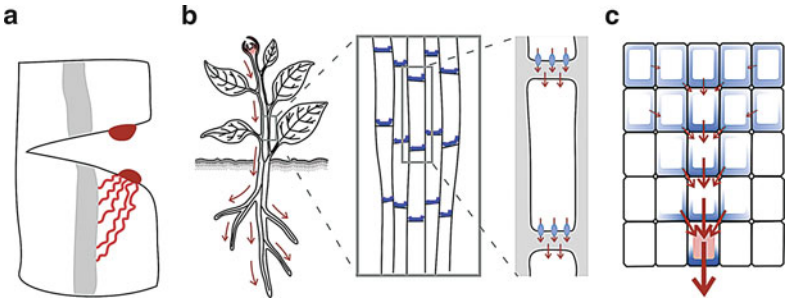

Fig. 2 Auxin-induced vascular-strand formation and polar auxin transport. (**a**) Induction of vascular strand formation by auxin application to a polarized piece of tissue. Auxin transport in this tissue occurs from top to bottom (shoot-to-root direction). The application of a droplet of transported auxin (*brown*), such as indole-3-acetic acid or 1-naphthaleneacetic acid, will generate vascular strands connecting the auxin source with preexisting vasculature (*gray*) further down towards the root pole. Note that an auxin droplet applied to the opposite flank of a cut will not generate a similar response. (**b**) Main features of auxin transport in adult plants. *Left panel*: major auxin transport occurs in the shoot-to-root direction (*arrows*), originating from—among other sources—young leaf and flower primordia (*brown*). *Middle panel*: within tissues, auxin is transported in a cell-to-cell fashion. *Middle* and *right panels*: specialized proteins facilitating the exit of auxin from a cell (*blue*) are primarily located towards the basal part of the plasma membrane. (**c**) Gradual acquisition of auxin transport polarity in a field of 25 cells. *Blue shading* indicates the distribution of plasma-membrane-bound auxin-efflux proteins, whose expression and basal subcellular localization is itself positively influenced by the auxin conductivity of a given cells. With this positive feedback in place, auxin efflux towards a common polarity in all cells becomes reinforced, and polar auxin transport becomes increasingly concentrated (*arrows* of increasing strength) to narrow files of highly conductive vascular precursor cells (*brown*) (Redrawn from Scarpella and Helariutta (2010) Vascular pattern formation in plants. Current Topics in Developmental Biology 91: 221–265, ©2010, with kind permission from Elsevier)

oriented away from the auxin source and towards the preexisting vasculature basal to the site of auxin application; third, it is continuous, as it generates uninterrupted files of vascular cells; fourth, it is spatially constrained, as it generates discrete bundles of vascular cell files rather than clouds of vascular cells around the application site; fifth, it is dependent on polar auxin transport, as it requires polarly transported auxins and is obstructed by inhibitors of polar auxin transport, suggesting that the mechanism underlying the auxin-induced vascular-differentiation response recruits the molecular machinery that polarly transports auxin. In the plant, auxin is primarily synthesized in immature apical organs, such as primordia of leaves and flowers (Normanly 2012; Zhao 2010), and is transported basally to the roots through the vascular strands (Michniewicz et al. 2007) (Fig. 2b). The apical–basal polarity of auxin transport is thought to derive from the localization of auxin efflux proteins at the basal end of auxin-transporting cells (Raven 1975; Rubery and Sheldrake 1974) (Fig. 2b). In fact, as a weak acid, auxin is negatively charged at the neutral intracellular pH and can only leave the cell through the action of specialized efflux proteins.

If the vascular strands that form in response to auxin application do so by recruiting the molecular machinery that polarly transports auxin, how can the cellular mechanism of polar auxin transport account for the unique properties of the auxin-induced vascular-differentiation response? To answer this question, the "auxin canalization hypothesis" proposed a positive feedback between auxin movement through a cell and localization of auxin efflux proteins to the site where auxin leaves the cell (Sachs 1981, 1991) (Fig. 2c). Auxin applied to plant tissues would initially move by diffusion with no preferred orientation, and auxin efflux proteins would be randomly distributed. By efficiently transporting auxin along the original apical–basal polarity of the tissue, the preexisting vasculature would act as auxin sink and orient auxin movement in neighboring cells, polarizing the localization of auxin efflux proteins in these cells. The initiation of polar auxin transport in these cells would be gradually enhanced by positive feedback between auxin transport and efflux protein localization. By draining auxin increasingly more efficiently and polarly, these cells would induce polar auxin transport and polarization of efflux protein localization in the cells above them and inhibit the same processes in their lateral neighbors. Reiteration of these events would result in auxin being preferentially transported through limited cell files which would eventually differentiate into vascular strands. During this process, chance localization of efflux proteins would be stabilized by positive feedback between auxin transport and efflux protein localization, resulting in random elements in the course of the selected cell files and deviations from the shortest routes for auxin transport. These predictions—not all necessarily intuitive—have been rigorously tested and supported by computer simulation of different mathematical models (reviewed in Garnett et al. 2010; Krupinski and Jonsson 2010; Santos et al. 2010; Smith and Bayer 2009; Wabnik et al. 2011).

The subcellular localization of the five plasma-membrane proteins of the PIN-FORMED (PIN) family of Arabidopsis marks the presumed auxin-efflux side of cells (Petrasek and Friml 2009). Because subcellular localization of PIN proteins is instrumental in defining the polarity of auxin transport (Wisniewska et al. 2006), such polarity can be inferred from the asymmetric subcellular localization of PIN proteins. Consistent with predictions of the auxin canalization hypothesis, local application of auxin to plant tissues activates PIN1 expression in broad domains that connect the applied auxin to the preexisting vasculature (Sauer et al. 2006). Within these broad domains, PIN1 localization is initially nonpolar. However, broad PIN1 expression domains eventually narrow to files of vascular cell precursors in which PIN1 polarity indicates auxin transport away from the site of auxin application and towards the preexisting vasculature. The molecular events underlying polarization of PIN1 localization are unclear. However, because auxin inhibits PIN1 endocytosis (Paciorek et al. 2005), PIN1 remains—or remains longer—where auxin leaves the cells, which could account for the positive feedback between auxin transport and localization of auxin efflux proteins postulated by the canalization hypothesis. These studies have not only added molecular details to conceptual models of auxin-induced vascular differentiation but have captured aspects of the process not necessarily implied by the original hypothesis. For example, the gradual

cessation of expression of efflux proteins in cells not selected for vascular differentiation, cessation which suggests feedback between auxin transport and efflux protein expression. The mechanistic basis for this feedback is unknown, but responsiveness of PIN expression to auxin levels (Heisler et al. 2005; Scarpella et al. 2006; Vieten et al. 2005) could be at its core.

3 Vascular Strand Formation in Embryo Development

3.1 A Short History of Early Embryonic Polarity

Vascular and whole-body architecture have a lot in common. In fact, prior to the emergence of leaves, the architecture of land plants can be described as two ramified systems of branching cylindrical organs, one above and one below ground, with vasculature at the core of each cylinder (Zimmermann 1930, 1952) (Fig. 3a). Although there are functional deviations from this rule, especially in leaves or organs undergoing secondary growth, the overarching scheme, particularly evident in embryos and newly emerging organs, seems to be that a central vascular primordium is established simultaneously with the emergence of a new organ. Likewise, when a new body axis is established in an early globular embryo, this is immediately associated with the formation of vascular precursor cells, whose axes are aligned with the new body axis (Scheres et al. 1995) (Fig. 3b).

In normal angiosperm development, polarities are passed on over generations in an uninterrupted sequence. The embryo-sac gametophyte produced by the mother plants is polar, and the egg cell within it, localized at the micropylar pole, is elongated and internally polarized (Mansfield et al. 1991). After fertilization, the first division of the zygote separates an apical cell, comprising the cytoplasmic part, from a somewhat larger basal cell, which is derived from the more vacuolated part of the zygote (Mansfield and Briarty 1991). Nearly all the globular embryo is derived from the apical cell, while most of the basal cell descendents will make up a filamentous suspensor. Although PIN protein localizations and mutant studies suggest that even these very early divisions seem to involve directional auxin transport, none of these polarities are stringently required for the establishment of polarity in the embryo proper. Occasional multiple, randomly polarized embryos along a single suspensor, for example, indicate that polarity can be established de novo in a very small embryonic cell mass and is then very stably maintained throughout all later stages of embryogenesis (Schwartz et al. 1994; Vernon and Meinke 1994; Zhang and Somerville 1997). Thus, there is no functional requirement for passing on polarities from one generation to the next, although this is the preferred mode of events.

While parallel divisions of suspensor cells keep elongating the single cell file of the suspensor, 90° shifts of division planes among descendents of the apical cell soon create a ball of eight cells (octant embryo) (Mansfield and Briarty 1991).

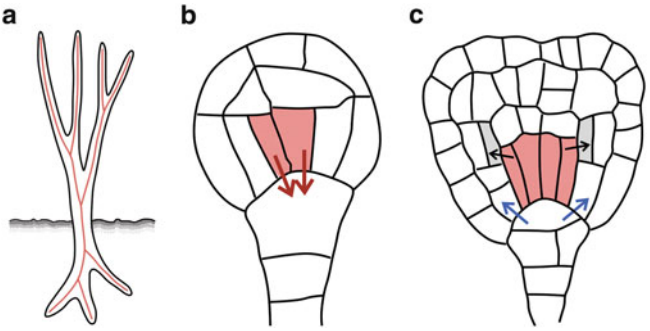

Fig. 3 Vascular strand formation in embryos. (**a**) Plants with broad leaves are thought to have evolved from simple branching systems. (**b**) Formation of a central procambium in the globular embryo. While the embryo is still perfectly globular, divisions of subepidermal cells are already occurring parallel or perpendicular to the apical–basal axis of the embryo. Further longitudinal divisions of the most central cells in the basal tier of the embryo lead to the formation of narrow, procambial cells (*brown*). *Arrows*, auxin transport from the procambium to the uppermost cell of the suspensor, where auxin is thought to accumulate. (**c**) By the triangular (early-heart) stage of embryogenesis, the procambial core influences patterning throughout the embryo. First, procambium-derived signals (*black arrows*) trigger the generation of a multilayered ground tissue (inner layer, *gray*). Second, auxin and possibly other signaling at the basal end of the procambial core specify the identity of the underlying lens-shaped cell—the hypophysis—from which the center of the embryonic root meristem will be derived and which in turn promotes stem-cell properties in the surrounding cells (*blue arrows*). Finally, the central procambium might be required as an efflux channel for a cotyledon-initiation-site-defining mechanism analogous to the one described in Fig. 4a (Redrawn Scarpella and Helariutta (2010) Vascular pattern formation in plants. Current Topics in Developmental Biology 91: 221–265, ©2010, with kind permission from Elsevier)

The next divisions occur again at maximum angle relative to the preceding ones, now at 45°, thereby separating a newly generated outer cell layer (protoderm, i.e. embryonic epidermis) from eight inner cells (dermatogen-stage embryo). It is in the divisions of these inner cells that the future axis of the embryo becomes evident. With all the new walls between the inner cells inserted along a single axis, the embryo is no longer isotropic but comprised of concentric cylinders, although its overall shape is still spherical (Scheres et al. 1995) (Fig. 3b). At the molecular level, this earliest sign of cellular axis formation is associated with the polar localization of PIN1 at the basal ends of the inner cells (Steinmann et al. 1999). Concentration of PIN1 seems to be particularly high in the innermost cells of the basal tier in embryos at this stage, which is the earliest indication of their specification to prospective vascular cells and the first subdivision of cell fates among the inner cells of the early embryo. The following divisions will increase the number of concentric cell layers, elongate the individual cell files, and elaborate the shoot and root end of the embryo axis (Mansfield and Briarty 1991; Scheres et al. 1995). However, all those future divisions will just extend and refine the axial pattern set up at this stage, using the early established axis as a positional reference (Scheres et al. 1994).

3.2 *From a Polar Axis to a Seedling Pattern*

The angiosperm seedling pattern can be formalized as ordered sequences of pattern elements: the shoot meristem, cotyledons, hypocotyl, and radicle along the apical-basal axis; vascular tissue, ground tissue, and epidermis along a central-peripheral axis (Capron et al. 2009). All these pattern elements arise in a remarkably short sequence of divisions after the establishment of the basic body axis and are recognizable already by the heart stage of embryogenesis (Fig. 3c). The proper positioning of all the apical–basal pattern elements depends on auxin transport, while, for example, the specification of the epidermis seems not to be affected even in embryos in which auxin transport is pharmacologically or genetically highly perturbed (Hadfi et al. 1998; Liu et al. 1993; Takada and Jurgens 2007). Moreover, dynamic patterns of polar PIN-protein localizations, data from experimental and genetic interference, and theoretical modeling provide continuously refined concepts for how feedback regulation of auxin transport could trigger a cascade of genetic interactions to put all major pattern elements in place (summarized in Capron et al. 2009). First, polar transport of auxin would not be expected to be uniform in all cell layers but subject to self-enhancing canalization in the core of the axis, which is consistent with particularly strong PIN1 expression in the innermost cells of the globular embryo (Steinmann et al. 1999) (Fig. 3b, arrows). Second, an auxin maximum at the bottom end of these files is observed in the embryo and in postembryonic root meristems and is instrumental for the specification of the root meristem center, which in turn induces stem-cell properties in the surrounding cells (Blilou et al. 2005; Friml et al. 2003; Sabatini et al. 1999; van den Berg et al. 1997; Weijers et al. 2005) (Fig. 3c, blue arrows). Mathematical modeling has illustrated how continuously maintained transport routes and associated robust cellular patterns in the root meristem can be maintained over extended periods of time (Grieneisen et al. 2007). Third, not only apical–basal patterning uses the central vascular core as a positional reference: also the radial pattern of ground-tissue cell layers is specified by an inductive signal from the center (Helariutta et al. 2000; Nakajima et al. 2001) (Fig. 3c, black arrows). The GRAS transcription factor SHORT ROOT is expressed in the vasculature and then moved to the surrounding ground tissue, where it induces periclinal cell divisions and acquisition of endodermal cell fate by cells of the inner of the two newly generated tissue layers. Fourth, lateral organ formation on shoot meristems has been shown to depend on initially randomly localized auxin self-regulated convergence in the epidermis of the peripheral zone of the meristem (Benkova et al. 2003; Heisler et al. 2005; Reinhardt et al. 2003). Mathematical modeling illustrates how the focusing of auxin leads to auxin depletion in surrounding epidermal areas and how this effective lateral-inhibition process can generate robust and natural phyllotactic patterns (Jonsson et al. 2006; Smith et al. 2006) (Fig. 4a). The first step in this auxin-transport-driven emergence of a phyllotactic pattern is the near-simultaneous generation of two lateral organs at opposite positions. Clonal analysis and PIN1 expression studies in early embryos suggest that the positions of the two cotyledons are established in

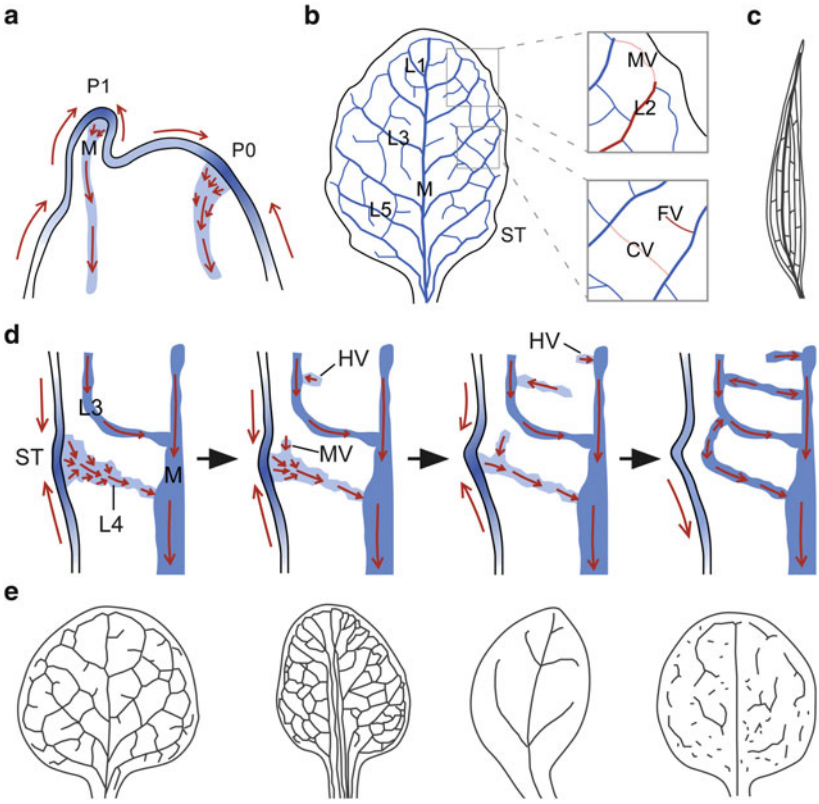

Fig. 4 Vein formation in leaf development. (**a**) Selection of leaf initiation sites (*blue gradient*, auxin concentrations; *arrows*, directions of auxin transport). A model based on the distribution of auxin transport proteins proposes that auxin converges in discrete spots in the epidermis of the peripheral zone of a shoot meristem, from where it seems to become internalized and drained basally by emerging vascular strands (*light blue*) in subepidermal tissues. As this mechanism depletes of auxin the epidermis surrounding a point of auxin convergence, new convergence points can only arise at maximum distance within the peripheral zone of the meristem. *P0* and *P1*, successive stages of leaf primordium development. (**b**) Types of Arabidopsis leaf veins. M, midvein (or first-order vein); L1–L5, lateral veins (or second-order veins); MV, marginal veins; CV, connected veins: veins attached at both ends to preexisting vasculature; FV, freely ending veins: veins attached to preexisting veins only at one end, while the other end terminates freely in the lamina; ST, serration tip. (**c**) Species-specific cues constrain the variety of possible vein patterns. Unlike dicot leaves, veins are arranged in a striate pattern in monocot leaves. (**d**) Successive stages of vein specification. PIN1 marker expression and procambium formation patterns suggest that vein patterns are the result of a gradual selection process that may initially involve all leaf cells. First- and second-order veins are initiated as broader PIN1 expression domains (*light blue*) beneath PIN1-expressing, presumed auxin-convergence points in the leaf margin. Those initially broad PIN1-expression domains narrow in an auxin-transport-dependent manner, with PIN1 subcellular polarities indicating auxin transport (*arrows*) towards preexisting vasculature. Sustained PIN1 expression in narrow domains is then congruent with the formation of procambium (*blue*), which is thought to be the ultimate readout of the selection process. Formation of higher-order veins (*HV* and *MV*) is preceded by similar PIN1-expression dynamics, except that here the initial domains gradually extend from the flanks of preexisting veins into the leaf lamina. Occasionally, this leads to extension towards preexisting

this way (Benkova et al. 2003; Friml et al. 2003; Steinmann et al. 1999; Woodrick et al. 2000). The patterning role of auxin transport in this process may not be restricted to the epidermis, as PIN1 expression studies suggest that auxin is internalized and drained basally through incipient vascular cell files (Benkova et al. 2003; Friml et al. 2003; Steinmann et al. 1999) (Fig. 4a). This, in turn, defines another organizing role of the central vascular core in the early embryo. In conclusion, there is increasingly detailed evidence linking major embryonic-patterning processes to the central vascular core, which occur at stages before any of those central cells have acquired differentiation characteristics of any functional conductive tissue. They seem to have acquired their cellular identity solely through their particularly high auxin-conductance and anchor the surrounding patterning events either as auxin conduits or through specific genetic programs that they have acquired as part of their early vascular identity.

3.3 Genes in Embryo Axis Formation

The genetics of embryo pattern formation goes far beyond auxin-transport-driven processes and has been summarized in a number of excellent recent reviews (De Smet et al. 2010; Jeong et al. 2011, 2012; Lau et al. 2010; Moller and Weijers 2009; Nodine et al. 2011; Peris et al. 2010; Zhang and Laux 2011). Nevertheless, it is fair to say that embryo polarity distortions can be genetically separated from other types of mutant defects and that their exploration has led to the identification of crucial proteins in auxin transport and auxin signal transduction. Only few members of the large and diverse group of embryo-defective mutants display massive axis defects, and in those cases the affected genes have been identified as core elements in auxin-transport or auxin-signaling genes. For example, the mutant with the most dramatic defect in apical–basal polarity, *embryo defective30*/*gnom* (*emb30*/*gn*; *gn* hereafter), does not disrupt any of the redundantly acting PIN proteins but a guanosyl-exchange factor required for the activity of adenosyl-ribosylation factor (Shevell et al. 1994; Steinmann et al. 1999). This ras-type phosphatase, in turn, has a specific role in a brefeldin A-sensitive vesicle transport pathway, through which PIN proteins are reshuffled to their proper locations in the plasma membrane (Geldner et al. 2001, 2003; Steinmann et al. 1999). In *gn* mutants, PIN proteins are still being produced but are not properly transported to their cellular target sites (Geldner et al. 2003;

Fig. 4 (continued) veins at the distal end, leading to a connected vein with two outwardly-directed PIN1-polarities (*arrows in opposite directions*), which are connected by a single bipolar cell. (**e**) Schematic drawings illustrate Arabidopsis leaf vein patterns under undisturbed (*left*), auxin-transport-inhibited (*second from left*), impaired-auxin-perception—as in an *mp* mutant—(*second from right*), and instable-continuity—as in the *van3* mutant—(*right*) conditions (Redrawn from Scarpella and Helariutta (2010) Vascular pattern formation in plants. Current Topics in Developmental Biology 91: 221–265, ©2010, with kind permission from Elsevier)

Kleine-Vehn et al. 2008; Steinmann et al. 1999). Through its importance for the function of various PIN proteins, *GN* is crucial for auxin transport in all parts of the plant. Mutant embryos can lose all aspects of apical–basal polarity (Mayer et al. 1993), similar to embryos grown in the presence of very strong inhibitors of auxin transport (Friml et al. 2003; Hadfi et al. 1998; Liu et al. 1993), but mutant cells remain viable and can divide indefinitely, yet without any organogenic potential (Mayer et al. 1993). Of all intracellular transport mutants, only *gn* mutants display entirely randomly oriented vascular cells. In the most extreme *gn* mutants, the embryo is perfectly ball-shaped with no sign of apical–basal polarity (Mayer et al. 1993). The phenotype documents that vascular differentiation and strand alignment can be uncoupled, as fully differentiated vascular cells can be observed in random orientation in *gn* mutants.

Many steps in auxin signal transduction are executed by very large protein families. Because of the very high degree of functional overlap, the functions of many members of each gene family are still unknown. The predominant family of transcription factors regulating auxin responsive genes, termed *AUXIN RESPONSE FACTORS* (*ARF*s), for example, comprises 22 members, of which only a few have been genetically characterized by loss-of-function mutations (Guilfoyle and Hagen 2007). The regulation of ARF activity occurs through interaction with negative nuclear regulators of the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) protein family. Auxin regulates ARF activity indirectly by promoting the interaction of AUX/IAA proteins with the $SCF^{TIR1/AFB}$ ubiquitin ligase, which targets them for proteolysis (Mockaitis and Estelle 2008). Reporter gene expression suggests that in this way dynamic patterns of auxin distribution are translated into correspondingly changing stabilities of AUX/IAA proteins and thereby of ARF activities (Leyser 2011). Within the large family of 29 *AUX/IAA* genes, deduction of gene function from loss-of-function phenotypes is even more difficult than in the ARF family, but there are many *AUX/IAA* gain-of-function alleles with defective $SCF^{TIR1/AFB}$ targeting sequences ("degron"). In such auxin insensitive mutants, the stability of a particular AUX/IAA protein is no longer diminished by high auxin concentrations, and the activity of ARFs remains unregulated.

Embryo axis defects are most pronounced in mutants of *MONOPTEROS/ARF5* (*MP/ARF5*; *MP* hereafter) (Berleth and Jurgens 1993; Hardtke and Berleth 1998). In apparent *mp* null mutants, one or two cotyledons and a shoot meristem are being formed, but the embryo axis comprising the hypocotyl and the radicle is missing. The mutant embryos are defective already at the triangular stage: the inner cells are not oriented in parallel files along the embryonic axis, and no vascular precursor cells are discernable. Other defects, such as a missing root meristem, failed ground-tissue separation, and a highly variable number and positioning of cotyledons, are consistent with their dependence on the presence of a central vascular core (Fig. 3c). Moreover, molecular evidence implicates auxin signaling through ARFs, among them MP, in the regulation of PIN1 and the root-stem-cell-promoting APETALA2 transcription factors of the PLETHORA family (Aida et al. 2004; Steinmann et al. 1999; Weijers et al. 2006). These

data suggest that the ARF-AUX/IAA is the most crucial signaling pathway in a self-regulatory auxin-patterning process during early embryogenesis. Consistent with a cell biological link between embryo axis formation and vascular strand formation, *mp* mutants show delayed and reduced vascular development in a variety of organs throughout the life cycle (Berleth and Jurgens 1993; Przemeck et al. 1996). At least one other ARF, NON-PHOTOTROPHIC HYPOCOTYL4/ARF7 (NPH4/ARF7; NPH4 hereafter), acts redundantly with MP in axis formation (Hardtke et al. 2004). In *mp nph4* double mutants, the residual formation of cotyledons, lateral shoot organs, and vascular strands, as it is observed in *mp* mutants, is completely obstructed, and overall embryo polarity is minimal. Specific functions of other ARFs at all stages of embryogenesis, including those preceding the formation of the embryo axis, are suggested by their specific and dynamic expression patterns (Rademacher et al. 2011). Nevertheless, the near absence of an embryo axis in *mp nph4* double mutants suggests that key ARFs for the relay of auxin in the establishment of embryo axis and vasculature have already be identified. Consistent with a role of auxin in their regulation, multiple mutations affecting the substrate-recognition protein of the $SCF^{TIR1/AFB}$ complex, TIR1 and its AFB paralogs, lead to diminished embryo axis formation capacity and *mp*-like mutant phenotypes (Dharmasiri et al. 2005). What are the key AUX/IAA proteins regulating ARFs in embryo axis formation and vascular strand formation? Gain-of-function mutations and overexpression studies suggest that overabundance of at least BODENLOS/IAA12 (BDL/IAA12), IAA18, IAA20, IAA30, and IAA31 can diminish vascular strand formation (Hamann et al. 1999, 2002; Ploense et al. 2009; Sato and Yamamoto 2008). While this result does not at all exclude the involvement of other AUX/IAA proteins in cell axis formation, the negative regulatory interaction between MP and BDL is well established (Hamann et al. 2002; Hardtke et al. 2004; Weijers et al. 2006), and *BDL* is the only *AUX/IAA* gene whose gain-of-function phenotype mimics the loss-of-function phenotype of *mp*. The combined action of MP and BDL does not only affect the formation of the first vascular strand—the axis core in the early globular embryo—but also the uppermost cell of the suspensor, the hypophysis, though this cell never becomes part of the embryo axis (Weijers et al. 2006). It is possible that specifying a hypophysis cell is the crucial orienting event aligning the newly formed embryo axis with the embryo as a whole and thereby perpetuates polarity across generations.

4 Leaf Vein Patterning

4.1 Basic Features of Vein Patterns

The auxin-convergence-defined spot on the flank of a shoot meristem can give rise to: (a) another meristem and thereby a new growth axis within a ramified shoot

system (Reinhardt et al. 2000); (b) a rotationally symmetric organ like a thorn; or (c) a leaf (Benkova et al. 2003; Heisler et al. 2005; Reinhardt et al. 2003) (Fig. 4a). Leaves come in all kinds of shapes, but they are typically flat and expanded, an adaptation to their most prominent role as solar panels. Leaf laminae of sometimes enormous sizes may grow out on both sides of the middle axis, and all their cells remain connected to the stem vasculature through complex networks of hierarchically thinner vascular strands, which in leaves are also called veins (Fig. 4b).

Vein patterns in monocots and dicots differ dramatically. A typical monocot vein network is comprised of major parallel veins along the length of the leaf that are cross-connected by thinner transverse veins, together forming a relatively rigid, nearly rectangular pattern (Nelson and Dengler 1997) (Fig. 4c). Genetic determinants influencing the architecture of monocot vein patterns have been summarized in a number of reviews (e.g., Nelson 2011; Scarpella and Meijer 2004), and a certain degree of responsiveness of the pattern to auxin-transport parameters has been reported (Sakaguchi et al. 2010; Scarpella et al. 2002; Tsiantis et al. 1999). Nevertheless, it is probably fair to say that the relative contributions of auxin transport mechanisms to the patterning of the monocot vein network are still relatively poorly defined.

In dicot leaves, veins can also be categorized into hierarchical orders (Candela et al. 1999; Nelson and Dengler 1997). Here they are arranged in a reiterative branching system, starting from one or more first-order veins, or midveins, which are continuous with the stem vasculature. Veins branching off from those are called second-order or secondary veins, from which veins of successively higher orders branch off (Fig. 4b). The second-order veins—the major veins branching from the midvein into the lamina—can end freely in the periphery of the leaf lamina, giving the overall vein pattern a superficial similarity to a drainage system, resembling a main river with its tributaries. Alternatively, and more frequently, the second-order veins form closed loops and are eventually connected to other veins at both ends (Fig. 4b). The patterns of lower-order (major) veins are often characteristic for a species or for a leaf type within a given species (Dengler and Kang 2001; Klucking 1995). The more irreproducible pattern of higher-order veins also includes veins that end freely in the ground tissue ("freely ending" veins) as well as veins that are connected to other veins at both ends ("connected" veins) (Candela et al. 1999; Nelson and Dengler 1997; Scarpella et al. 2004) (Fig. 4b). Typically, veins of the highest orders have the largest percentage of freely ending veins. Vein patterns have fascinated artists, biologists, and mathematicians for centuries, and more recently interdisciplinary research between the latter two groups has led to an extremely productive exchange between mathematical modeling and ever more sophisticated observation and experimentation technology. The outcome of this interaction has been summarized and updated in a number of recent reviews, the study of which will provide the reader with many fascinating perspectives of possible mechanisms feeding into the definitions of vein pattern (Garnett et al. 2010; Krupinski and Jonsson 2010; Santos et al. 2010; Smith and Bayer 2009; Wabnik et al. 2011). In this chapter, however, we will focus on auxin-transport-related experimental observations and conclusions that are immediately derived from them.

4.2 The Origin of Vein Patterns

Leaf development is responsive to a number of external parameters. For example, after wounding or when grown under constrained conditions, the shape of a leaf can be extremely abnormal (Jost 1942; Sachs 1988, 1989). Remarkably, the path of veins is guided by cues sufficiently flexible to create functional networks in perfect adaptation to whatever the respective leaf shape is. From a developmental point of view, this is the more impressive because the paths of vascular strands are selected extremely early and from an apparently uniform layer of subepidermal ground meristem cells, which are then turned into procambial cells (Foster 1952; Pray 1955). The procambium is the meristematic tissue that gives rise to the various types of vascular cells (Esau 1943), which have to be aligned in continuous strands in order to act as conductive tissues. What ensures that ground meristem cells are selected towards procambial fate in continuous lines, and what ensures that these lines are connected to preexisting vasculature? Further, how can one reconcile the precision of the selection mechanism with its obvious adaptive capacity?

With some exceptions, the mathematical models defining suitable self-organizing space-filling mechanism for network formation can very broadly be subdivided into two major categories. In distance-based models, cells communicate their relative positions and distances from reference points through the exchange of hypothetical molecules in such ways that lines of adjacent cells are preferentially selected (Meinhardt 1982; Meinhardt and Gierer in press). These models are attractive because they can explain the formation of closed networks just as easily as that of open ramified systems, but they are not strictly dependent on continuity. However, such models leave open why there is near-perfect continuity of vascular strands already at the procambial stage (Mattsson et al. 1999; Sawchuk et al. 2007; Scarpella et al. 2004). In signal-flow models, a critical signal substance is passed on through the selected cells themselves, and thus interrupted veins should be inherently impossible (Sachs 1981, 1989, 1991) (Fig. 2c). Flow models explain continuity at all stages but have difficulties explaining the specification of closed networks. Of course, in reality, nobody expects a single concept to cover all features of all vein patterns. Instead, the merit in model formalization lies in disentangling which set of features within a complex pattern can be attributed to a formulated mechanism, and for which other unexplained properties other principles still need to be found.

Genetic and experimental studies have identified various influences on leaf vein patterning over the past decades. Within this context, several lines of research have generated a comparatively consistent picture of the role of auxin transport within the patterning process. First, auxin-triggered regenerative vascular-strand formation and its mathematical interpretation in variations of the canalization hypothesis have provided a stunning diversity of examples in which vascularization responses fit into a predictable scheme. Second, responses of leaf vein patterns to genetically or experimentally induced changes of auxin-transport or auxin-response properties within the developing tissue have been consistent with predictions of auxin flow

models. Third, visualization of vein development using molecular cell-state markers has revealed, among other things, the derivation of connected veins from freely ending veins. Fourth, the visualization of auxin flow routes and directions in normal and perturbed vein development has provided possible explanations for the generation of connected veins through a flow-based mechanism. Fifth, the auxin-flow-based mechanisms for dicot vein development have become part of a larger concept of auxin-flow patterning.

4.3 Auxin Application Inducing Vascular Strands

As pointed out in Sect. 1, the mere induction of a vascular strand by local auxin application reveals a number of remarkable features, which together are strongly suggestive of a flow mechanism that, as Tsvi Sachs put cautiously, "could not be experimentally separated from auxin" (Sachs 1981) (Fig. 2a, c). Experimental evidence in support of a flow-based self-organizing mechanism that would inherently restrict auxin transport to preferred narrow routes was obtained by Sachs and coworkers and by other groups in a number of species and organs as well as under a large diversity of experimental challenges (Flaishman et al. 2003; Jacobs 1952; Jost 1942; Roberts 1960; Roberts and Fosket 1962; Sachs 1981). These included the obstruction of vein formation by auxin transport inhibitor, the convergence of vascular strands induced by appropriately spaced auxin sources, the formation of oblique routes of auxin transport around obstacles and incisions, and many others. In 2006, a number of those experiments were repeated in plants in which PIN proteins were visualized by immunohistochemistry, confirming the dynamics of auxin-transport routes and polarities (Sauer et al. 2006). In the same year, veins were also induced by auxin application to leaf primordia, and a cellular PIN1 marker visualized the basal polarity and the narrowing, self-restricting properties of PIN expression domains during the process (Scarpella et al. 2006). To this day, these auxin application studies provide the most direct link between the patterning process and the chemical identity of the inducing compound as the natural auxin indole-3-acetic acid.

4.4 The Influence of Cellular Parameters on Vein Formation

If auxin transport is instrumental and self-regulatory in vein patterning, there should be reproducible and interpretable consequences of a change in either parameter. Fortunately, even relatively severe changes of either auxin transport or auxin sensitivity do not prevent leaf formation per se, so that the consequences on the leaf vein pattern can be studied. Reduced auxin transport during early stages of leaf development, whether caused by chemical inhibitors or mutations in genes like *PIN1*, affects the formation of all veins (Mattsson et al. 1999; Sieburth 1999)

(Fig. 4e). Major veins become thicker and more abundant in the periphery of the leaf. Upon strong inhibition of auxin transport, vascular differentiation becomes progressively restricted to the leaf periphery, which has been interpreted to suggest that major auxin sources should be located in this area at the phenocritical stages of early leaf outgrowth.

If auxin transport inhibition keeps auxin closer to its sources and leads to vascular differentiation in the periphery, normal auxin transport in combination with reduced auxin-responsive cell differentiation should lead to restriction of vascular differentiation to the center, where converging flows of auxin from the entire leaf would result in the relatively highest auxin concentrations. Consistent with this interpretation, the leaf vascular system of the auxin response mutant with the strongest reduction of vascular tissue, *mp*, is reduced to the midvein and the most basal part of second-order veins, while the distal parts of second-order veins and all higher-order venation are either extremely delayed in their development or completely absent (Hardtke and Berleth 1998; Przemeck et al. 1996) (Fig. 4e).

Disrupted vein mutants, among them *vascular network3/scarface/forked2* (*van3* hereafter), have been reported and seem to contradict flow-based vein specification (Carland and Nelson 2009; Deyholos et al. 2000; Koizumi et al. 2000, 2005; Sieburth et al. 2006; Steynen and Schultz 2003). However, closer inspection of PIN1 expression domains in *van3* mutants suggests that the primary defect lies in the stable maintenance of PIN1 expression domains rather than in their discontinuous specification (Scarpella et al. 2006).

4.5 The Ontogeny of Leaf Veins

In the absence of molecular markers, the ontogeny of leaf veins is hard to trace. Overt differentiation characteristics, such as wall reinforcements in developing vessels, are inappropriate markers for visualizing events close to the recruitment of ground meristem cells into procambial strands. Procambial characteristics, such as narrow shape and dense cytoplasm, on the other hand, are difficult to visualize along the often curved and oblique route of procambial strands. Thus, an authentic time course of vein pattern formation became feasible only with the availability of molecular markers of defined early vascular cell states (Sawchuk et al. 2007; Scarpella et al. 2004). Molecular markers of the procambial state became first available in Arabidopsis, but it turned out that this state was acquired nearly simultaneously along the entire lengths of, for example, a second-order vein loop, which is not easy to reconcile with a flow-based mechanism. Simultaneous formation of procambial cells along the entire length of a vein, however, is contrasted by the expression of equally reliable but even earlier expression markers (Kang and Dengler 2004; Sawchuk et al. 2007; Scarpella et al. 2004). Expression of the Arabidopsis homeobox–leucine zipper gene *ATHB8* (Baima et al. 1995) precedes procambium formation but is turned on in a polar fashion (Kang and Dengler 2004; Scarpella et al. 2004). In each new vein, expression commences at the basal

attachment point to a preexisting vein and progresses towards the distal end. This polar extension does not seem to be the peculiarity of this singular marker, but seems to reflect a cell state of functional relevance. If growth conditions interfere with the completion of vein specification, normally connected veins remain freely ending, attached to preexisting vasculature at the spot of first *ATHB8* expression (Scarpella et al. 2004). Observation of similar regularity in different types of veins (Sawchuk et al. 2007; Scarpella et al. 2004) suggests that most, if not all, connected veins are generated through ongoing extension of initially freely ending veins.

4.6 Dynamic Routes of Auxin Flow in Leaf Primordia

Even the early marker *ATHB8* is preceded by the expression of the auxin-efflux protein PIN1, which seems to visualize the earliest events around vein specification (Scarpella et al. 2006). At least two observations make it unlikely that any other marker may precede PIN1 in vein specification. First, PIN1 is genetically and cell biologically linked to the process of auxin transport canalization in the leaf lamina. Second, narrow PIN1-marked routes of auxin transport presage the formation of specific vascular strands already in primordia with surprisingly few cells. At any earlier stage, there would simply be not enough cells from which prospective vascular cells could be selected.

Dynamic PIN1 expression patterns reveal at least six rules for auxin-flow-based vein patterning (Scarpella et al. 2006; Wenzel et al. 2007) (Fig. 4a, d). First, just like *ATHB8* expression, PIN1 expression extends distally from vein attachment points. Second, as a drainage concept would predict, PIN1 polarities in extending, freely ending domains are invariably basal, that is oriented towards preexisting vasculature. Third, in this marker setup, the controversial issue of how to reconcile auxin-flow polarity and the formation of connected veins should be directly observable. The reproducible dynamics deduced from observations in staged leaf primordia indicates that connected veins are the results of fusions of two freely ending domains connected distally with a cell of dual polarity, termed “bipolar” cell. The regularity with which single, bipolar cells are observed, whose polarities match the opposite polarities of the two adjoining domains, supports the notion of a universal mechanism for the formation of connected veins. Fourth, irrespective of the generality of the fusion-based mechanism for the generation of connected veins, the PIN1 expression data show that veins become connected already at very early stages, with correspondingly few cells. From a mathematical point of view, this increases the probability of vein fusions dramatically. Fifth, PIN1 expression domains narrow during vein development, and only narrow, sustained routes of auxin transport give rise to procambial strands. The narrowing process is dependent upon auxin drainage, as auxin-transport inhibition delays it dramatically. It is possible, and conceptually likely, that at an early stage all subepidermal cells participate in the auxin-flow-route-finding process. Sixth, the formation of first- and second-order

veins is distinct from that of higher-order veins. Just like the formations of first-order veins, those of the second order are initiated through auxin convergence at the leaf epidermis (Fig. 4a, d).

4.7 The Flat Meristem Concept

If the formation of second-order veins, just like that of midveins, is associated with epidermal convergence points, what exactly is the distinction between those two apparently cell biologically similar processes (Fig. 4a, d)? While the formation of the midvein occurs in conjunction with a massive local outgrowth on the flanks of a rotationally symmetric shoot meristem (Bayer et al. 2009; Benkova et al. 2003; Heisler et al. 2005; Reinhardt et al. 2003; Scarpella et al. 2006; Wenzel et al. 2007), the formation of a second-order vein occurs on a flat leaf primordium, which restricts auxin convergence to a single cell file, the leaf epidermal margin (Scarpella et al. 2006; Wenzel et al. 2007). Further, no local outgrowth is necessarily linked to areas around second-order veins. For example, in Arabidopsis leaves, the growth of the lamina is not correlated to second-order vein positions, and an overall elliptic leaf is generated. However, in highly serrated leaves, and even more in compound leaves, the similarities between second-order vein formation and leaf initiation are striking. Leaf primordia in the close relative of Arabidopsis *Cardamine hirsuta*, for example, resemble their Arabidopsis counterparts at very early stages but show uneven lamina growth soon afterwards (Barkoulas et al. 2008). Each of the laterally outgrowing lamina areas develops into a clearly separated leaflet, and this process occurs beneath a marginal convergence point and in association with the formation of a second-order vein. When a compound leaf is fully grown, its leaflets look a lot like regular leaves comprising midveins and an elaborate vein pattern. Thus, the original primordium of a compound leaf has much in common with a shoot meristem, except that it is flat and that it generates only a defined number of leaflets. These features may have great advantages: the flat architecture makes it more amenable to confocal microscopic analyses of internal tissues, and mathematical modeling may benefit from the reduced dimensionality. In this special type of "flat" meristem, auxin convergences within a single cell file and their vascular readout occur in a single plane.

5 Continuity of Vein Formation

Continuity of vascular strands is a stringent requirement for transport function, and the auxin canalization hypothesis predicts that vascular strands, as expression of a continuous auxin-transport stream, should form without discontinuities. Therefore, continuous cell-to-cell transport of auxin should apparently be refuted as an organizing principle in all discontinuous vein networks. Mutation of the *VAN3*

gene of Arabidopsis results in highly fragmented vein networks (Carland and Nelson 2009; Deyholos et al. 2000; Koizumi et al. 2000, 2005; Sieburth et al. 2006; Steynen and Schultz 2003) (Fig. 4e), a defect enhanced by mutation in the three Arabidopsis genes most related to *VAN3* (Naramoto et al. 2010; Sieburth et al. 2006). However, vein fragments in *van3* leaves seem to be largely formed along the same paths where they would normally differentiate continuously.

Consistent with this observation, subepidermal PIN1 expression domains are initially continuous in *van3* leaves (Naramoto et al. 2009; Scarpella et al. 2006). However, continuity of PIN1 expression domains is fragile in *van3*, leading to discontinuous PIN1 expression domains at later stages of vein development. This observation suggests that the primary defect in *van3* is the maintenance, rather than the formation, of vein continuity. But what is the molecular nature of the fragility in vein continuity of *van3*? At early stages of vein development, continuous PIN1 expression domains initially comprise very few cells but extend by intercalary cell division over time (Scarpella et al. 2006). In wild type, any cell inserted into a continuous PIN1-expression domain by cell division would be expected to adopt position-dependent PIN1-polarity and auxin-transport properties, thereby maintaining continuity. Because PIN1 is localized to the emerging cell plate during cell division (Geldner et al. 2001), PIN1 must be relocated to the appropriate plasma membranes of daughter cells after each division. It is possible that VAN3 activity is required for PIN1 relocation after cell division; in *van3*, failure to integrate PIN1 polarity of newly formed cells with that of neighboring ones would then result in fragmentation of PIN1 expression domains. Consistent with this interpretation, VAN3 encodes an ADP-ribosylation-factor GTPase-activating protein (ARF GAP) (Koizumi et al. 2005; Sieburth et al. 2006), which promotes conversion of activated ARF–GTP into ARF–GDP, conversion that is necessary for vesicle fusion with specific target membranes (Chavrier and Goud 1999). As it would be expected from such function, the localization of VAN3 to the proper membrane compartment turns out to be crucial for continuous vein formation. The activity of the COTYLEDON VASCULAR PATTERN2 (CVP2) and CVP2-LIKE1 (CVL1) inositol polyphosphate 5′-phosphatases (5-PTases) of Arabidopsis is required for VAN3 subcellular localization, and simultaneous mutation of *CVP2* and *CVL1* results in collapse of continuity of PIN1 expression domains and in discontinuous vein networks (Carland and Nelson 2009; Naramoto et al. 2009). CVP2 and CVL1, and possibly other 5-PTases (e.g., At5Pase13) (Lin et al. 2005), function redundantly to generate phosphatidylinositol 4-monophosphate (PI4P), the phosphoinositide species bound by VAN3 (Carland and Nelson 2009; Koizumi et al. 2005; Naramoto et al. 2009). VAN3 could thus be recruited to the proper membrane compartment by its affinity for PI4P-enriched membrane domains. While the affinity that many VAN3-related proteins have for membrane phosphoinositides seems insufficient to drive their localization (Lemmon and Ferguson 2000), such affinity may be increased by interaction with other membrane-localized proteins. For VAN3, one such protein could be VAN3-BINDING PROTEIN1/FORKED1 (VAB1/FKD1), whose localization overlaps with that of VAN3 and whose function seems required for proper localization of VAN3 (Hou et al. 2010; Naramoto et al. 2009).

As *van3* single mutants and *cvp2 cvl1* double mutants, mutants in the Arabidopsis gene *COTYLEDON VASCULAR PATTERN1/STEROL METHYLTRANSFERASE2* (*CVP1*/SMT2) leads to vein network fragmentation (Carland et al. 1999, 2002), a defect that is enhanced by mutation of the functionally redundant SMT3 (Carland et al. 2010). Sterol methyltransferases regulate the ratio of brassinosteroids to sterols; in wild type, this ratio is shifted towards the production of sterols, but the ratio is inverted in the sterol-depleted *cvp1*. Sterols are synthesized at the endoplasmic reticulum and transported to the plasma membrane, where they typically accumulate (Boutte and Grebe 2009). In animals, membranes contain sterol-enriched domains that associate with specific proteins to compartmentalize cellular processes (Lingwood et al. 2009). Currently, there is no in vivo evidence that sterols and specific proteins cluster together in membrane domains of plant cells (Boutte and Grebe 2009). However, PIN polarity is disrupted in sterol-deficient mutants (Men et al. 2008; Pan et al. 2009; Willemsen et al. 2003), and in at least one of them PIN proteins fail to relocate to the appropriate plasma membranes of daughter cells after division (Men et al. 2008). Therefore, it is possible that, as in *van3* and *cvp2 cvl1*, vein continuity defects in *cvp1* and other sterol-deficient mutants result from failure to integrate PIN1 polarity of newly formed cells with that of neighboring ones.

In conclusion, evidence from vein continuity mutants is not inconsistent with an instrumental role of auxin transport along continuous cell files in defining sites of vascular strand formation. While ultimately required for vascular function, continuity of vascular strands seems to be dispensable for vascular differentiation. As a consequence, islands of differentiated vascular cells can be observed easily in vascular mutants and will enable a more comprehensive understanding of the cellular prerequisites of vein continuity.

6 Conclusions and Perspectives

Although new visualization tools, especially those indicative of auxin-flow routes and directions, have revolutionized leaf vein patterning research in the past years and have helped formulating a few internally consistent mechanistic concepts, we should remain aware that most of the cell biology of auxin transport and of the regulatory hierarchy of vascular cell specification is still in an opaque area only locally illuminated by the use of few, exceptional, better explored genes. There is justified hope that the consistent regularities summarized above provide a viable entry point into the complexity of leaf vein patterning, but it will require the application of more sophisticated cell biological visualization tools in a variety of species and leaf types, as well as a better interdisciplinary integration of comparative anatomical studies with molecular ones in order just to achieve a widely accepted conceptual consensus on a mechanistic basis underlying the vast diversity of leaf vein patterns. Even once such consensus is obtained, more precise quantification of all observed parameters and full live-imaging of the dynamic specification

processes will be necessary for a comprehensive mathematical description of the vein patterning process. The gain of such tremendous efforts will not only lie in a more profound understanding of the world's most widely used solar panels but also in the implications of the results on our understanding of shoot and embryo patterning.

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Regulation of Polar Auxin Transport by Protein–Protein Interactions

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Abstract Work over the last years has uncovered that during the highly integrative process of polar auxin transport, dynamic interactions of membrane proteins with other membrane or soluble proteins or modulatory drugs are providing a high degree of flexibility. This overall concept is supported by the recent release of a first, partial Arabidopsis interactome by the Arabidopsis Interactome Mapping Consortium. In this context, we have summarized the current knowledge of post-transcriptional regulation of auxin transport with an emphasis on protein–protein interaction and protein phosphorylation. We suggest a novel protein–protein interaction feedback loop of auxin transport. Further, we summarize evidence that this interaction loop is tightly interconnected with a previously described PIN polarity loop via AGC3 kinases represented by PINOID. These data are compatible with the view of a putative multi-protein auxin efflux complex that is building the basis for a plastic and economic control of auxin streams during PAT.

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1 Introduction

Gradients of the intercellular signaling compound auxin are the primary determinant for the establishment and maintenance of plant polarity in respect to their development, growth, and physiology (Robert and Friml 2009; Vanneste and Friml 2009). Although the exact mechanism how indol-3-acetic acid (IAA), the most common auxin, is transported from one plasma membrane domain to the next is still controversial (Schlicht et al. 2006), the intercellular distribution of IAA provided by polar auxin transport (PAT) is a unique process so far not found for other signaling molecules or in non-plant species (Vieten et al. 2007).

The chemiosmotic model has predicted the existence of secondary active auxin transporters that are thought to be directly or indirectly energized by the proton gradient established by the H^+-ATPase (Vanneste and Friml 2009). Despite the fact that also novel auxin transporters have been recently found (Barbez et al. 2012), until now, members of three distinct transporters families have been convincingly implicated to be directly involved in polar auxin transport: AUXIN RESISTANT 1/LIKE AUX1 (AUX1/LAX) uptake symporters, PIN-FORMED (PIN) efflux carriers, and P-GLYCOPROTEIN/MULTIDRUG RESISTANCE (PGP/MDR/ABCB) efflux pumps. A subgroup of ER-localized members of so-called short PINs, such as PIN5 and PIN8, have recently been shown to be implicated in cellular homeostasis and not in PAT (Bosco et al. 2012; Mravec et al. 2009; Ding et al. 2012; see Chap. 2 for details).

Importantly, due to the chemical features of IAA allowing partial diffusion into cells, exporters are the primary control units for PAT (Geisler and Murphy 2006). Accordingly, their activity has been shown to be tightly controlled on both the transcriptional and posttranscriptional levels (Titapiwatanakun and Murphy 2009). In the last years, members of the described PIN and ABCB family apparently independently—but also coordinately—function as the molecular machines that establish and maintain these gradients according to a revised version of the chemiosmotic model (Bandyopadhyay et al. 2007; Geisler and Murphy 2006).

In this chapter, we will focus mainly on the posttranscriptional regulation of PINs and ABCBs with a special focus on protein–protein interaction in respect to its impact on individual transport capacities. We suggest a novel regulatory, interaction feedback loop. However, we will also integrate very recent findings indicating that ABCB activity is directly controlled by protein phosphorylation, indicating that regulatory phosphorylation/polarity and interaction loops are interconnected.

2 Posttranscriptional Regulation of Auxin Catalysts

Based on the "canalization concept" originally hypothesized in order to explain vascular patterning of the leaf, a positive feedback loop between auxin flux and the cell's auxin transport capacity has been proposed (Sachs 1969; Stoma et al. 2008). Many of the involved molecular components have been identified, including auxin

transport catalysts and mechanisms by which auxin itself feeds back on their polar locations (reviewed in detail in Vieten et al. 2007). However, it should be stressed that until now the proposed auxin flux sensor has not yet been identified in plants (Merks et al. 2007).

Recent work on the posttranscriptional regulation of auxin catalysts has mainly focused on the trafficking pathways of PIN proteins (and also of AUX1) that have been studied intensively, and endosomal cycling is thought to play an essential role in PIN localizations (Feraru and Friml 2008; Jurgens and Geldner 2007; Kleine-Vehn and Friml 2008). Compared to PINs, the trafficking routes of ABCBs have not yet been analyzed in detail. However, keeping the functional ABCB–PIN interactions on the plasma membrane (see below) and the BFA sensitivity of the ABCB1 plasma membrane location (Titapiwatanakun et al. 2009; Wu et al. 2010) in mind, one might speculate that ABCBs follow known PIN routes: ABCB1, like PIN1, is internalized into endosomal compartments upon BFA treatments, unlike ABCB19 that is widely BFA insensitive and follows apparently a trafficking pathway that is distinct from PIN1/ABCB1 (Titapiwatanakun et al. 2009). Finally, plasma membrane locations of ABCB1, ABCB19, and ABCB4 but not PIN1 or PIN2 (Bouchard et al. 2006) have been shown to be dependent on the action of the FKBP42, TWISTED DWARF1 (TWD1), although the underlying mechanism is not yet clear (Wu et al. 2010).

The current picture that emerges is that PIN (and most probably also AUX1/LAX) transporters both obviously providing the majority of bulk PAT streams are mainly regulated by influencing their subcellular location and polarity, while ABCBs that show widely nonpolar locations and are more stable on the plasma membrane are obviously controlled by regulation of their catalytic activity. The underlying mechanisms for regulatory modules, protein–protein interaction/modulatory drugs, and protein phosphorylation are summarized in more detail in the following.

3 Regulation of Auxin Catalysts by Protein–Protein Interaction

Work over the last years has revealed that during the highly integrative process of PAT, dynamic interactions of membrane proteins with other membrane or soluble components (hereafter referred to as *PAT* modulators) are thought to provide a high degree of flexibility that usually characterizes higher plants. This overall concept is supported by the recent release of a first, partial Arabidopsis interactome by the Arabidopsis Interactome Mapping Consortium (2011). Apparently, the Arabidopsis interactome reveals a strong enrichment of a few network communities, including those for transmembrane transport and vesicle trafficking. Strikingly, the largest transmembrane transport community shares a high amount of proteins with the vesicle-trafficking community, suggesting a strong physical and functional overlap and interaction. While the Arabidopsis interactome, in contrast to those of yeast, human, *C. elegans*, and *Drosophila*, awaits its completion also for Arabidopsis protein–protein interaction (PPI) tools and experimental and theoretical large-scale

protein interaction maps have been developed (Chen et al. 2012; De Bodt et al. 2012; Geisler-Lee et al. 2007; Lee et al. 2010; Li et al. 2011). In agreement with the overrepresentation of Arabidopsis transporter proteins (over 1,200 proteins) have been assigned as transporters although only 267 transporters have been as such characterized (Lalonde et al. 2010). A focus was laid on the elucidation of membrane proteins and their signaling networks (Chen et al. 2012; Lalonde et al. 2010). However, previous experimental work on PAT in respect to protein–protein interaction has focused on functional interactions between ABCBs and the immunophilin-like FKBP42, TWISTED DWARF1 (TWD1) on the one hand and on the interaction between ABCBs and PINs on the other.

3.1 TWD1–ABCB Interaction

Work over the last decade has established the physical and functional interaction of the immunophilin-like protein FKBP42, called TWISTED DWARF1 (TWD1)/ULTRACURVATA 2 (UCU2) (Perez-Perez et al. 2004), with ABCBs, ABCB1, and ABCB19 (Geisler and Bailly 2007). TWD1/FKBP42 belongs to the FK506-binding protein (FKBP) subfamily of PPIases (*cis–trans* peptidyl-prolyl isomerases), which are thought to catalyze the *cis–trans* isomerization of *cis*-prolyl bonds (Geisler and Bailly 2007). Many FKBPs have a PPIase (rotamase) activity, creating the impression that FKBPs function primarily in protein folding. However, extensive research during the past decade has elucidated two independent functions for FKBPs (1) a PPIase activity classically inhibited by binding of clinically relevant immunosuppressant drugs, such as FK506 (tacrolimus) or rapamycin (sirolimus), and (2) a chaperone function that is independent of the PPIase activity and unaffected by immunosuppressant drugs (Barik 2006; Blecher et al. 1996; Harrar et al. 2003).

The *twisted dwarf1* T-DNA insertional mutant (*twd1-1*) has been isolated in a mutant screen for developmental phenotypes. In order to understand the pleiotropic developmental growth phenotype of *twd1*, characterized by a dwarf plant size, reduced cell elongation, disoriented growth of all organs, and misshapen epidermal cells (causing a twist), a yeast two-hybrid screen for putative interactors using the soluble portion of TWD1 as bait was performed (Geisler et al. 2003). The rationale to do so was based on the fact that TWD1 owns no detectable PPIase activity using standard test substrates but contains three repetitions of a so-called tetratricopeptide repeat (TPR). This qualified TWD1 as a multi-domain (high-molecular weight) FKBP, containing typically up to three N-terminal putative FK506-binding domains (FKBDs), typically followed by a TPR domain and a calmodulin-binding domain (CaM-BD; see Fig. 1), both known to mediate protein–protein interactions to calmodulin and heat-shock proteins, respectively (Geisler and Bailly 2007). Both calmodulin and HSP90 in vitro binding to TWD1 was demonstrated although the physiological relevance of these interactions is entirely unclear (see Sect. 6).

The two-hybrid screen for TWD1 interactors resulted, besides HSP90, in the identification of C-terminal nucleotide-binding domains (NBDs) of ABCB1 and

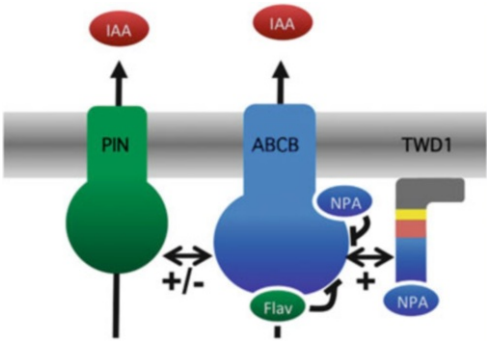

Fig. 1 Model summarizing the effect of PIN1–ABCB and TWD1–ABCB interactions on auxin export. ABCB and PIN proteins are able to function as independent auxin export catalysts; however, tissue-specific ABCB–PIN pairings alter positively and negatively (+/−) IAA export, NPA sensitivity, substrate specificity, and eventually also transport directionality of ABCBs. Interaction is provided between the cytoplasmic loop of plasma membrane-bound PINs interfering with the NBD2 of ABCBs. TWD1 functions as a positive (+) modulator of ABCB activity by interaction between the TWD1 FKBD (*blue*) and the ABCB NBD2. Interaction is thought to be either disrupted by NPA binding to the TWD1 FKBD (*blue*) and the ABCB interface between NBD and intracellular loops (Bailly and Geisler, unpublished) or by flavonoid binding to the ABCB NBDs (Conseil et al. 1998), resulting in ABCB inhibition

members of the ABCC/MRP family. TWD1–ABCB1 interaction was verified by using in vitro pull-down assays, NPA- and TWD1-HA affinity chromatography (Geisler et al. 2003) and bioluminescence resonance energy transfer in yeast (BRET; Bailly et al. 2008). Mapping of interacting domains demonstrated unexpectedly that not the C-terminal TPR domain but the N-terminal putative PPIase domain (FKBD) provided this interaction (Geisler et al. 2003). Interaction was not affected by immunosuppressant drugs, suggesting, together with the absence of a detectable PPIase activity, an evolutionary shift of function toward protein–protein interaction (Geisler and Bailly 2007; Geisler et al. 2003). Both interaction and ABCB regulation are provided by the N-terminal FKBD, as the soluble FKBD upon co-expression in yeast can functionally replace the full-length TWD1 (Bailly et al. 2008).

Phenotypic *twd1* analysis revealed reduced hypocotyl lengths in the dark and under light, elongated root length (in the dark), and an obvious overlap between *twd1* and *abcb1abcb19* loss-of-function alleles, especially at early stages, suggesting a regulatory impact of TWD1 on ABCB1/B19-mediated auxin transport capacities (Geisler and Bailly 2007; Geisler et al. 2003). Co-expression of ABCB1 with TWD1 (but not with Arabidopsis FKBP12) in yeast reduced ABCB1 IAA export activity to vector control levels as shown by transport and growth assays (Bouchard et al. 2006). This was surprising as it is the opposite of what one would have expected from previous in planta data. TWD1 has also a negative impact on ABCB1 activity when co-expressed in the heterologous plant system *N. benthamiana* (Henrichs et al. 2012).

However, in mammalian HeLa cells, TWD1 has an activating effect on ABCB1, suggesting that a third factor might be absent in yeast and tobacco (Bouchard et al. 2006). In this respect, TWD1 might compete for ABCB1 activation by high levels of yeast (or tobacco) FKBP12, the first shown to activate ABCB1 and mouse ABCB3/MDR3 (Hemenway and Heitman 1996). Assuming higher affinity and/or abundance of heterologous FKBP12 compared to TWD1, this would result in a net reduction of ABCB1-mediated auxin transport (Bailly et al. 2008; Henrichs et al. 2012). This concept is supported by the recent finding that yeast FKBP12 is able to widely complement *twd1* (Henrichs et al. 2012). Moreover, TWD1 does apparently not compete for endogenous FKBP12 activation in Arabidopsis as AtFKBP12 has no significant effect on ABCB1 activity (Bouchard et al. 2006).

However, a positive regulatory in planta role on ABCBs and in PAT was indicated by reduced reflux capacities in *abcb* and *twd1* roots measured by employing an IAA-specific microelectrode (Bouchard et al. 2006) and by demonstrating that locations of PIN1 and PIN2 were unchanged (Wu et al. 2010). This concept was underlined by the finding that *twd1* roots, similarly to those of *abcb1 abcb19* (but not *abcb1* and *abcb19* single) mutants, reveal elevated levels of free IAA (especially in mature parts) and altered gravitropic responses (Bailly et al. 2008). Together with the major predicted roles of ABCB1 and ABCB19 in basipetal (ABCB1) and acropetal (ABCB19) auxin transport in roots (Bandyopadhyay et al. 2007; Blakeslee et al. 2007; Geisler and Murphy 2006), this suggests TWD1 to function as a central regulator of ABCB-mediated long-range auxin transport controlling plant physiology and development (Bailly et al. 2006; Geisler and Bailly 2007).

The crystal structures of the TWD1 FKBD and the full-length protein without membrane anchor have been determined (Granzin et al. 2006; Weiergraber et al. 2006). Modeling of FK506 docking indicates that, consistent with experimental data, TWD1 (like HsFKBP38) sterically excludes immunosuppressant drugs like FK506 (Bailly et al. 2006). Even more informative is that FK506 binding positively correlates with the presence of a PPIase activity. Therefore, the current picture that emerges is that stress-related FKBPs have apparently maintained a conserved PPIase activity to fulfill their proposed chaperone function, whereas others (such as human FKBP38, TWD1, or PAS1) have lost (or only retained low) PPIase activity (Geisler and Bailly 2007). A structural shift toward functionality in protein–protein interaction was provided by recent NMR assignments of the FKBP-type PPIase domain of FKBP42. Signal intensities revealed an additional structure element that is atypical for such FKBP domains (Burgardt et al. 2012).

In silico modeling of the protein–protein interaction with key interacting partners, HSP90, ABCB1, and ABCC1, has facilitated the prediction of docking sites at the molecular level. Although the docking domains of TWD1 that interact with the nucleotide-binding fold of ABCB- and ABCC-like ABC transporters are different (FKB and TPR domains, respectively), both transporters use overlapping surface areas on the transporters, suggesting a new paradigm for the regulation of

ABC transporter activity (Granzin et al. 2006). Co-crystallization of FKBPs with interacting partners will be the method of choice to understand TWD1-ABC transporter at the molecular level.

TWD1 is surprisingly difficult to solubilize by detergents, which has initially supported the prediction of a glycophosphatidyl inositol (GPI) anchoring (Geisler et al. 2003). However, no GPI moiety has been detected biochemically (Murphy et al. 2002), and TWD1 has not been identified in any of the proteomic approaches on raft-like structures (Morel et al. 2006). Recently, based on NMR analysis of a C-terminal TWD1 peptide, a perpendicular orientation of the TWD1 anchor forming a so-called amphipathic in-plane membrane (IPM) anchor has been predicted (Scheidt et al. 2007).

Using electron microscopy on HA-TWD1-OX plants, TWD1 has, besides on the plasma membrane, additionally been localized to the tonoplast (Kamphausen et al. 2002). This is of relevance because, as mentioned above, TWD1 has been shown to functionally interact with ABCC1/MRP1 and ABCC2/MRP2 (Geisler et al. 2004). Moreover, recently employing a C-terminal CFP tag fused to a genomic construct expressing TWD1 under its own promoter, a convincing location that was restricted solely to the ER was found (Wu et al. 2010). Like the previous HA-tagged cDNA construct expressing TWD1 under the constitutive, also this genomic construct fully complemented the twisted syndrome. This conflict is even more puzzling as expression of TWD1 from the TWD1:TWD1–CFP construct resulted in a clear plasma membrane location in tobacco leaves but no obvious ER signal (Henrichs et al. 2012). In summary, it appears that TWD1 resides on multiple subcellular compartments in analogy to its mammalian ortholog, FKBP38 (Edlich and Lucke 2011; Shirane and Nakayama 2003).

Interestingly, based on the finding that ABCB1-, B4-, and B19-GFP are retained on the ER in *twd1*, a chaperone function independent of a PPIase activity for TWD1 in plasma membrane secretion of ABCBs was re-proposed (Wu et al. 2010). These data are in conflict with a previous work showing that ABCB1 resides on plasma membrane fractions after sucrose density gradient centrifugation (Bouchard et al. 2006). However, ER retention of ABCB1/B4/B19 suggests an alternative TWD1 functionality that implies that the twisted dwarf phenotype is caused not by a lack of ABCB activation but their delocalization. Obviously, these two scenarios are not exclusive. Moreover, one might also imagine that in the absence of TWD1, inactive ABCBs are removed from the plasma membrane and that ER locations represent degradation locations. In any case, a comparison of *twd1* with *abcb1,b4,b19* plants as well as an analysis of TWD1–ABCB4 interaction is highly desirable.

Regardless of the mode of membrane anchoring and the intracellular compartment of interaction, a relevant question is why the need for membrane anchoring of TWD1 at all? It has been suggested that FKBP38 acts as a mitochondrial docking molecule that concentrates two anti-apoptotic membrane proteins at the mitochondria (instead of the ER), thus preventing apoptosis (Edlich and Lucke 2011). A similar mechanism might be involved in TWD1 regulation of ABC transporters both on the tonoplast and plasma membrane: Membrane anchoring might thus increase the probability of contacts by reducing the spatiality of TWD1

diffusion. Furthermore, restraining the mobility of TWD1 by membrane anchoring might serve as a means to decouple the regulation of transporters located on different membranes (Scheidt et al. 2007).

3.2 ABCB–PIN Interaction

Previous work established that ABCB and PIN proteins are able to function as independent auxin export catalysts (Petrasek et al. 2006). However, both transporter classes lack the degree of substrate specificity seen in planta when expressed in heterologous systems (Blakeslee et al. 2007). Moreover, *abcb1* and, to an even higher degree, *abcb19* and *pin1* mutant roots show a high degree of unspecific basipetal BA transport not found for wild type (Blakeslee et al. 2007). This, together with the fact that ABCB1, B19, and PIN1/P2 show widely overlapping root expression profiles, suggested functional interaction between these auxin efflux catalysts (Blakeslee et al. 2007).

Subcellular co-localization, yeast two-hybrid interaction, and co-immunoprecipitation analyses provided clear evidence for distinct ABCB1,19-PIN1,2 pairings (Blakeslee et al. 2007; Rojas-Pierce et al. 2007). However, it should be mentioned that not all possible ABCB1/B19–PIN1/P2 combinations have been clearly proven and that the hardest set of evidence exists for ABCB19–PIN1 and ABCB1–PIN2 interactions (Blakeslee et al. 2007). Not surprisingly, these interactions correlate with their proposed overlapping functions in apical and basipetal auxin transport, respectively. As was the case for ABCB/TWD1 pairs, interaction of ABCBs and PINs employs the C-terminal NBDs of the ABCBs binding the central cytoplasmic PIN loops (Blakeslee et al. 2007).

Co-expression of ABCBs in HeLa cells with PIN1 increased export, NPA sensitivity, and substrate specificity of ABCB1/B19, while PIN2 had only significant effects on ABCB specificity. Similar results were found with ABCB1 co-expressed with PIN1 and PIN2 (Blakeslee et al. 2007). Functional interaction was supported by synergistic *abcb19 pin1* plant phenotypes and *abcb1 abcb19 pin2* root agravitropism (Blakeslee et al. 2007). Interestingly, dynamics of PIN1 cycling are reduced in ABC19 locations, and PIN1 plasma membrane location was more easily perturbed in *abcb19* roots, suggesting that ABCB19 stabilizes PIN1 in plasma membrane microdomains (Titapiwatanakun et al. 2009).

As mentioned already, based on interaction and transport studies, there was no indication for ABCB–AUX1 interaction (Blakeslee et al. 2007). However, functional co-expression of PIN1 reversed the import direction of ABCB4 in HeLa cells, while PIN2 enhanced ABCB4 activity (Blakeslee et al. 2007). However, trials to verify PIN–ABCB4 interaction failed so far (Titapiwatanakun et al. 2009).

In summary, these data provide evidence for independent ABCB and PIN transport mechanisms but also tissue-specific ABCB–PIN pairings that function interactively. In these, PIN proteins seem to add a vectorial dimension to ABCB-mediated nonpolar cellular auxin export required for PAT.

3.3 PAT Modulators

The auxin efflux complex is thought to consist of at least two proteins: a membrane integral transporter and regulatory subunit binding the noncompetitive, synthetic auxin efflux inhibitor, 1-*N*-naphtylphtalamic acid (NPA), qualifying it as NPA-binding protein (NBP) (Bernasconi et al. 1996; Cox and Muday 1994; Luschnig 2001; Michalke et al. 1992). Until today the identity, number, and affinity of putative NBPs are still controversial (Cox and Muday 1994; Michalke et al. 1992; Sussman and Gardner 1980), but there is apparently a consensus that PIN proteins do not itself act as NBPs (Lomax et al. 1995).

The regulatory impact of flavonoids, a class of plant-derived secondary compounds, on PAT was initially based on their ability to compete with NPA for transporter binding sites (Jacobs and Rubery 1988; Lomax et al. 1995; Morris 2000). This concept is further supported by auxin-related phenotypes of Arabidopsis mutants with altered flavonoid levels (Buer et al. 2007; Peer and Murphy 2006, 2007; Taylor and Grotewold 2005), although fundamental physiological processes occur in the absence of flavonoids. Aglycone molecules, such as quercetin and kaempferol, have been shown to inhibit PAT and consequently to enhance localized auxin accumulation (Brown et al. 2001). Currently they are seen as transport modulators (Peer and Murphy 2007); nevertheless, the mechanisms by which flavonoids physically interfere with auxin efflux components are not yet clear.

ABCB1 and ABCB19 have been identified—together with TWD1—in high-affinity fractions as NBPs (Geisler et al. 2003; Murphy et al. 2002; Noh et al. 2001), which obviously does not directly prove that all three are high-affinity NBPs (see below). However, it was shown that high micromolar NPA concentrations cause inhibition of auxin efflux catalyzed by ABCB1 and ABCB19 (Bouchard et al. 2006; Terasaka et al. 2005). NPA binding studies using microsomes prepared from *abcb* mutants and yeast or HeLa cells expressing ABCB1 and ABCB19 verified this assumption (Benjamins et al. 2001; Kim et al. 2010; Noh et al. 2001; Rojas-Pierce et al. 2007).

ABCB1 and ABCB19 both bind NPA but own apparently different NPA affinities. This has led to the idea that ABCB19 represents the major target of NPA (Rojas-Pierce et al. 2007). Using plasma membrane-enriched microsomes from loss-of-function plants, it was shown that *abcb19* but not *abcb1* (or *pin1*) showed significantly reduced NPA binding, although the synthetic ATI, gravacin, removed NPA to similar wild-type levels (Rojas-Pierce et al. 2007).

By means of chemical genomics, another auxin transport inhibitor, called BUM (2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid), was identified by its potential to efficiently block auxin-regulated plant physiology and development (Kim et al. 2010). In many respects, BUM resembles the functionality of NPA but has an IC_{50} that is roughly a factor 30 lower. Physiological analysis and binding assays identified ABCBs, primarily ABCB1, as key targets of BUM and NPA, while PIN proteins were shown not to be directly affected. BUM is complementary to NPA by

having distinct ABCB target spectra and impacts on basipetal polar auxin transport in the shoot and root. In comparison to gravacin, it lacks interference with ABCB membrane trafficking.

Two findings were suggesting that TWD1–ABCB interaction was disrupted by NPA: First, concentrations needed to block ABCB transport activity expressed in heterologous systems were far higher than what was needed in planta. And, second, excess washing with NPA excluded TWD1 but not ABCBs from NPA chromatographies (Geisler et al. 2003). Using a yeast-based BRET (bioluminescence resonance energy transfer) system, NPA and BUM, but not competitive ATIs, TIBA, or CPD, were shown to disrupt TWD1–ABCB1 interaction (Bailly et al. 2008; Kim et al. 2010). Further, all flavonoids tested disrupted the interaction (Bailly et al. 2008), while gravacin (Rojas-Pierce et al. 2007) had no significant effect on the interaction (Kim et al. 2010) verifying the idea that TWD1 is probably not a target of it. The flavonol quercetin ($IC_{50} \approx 200$ nM) was the most efficient and, surprisingly, was also active as glucoside (Bailly et al. 2008). Yeast IAA export assays in the presence of ATIs verified the BRET measurements on the transport levels. Mutant analysis indicated that the TWD1 FKBD is responsible for both interaction and drug regulation of ABCB1. This assumption was confirmed by specific NPA binding studies using whole yeast, highly pure FKBD, and plant microsomes prepared from *TWD1* loss- and gain-of-function mutants (Bailly et al. 2008). Using gravitropism analysis, imaging of auxin fluxes upon gravistimulation, and measuring root auxin fluxes, this concept was further substantiated by the finding that *twd1* and to lesser amount also *abcb1 abcb19* (but not the single *abcb* loss-of-function alleles) were NPA insensitive (Bailly et al. 2008).

These data are in agreement with the current concept that the efflux complex consists of at least two proteins: a transporter and an NPA-binding regulatory subunit (Luschnig 2001; Morris 2000; Petrasek et al. 2003). ABCBs represent apparently integral membrane-embedded NBPs identified by Bernasconi et al. (1996) and Ruegger et al. (1997). Therefore, TWD1 might be the peripheral NBP (Cox and Muday 1994), which is in line with the recently proposed perpendicular orientation of the TWD1 C-terminus. However, this perception is also supported by the fact that the NBP has been suggested to be required for auxin efflux transporter positioning (Gil et al. 2001). Interestingly, a low-affinity NPA binding site has been associated with the transporter because its block results in transport inhibition, while the high-affinity site does not interfere directly with auxin transport (Michalke et al. 1992). In this respect, it will be of high interest to quantify NPA binding affinities for TWD1 and ABCBs.

On the other hand, these data suggest a novel mode of drug-mediated regulation of ABCB activities via an interacting FKBP: The TWD1 FKBD owns a receptor-like function and is therefore capable of integrating negative (ATI) inputs on ABCB1 (see Fig. 1). This concept is supported by in silico docking analysis of ATIs on the crystal structure of the FKBD, providing indication for an ATI binding pockets (Bailly and Geisler, unpublished). Interestingly, this pocket is overlapping with surfaces thought to dock to the ABCB1 NBD2 (Granzin et al. 2006), providing a mechanistic ratio for disruption of TWD1–ABCB interaction. Based on computational binding, NPA

docks to ABCB pockets flanked by coupling helices and Q loops of NBD1 and NBD2 at the NBD–ICL interface (Kim et al. 2010). One could easily imagine that NPA blocks efficiently the main mechanistic of the transporter during transition of conformational changes between the NBDs and the ICLs.

Recent data supported that TWD1 does not bind flavonols, like quercetin, itself (Henrichs et al. 2012); therefore, their potential to disrupt TW1–ABCB interaction suggests that they bind to plant ABCBs. In agreement, flavonoids function as inhibitors of plant (Geisler et al. 2005; Terasaka et al. 2005) and mammalian ABCBs (Morris and Zhang 2006), most probably by mimicking ATP and competing for ABCB nucleotide-binding domains (Conseil et al. 1998). Different targets and binding domains clearly indicate distinct modes of actions for NPA and flavonols and question the simplified view that flavonols act as plant-endogenous NPA homologs.

The eligible question that arises still is why nature invented second NPA binding affinities on ABCBs besides TWD1. The simplest answer to this might be that there is apparently a need for ABCB regulation by ATIs in the absence of TWD1.

4 Regulation of Auxin Transporter Activity by Protein Phosphorylation

4.1 PIN Phosphorylation

Several studies indicate that reversible protein phosphorylation is an important regulatory mechanism for PAT. As mentioned above, endocytotic cycling represents a highly regulated mechanism for polar PIN locations that among others has been shown to be regulated by protein phosphorylation events (Friml et al. 2004; Michniewicz et al. 2007). Flowering plants do not contain orthologs of animal protein kinase A (PKA), cyclic GMP-dependent protein kinase (PKG), or protein kinase C (PKC). However, a family of so-called AGCVIII Ser/Thr protein kinases, named after their mammalian homologs, is thought to own similar function in growth factor signal transduction (Galvan-Ampudia and Offringa 2007).

The Ser/Thr protein kinase PINOID (PID), belonging—together with PID2, WAG1, and WAG2—to the AGC3 clade of AGCVIII kinases (Galvan-Ampudia and Offringa 2007), is an important regulator of this process and was shown to function as molecular switch of PIN locations (Friml et al. 2004; Kleine-Vehn et al. 2009; Michniewicz et al. 2007; Rakusova et al. 2011; Zhang et al. 2010). Loss-of-function alleles reveal a *pinoid* (*pin1*-like) plant phenotype (Benjamins et al. 2001), while plants overexpressing PID reveal defects in gravitropism and a loss of root meristem organization probably due to auxin depletion (Friml et al. 2004). In *pid* inflorescences, PIN1 has been shown to be shifted from the upper to the lower side, explaining similar phenotypes found for *pin1* and *pid*, while in PID-OX plants, PIN2

and PIN4 were shown to be mistargeted (Friml et al. 2004). Moreover, polarization of PIN3-dependent auxin transport for hypocotyl gravitropic response was shown to be as well controlled by PID (Rakusova et al. 2011).

Recently, PID, WAG1, and WAG2 were shown to phosphorylate PIN carriers at a conserved TPRXS(N/S) motif in the central hydrophilic loop, leading to PIN recruitment into the apical recycling pathway (Dhonukshe et al. 2010; Huang et al. 2010). Moreover, disruption of PID and its three closest homologues completely abolishes the formation of cotyledons (Cheng et al. 2008). These findings, together with the fact that WAG1 and WAG2 are apolar and plasma membrane-associated, suggest that AGC3 kinases act in the same or in a parallel regulatory pathway of PAT (Santner and Watson 2006).

The current model suggests that PID, together with the trimeric serine–threonine protein phosphatase 2A (PP2A), antagonistically determines the fate of PIN cargoes for trafficking to the appropriate membrane by (de)phosphorylating conserved motifs of the hydrophilic loop of PIN proteins (Dhonukshe et al. 2010; Ding et al. 2011; Huang et al. 2010; Kleine-Vehn et al. 2009) (see Fig. 4 and Chap. 5 of this series for more details). In summary, this suggests a posttranscriptional polarity loop via antagonistic action by PID/PP2A (Benjamins and Scheres 2008; see Fig. 3). However, it is an open question if altered PIN polarity is indeed directly caused by PIN phosphorylation or not simply the consequence of altered PIN activity. The latter has been suggested for D6 protein kinase (Zourelidou et al. 2009).

4.2 ABCB Phosphorylation

Several lines of clinical evidence suggest ABCBs as general targets for phosphorylation-dependent regulation in a so-called linker region. This linker region connects the N- and C-terminal NBDs of ABCBs and has been shown to regulate ABCB by multiple phosphorylation events catalyzed by PKC (Chambers et al. 1990; Conseil et al. 2001). Linker phosphorylation modifies ABCB transport and associated ATPase activity (Szabo et al. 1997). An accumulation of serine residues was identified to be phosphorylated by PKA and/or PKC (Chambers et al. 1990; Conseil et al. 2001; Orr et al. 1993) that regulate the drug transport properties (Szabo et al. 1997). Employing different phospho-proteomics approaches, plant ABCB proteins have recently (among other ABC transporters) shown to be phosphorylated (Benschop et al. 2007; de la Fuente van Bentem et al. 2006; Nuhse et al. 2004; Peck 2006).

The first proof that also plant ABCBs are controlled by reversible protein phosphorylation came from the finding that the AGC4 kinase, PHOTROPIN1 (phot1), was shown to interact with both NBDs of ABCB19 but not with the NBD2 of ABCB1 (Christie et al. 2011). Interestingly, interaction was blocked by light irradiation as would be expected for a blue-light receptor kinase. In vitro phosphorylation experiments verified ABCB19 as phot1 kinase substrate, although phosphorylated domains and residues remain exclusive. Using co-expression in

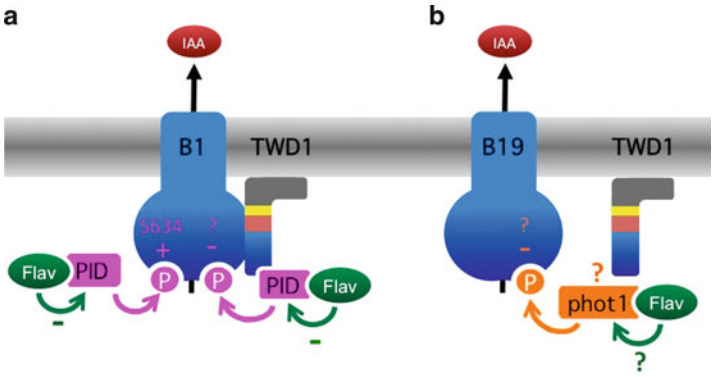

Fig. 2 Model summarizing the analogous regulatory impact of AGC3 kinases, PID and phot1, on ABCB1 and ABCB19 activities. (**a**) In the absence of TWD1, PID has a positive (+) regulatory effect on ABCB1 activity, most probably by S634 linker phosphorylation. In its presence, PID phosphorylates an unknown residue (?) resulting in block of auxin efflux (−). Flavonols (Flav), such as quercetin, bind to PID resulting in PID inhibition and block of ABCB regulation by PID. (**b**) Phosphorylation of the NBDs of ABCB19 by phot1 marks inhibition of transport activity, suggesting an analogous mode of ABCB regulation via protein phosphorylation where TWD1 would function in recruiting individual AGC3 kinases. Please note that TWD1–phot1 interaction awaits confirmation but is supported by the finding that TWD1–ABCB19 interaction, shown to as well positively enhance B19 activity, was ameliorated in phot1 plants. Functional domains of TWD1 are in *blue* (FKBD), *red* (TPR), *yellow* (calmodulin-binding domain), and *gray* (in-plane membrane anchor); *question marks* label functionalities that need experimental approval

HeLa cells, auxin efflux activity of ABCB19 but not of B1 was specifically shown to be inhibited by phot1 co-expression, in a mode that is dependent on the phot1 kinase activity and that is accelerated by light irradiation (see Fig. 2b).

Interestingly, TWD1–ABCB19 interaction, shown as well to positively enhance B19 activity, was ameliorated by light irradiation but enhanced in phot1 plants, supporting the concept that phot1-catalyzed ABCB19 phosphorylation blocks TWD1–ABCB19 interaction. Alternatively, phot1 might simply compete for TWD1 docking surfaces at the ABCB19 NBD2.

Using co-immunoprecipitation and shotgun LC–MS/MS analysis, PID was identified as a valid partner in interaction with TWD1. PID interaction was verified by BRET analysis in planta and in vitro pulldowns. In vitro and yeast expression analyses indicated that PID specifically modulates ABCB1-mediated auxin efflux in an action that is dependent on its kinase activity and that is reverted by quercetin binding and thus inhibition of PID auto-phosphorylation. ABCB1/PID co-transfection in tobacco revealed that PID enhances ABCB1-mediated auxin efflux in the absence of TWD1, while PID had a negative impact on ABCB1 in yeast. As discussed above, the most likely explanation is that ScFKBP12 is able to functionally complement TWD1 in yeast as has been suggested for TWD1 modulation of ABCB1 (Bailly et al. 2008; Bouchard et al. 2006). Interestingly, triple ABCB1/PID/TWD1 co-transfection in tobacco revealed that PID blocks ABCB1-mediated auxin efflux in the presence of TWD1 (see Fig. 2a), suggesting that TWD1 might function as a recruiting factor for

ABCB1 phosphorylation. The fact that ABCB1 phosphorylation in the presence of TWD1 has the opposite effect on ABCB1 transport capacity than TWD1–ABCB1 interaction per se argues for the idea that protein phosphorylation is not the primary mode of TWD1 activation. Obviously, both modes of ABCB1 regulation—directly via TWD1 interaction and PID phosphorylation—might also take place in parallel or in competition, resulting in fine-tuning of ABCB activity as reported for mammalian ABCBs. Alternatively, ABCB1 phosphorylation in the presence of TWD1 might disrupt TWD1–ABCB1 interaction, leading to ABCB1 inhibition (see below).

Phospho-proteomics analyses identified S634 as a key residue of the regulatory ABCB1 linker, which was verified by mutation analyses in yeast and tobacco. In the absence of TWD1, PID does phosphorylate S634, resulting in ABCB1 activation. On the other hand, negative ABCB1 regulation in the presence of TWD1 argues for a second, PID-specific ABCB1 phosphorylation site that does not essentially need to be part of the linker.

Currently, PID is seen as a positive regulator of NPA-sensitive PAT (Lee and Cho 2006). This is based on the correlation of the *pid* mutant shoot phenotype that—in analogy to the more drastic one of *pin1* (Palme and Galweiler 1999)—can be widely phenocopied by NPA treatment (Wisniewska et al. 2006) and supported by the fact that *pid* shoots (Bennett et al. 1995) and roots (Sukumar et al. 2009) show reductions of acropetal and basipetal PAT, respectively. A current study, however, shows that PID phosphorylation of the ABCB1 linker might modulate not only ABCB1 activity but also NPA binding capacities (Henrichs et al. 2012). This implies that enhanced (reduced) NPA (quercetin) binding to *PID* gain-of-function microsomes might be a direct result of altered ABCB1 phosphorylation at S634 by PID. These findings, however, also suggest that the *pinoid* phenotype and repression of PID-OX defects by NPA are at least to a certain magnitude taken over by PIN-independent transport mechanisms, such as ABCBs. This is also supported by additive, drastic developmental defects of *pin1 pid* alleles (Furutani et al. 2007). NPA action might be therefore mediated by closely related AGC3 kinases, like PID2 or WAG1/WAG2, that have been shown to share the regulation of identical NPA-sensitive PAT pathways (Dhonukshe et al. 2010; Santner and Watson 2006).

Protein phosphatases for ABCB1 and B19 dephosphorylation have not yet been identified, but indirect evidence suggests that PP2A, RCN1, might be involved: *rcn1 abcb1 abcb19* triple mutants exhibited strong embryonic and postembryonic auxin-related phenotypes (Mravec et al. 2008). Moreover, PP2C (At2g30020) was identified as putative TWD1 interactor (Henrichs et al. 2012).

In summary, these two analogous sets of data on ABCB19 and ABCB1 regulation by AGC kinases, phot1 (Christie et al. 2011) and PID, imply that AGC kinases, besides their function as a molecular switch of PIN polarity, have a direct impact on auxin efflux (ABCB) activity. Although phosphorylated residues in ABCB19 by phot1 have not yet been identified and a phot1–TWD1 interaction has not been proven (see Fig. 2), both modes of actions show common features: In the presence of TWD1, both phosphorylation events catalyzed by phot1 and PID lead to an inhibition of ABCB activity. In both cases, ABCB phosphorylation might result in a block of ABCB–TWD1 interaction, which would be a plausible ratio for a loss of

functionality. However, this mode of action, although intriguing and worth testing, is in contrast to the current picture that ABCB–TWD1 interaction is of transient nature, which is obviously supported by the low TWD1/ABCB expression stoichiometry (Bailly et al. 2008, Wang et al. 2013). Moreover, this regulatory circuit would suggest a paradox situation in which TWD1 recruits its individual AGC kinase for ABCB phosphorylation, resulting in TWD1–ABCB separation (Fig. 2).

5 Interaction and Polarity Loops Are Interconnected

Auxin transport is thought to employ two main regulatory feedback loops, a transcriptional loop and a posttranscriptional PIN polarity loop (Benjamins and Scheres 2008). In the polarity loop, PIN polarity is regulated by its phosphorylation status provided by protein kinases and phosphatases, represented by PID and PP2A (see Sect. 4; Fig. 3b).

However, a whole series of recent data imply a third protein–protein interaction feedback loop of auxin transport (see Fig. 3a). Therein, distinct ABCB–PIN and ABCB–TWD1 pairings of a putative multi-protein auxin efflux complex are building the basis for a plastic control of auxin streams during PAT. This complex probably does not involve direct TWD1–PIN interaction, which is also supported by the finding that expression and locations of PIN1 and PIN2 are unchanged in *twd1* (Bouchard et al. 2006; Wu et al. 2010).

Endogenous auxin transport inhibitors, that for flavonoids have been suggested and partially also verified, are also part of this regulatory network. The integrative, sometimes even confusing, modulatory effect of flavonoids on auxin transport might result from combinatory effect on ABCB activity and its interaction with TWD1 and additionally on PIN gene expression and cellular trafficking (Peer et al. 2001; Peer and Murphy 2006, 2007; Santelia et al. 2008). Very recent findings suggest that polarity and interaction loops are interconnected via the action of AGC kinases, such as PID (Fig. 3b): Besides decoding PIN polarity, PID also interacts with TWD1, thus negatively regulating ABCB1 activity (Henrichs et al. 2012). At present it is unclear if ABCB1 inhibition is of direct nature or causes indirectly by loss of interaction with TWD1.

Interestingly, flavonoids, such as quercetin, but not NPA are able to interfere with both interaction and polarity loops on different levels (1) direct inhibition of ABCB activity, (2) disrupting TWD1–ABCB interaction, and (3) inhibition of PID that itself has an indirect impact on ABCB1 phosphorylation/activity or PIN polarity. As such, these partially opposite effects reflect pretty well the complexity of flavonol action.

In summary, it appears that auxin controls besides its own biosynthesis and homeostasis also its own transport by interconnected regulatory feedback loops (Benjamins and Scheres 2008). Auxin, in interplay with ATIs, is able to compose the set of its own regulatory machinery according to the developmental stage of the

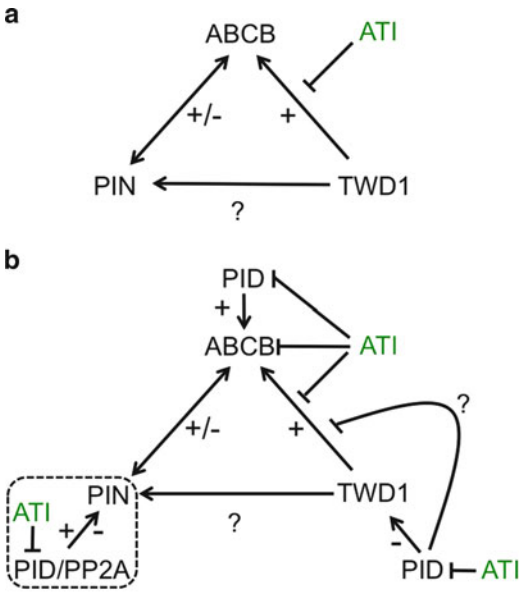

Fig. 3 Interaction and polarity loops of ABCB and PIN-mediated auxin transport are interconnected. (**a**) In a novel interaction loop, TWD1 and PIN proteins positively contribute to ABCB-mediated auxin transport by protein–protein interaction. Polar PINs provide vectorial auxin streams, while TWD1 acts a positive modulator of nonpolar ABCB fluxes. ATIs, such as flavonols, disrupt ABCB1–TWD1 interaction probably by binding to ABCB1, resulting in transport inhibition. *Arrows* denote positive and *bars* negative regulation at the transport level. (**b**) In a previously suggested polarity loop (*dashed line*; modified from Benjamins and Scheres (2008)), PIN polarity is coordinated by the phosphorylation status of PINs controlled by PID/PP2A action. PID interferes with all major components of interaction and polarity loops in an action that is inhibited by ATI/quercetin binding. Note that the negative impact of PID on TWD1–ABCB1 interaction by ABCB1 phosphorylation has not yet been demonstrated

cell and the environmental conditions of the plant. As a consequence, individual transporter and regulatory proteins interact, interfere, and regulate each other in order to allow a fine-tuning of an auxin distribution pattern.

6 Outlook

Upstream events regulating PID—and most probably also TWD1—activity reveal interesting links to known but widely unclear regulatory mechanisms of the auxin transport machinery: Since the 1970s, it is well known that gravity perception and PAT are tightly controlled by intracellular calcium levels (Dela Fuente and Leopold 1973; Toyota et al. 2008a). However, the order and relationship between both signaling pathways are unclear. However, recent work provided evidence that gravistimulation-induced calcium increases constitute an upstream event of PAT (Toyota et al. 2008b).

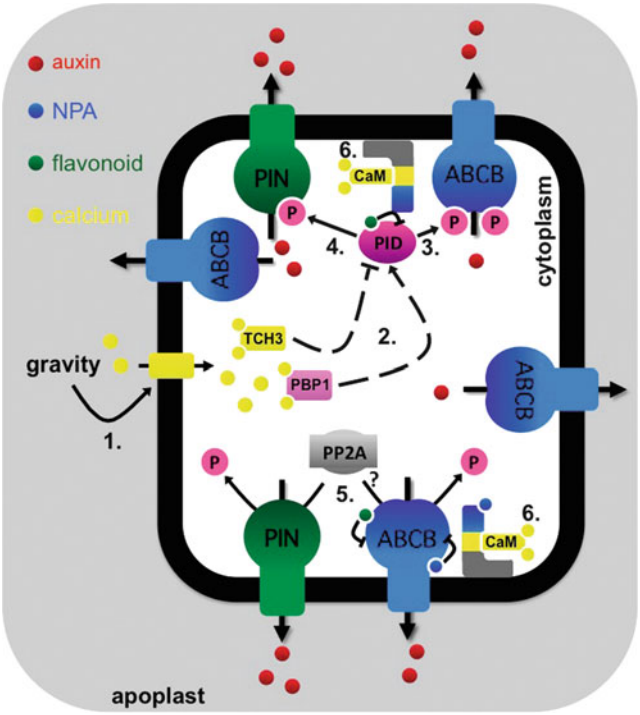

Fig. 4 Overview of protein–protein interactions regulating PIN- and ABCB-mediated auxin efflux. As explained in detail in the text, reversible protein phosphorylation by AGC3 kinases, represented here by PID, have a dual effect on PIN polarity (4) and ABCB transport capacity (3). PID activity is calcium-dependently regulated inversely by the calcium-binding proteins pinoid-binding protein1 (PBP1) and the calmodulin-like TOUCH3 (TCH3) (2). Dephosphorylation of PIN and ABCB proteins by protein phosphatases, such as phosphatase PP2A (5), leads to a top-to-bottom switch of PIN proteins and reversal of PID-mediated ABCB regulation. Note that many of these regulatory processes are directly or indirectly controlled by changes of intracellular calcium concentration provided by plasma membrane calcium channels that itself are under control of auxin-related responses, such as gravitropism (1). Further, besides PID also TWD1 was shown to bind calmodulin with its C-terminal calmodulin-binding domain (6) although the functional relevance of this interaction is still unknown

This is supported by the finding that PID is negatively and positively regulated by protein–protein interaction with calcium-binding proteins, TCH3 and PBP1, respectively. TCH3 is a calmodulin-related protein, and as a consequence, calmodulin inhibitors enhance PID activity (Sistrunk et al. 1994). In this respect, it might be worth recalling that TWD1 itself is calmodulin binding, although the in vivo relevance of this interaction is still unclear (Fig. 4) (Geisler et al. 2003; Kamphausen et al. 2002). Studies on the human homologue of TWD1, FKBP38, show that anti-apoptotic function of HsFKBP38 requires a priori activation by calmodulin activating the *cis–trans* peptidyl-prolyl isomerase (PPIase) activity of HsFKBP38 (Edlich et al. 2005, 2007). Taking the human FKBP38 as an example, a

scenario becomes likely that conformational changes of TWD1 are induced by calcium-dependent binding of a calmodulin-like proteins, such as TCH3, that might affect directly ABCB activity or PID activity and thus indirectly ABCB auxin transport.

Although several lines of evidence also indicate a function for WAG kinases during auxin transport, the regulatory impact of WAGs on the auxin transport machinery remains less clear. Enhanced NPA sensitivity, the fact that WAG kinases, like PID, are polar and plasma membrane-associated, and their enhanced expression in the root tips suggest that PID and WAG kinases act in the same or in a parallel pathway (Santner and Watson 2006), probably by regulation of NPA-binding proteins, like ABCB1, or interactors, like TWD1. Taking into account that members of the AGC3 clade show redundancy during development and toward their calcium-dependent regulation by TCH3 and PBP1 (Dhonukshe et al. 2010; Huang et al. 2010; Robert and Offringa 2008), the specific impact of individual AGC3 kinases in regulation of auxin transport needs to be explored. Therefore, the effect of WAG kinases on PIN protein polarity and ABCB activity needs to be investigated. WAG kinases are rapidly downregulated by light and show a more pronounced effect on root growth; they probably play a role during gravitropism or root development, responses where PID plays a limited role.

Finally, and connected to the above, there is a need to understand how nature was able to dually utilize and separate functionality of AGC kinases, such as PID, for regulation of ABCB activity and PIN polarity. PID coevolved relatively early during plant evolution together with PINs during the origin of land plants, while phot1 seemed to have appeared later during development of seed plants.

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The Role of Auxin Transport and Distribution in Plant Gravimorphogenesis

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Abstract Auxin is one of plant hormones that regulate various aspects of plant growth and development. It has long been proposed that auxin redistribute in response to gravity, which regulates gravimorphogenesis in plants. Recent molecular genetic analysis of *Arabidopsis thaliana* demonstrated that the gravity-regulated auxin redistribution is brought by auxin transport mediated by auxin efflux carriers. On the other hand, we have shown that the gravity-regulated morphogenesis, peg formation, of cucurbit seedlings is also controlled by auxin redistribution. Namely, cucumber (*Cucumis sativus* L.) seedlings have ability to develop a peg on each side of the transition zone between hypocotyl and root but develop one peg on the lower flank of the gravistimulated transition zone because its development on the upper flank is suppressed when the seedlings were grown in a horizontal position. The peg suppression occurs due to a reduction of auxin level on the upper flank. This auxin redistribution appears to involve a cucumber auxin efflux carrier CsPIN1 whose localization changes in response to gravistimulation. Here, we attempt to compare the mechanisms for gravimorphogenesis of cucumber seedlings and for gravity response in Arabidopsis seedlings.

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1 Plant Responses to Gravity

Most plants are sessile organisms and have to spend their entire life cycle at the site of seed germination. The sessile nature of terrestrial plants resulted in the development of various mechanisms to ensure plants survive in stressful environments. For example, plants display tropisms in response to various environmental stimuli. Charles Darwin and his son Francis described some of these responses more than a century ago in their book *The Power of Movement in Plants* (Darwin and Darwin 1881). They noted that plants have the ability to sense their circumstances and orient themselves for optimal growth and development. To date, plants have been shown to respond to diverse environmental signals such as gravity, light, touch, and moisture gradients (Muday 2001; Correll and Kiss 2002; Massa and Gilroy 2003; Takahashi et al. 2009). Over time, plants have adapted to their surroundings with a high degree of plasticity, affording them the ability to respond to ever-changing conditions that provide constant stimulation. One of the most important adaptations that plants have evolved is the ability to sense gravitational forces and adjust their growth patterns accordingly. This gravity-dependent growth and development in plants is called gravimorphism or gravimorphogenesis (Wareing and Nasr 1958; Takahashi 1997; Hoson and Soga 2003). A typical example of gravimorphogenesis is gravitropism, which enables plants to precisely orient their photosynthetic organs to light and develop a root system to anchor themselves and to absorb water and nutrients (Blancaflor and Masson 2003). Through the study of *Arabidopsis thaliana*, the plant hormone auxin was shown to be crucial for the process of gravitropism (Muday 2001; Takahashi et al. 2009). To comprehensively understand the mechanisms of gravimorphogenesis in plants, however, we have to compare the molecular mechanisms for gravimorphogenesis not only in Arabidopsis but also in various other plants. Peg formation in the Cucurbitaceae family was previously shown to be a unique form of gravimorphogenesis, and we showed that auxin plays an important role in the gravity-regulated peg formation (Takahashi 1997; Watanabe et al. 2012). Comparing the molecular mechanisms of gravitropism of Arabidopsis with those of gravimorphogenesis of cucumber seedlings would enhance our understanding of the shared or divergent mechanisms underlying gravimorphogenesis in plants. In this chapter, we will review the mechanisms for shoot and root gravitropism in Arabidopsis and those for gravimorphogenesis in cucumber seedlings, with a particular focus on the role of auxin. We will then discuss both common mechanisms and species-specific mechanisms.

2 Gravitropism Mechanisms in Arabidopsis Seedlings

2.1 Root Gravitropism

Root gravitropism involves a succession of physiological steps (1) gravity perception, (2) gravity signal occurrence (primarily mediated by the columella cells of the

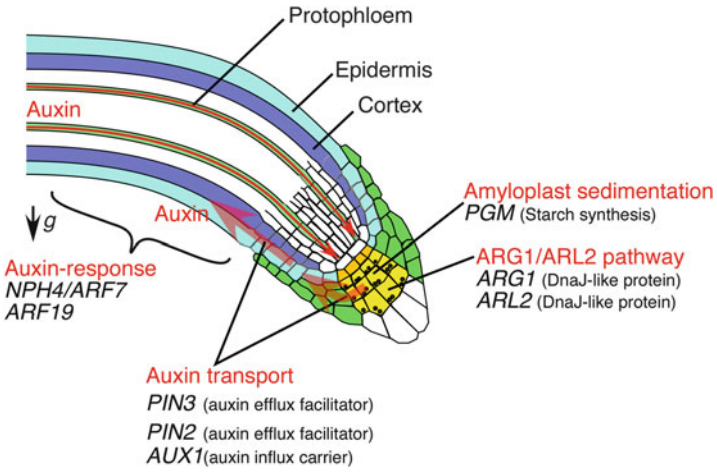

Fig. 1 The molecular mechanisms involved in gravitropism in *Arabidopsis* roots. In *Arabidopsis* roots, gravity perception primarily involves the sedimentation of amyloplasts within specialized cells (statocytes) located in the columella region of the root cap. Amyloplasts store starch grains within their stretched membranes, and *PHOSPHOGLUCOMUTASE* (*PGM*) is involved in starch biosynthesis; thus, this is important for amyloplast sedimentation. DnaJ-like proteins ARG1 and its paralog ARL2 also act in root statocytes to facilitate gravitropism (Boonsirichai et al. 2003; Guan et al. 2003). In gravistimulated roots, auxin is redistributed asymmetrically across the root cap and transmitted to the elongation zone where it promotes a gravitropic curvature. This redistribution of auxin is mediated by auxin influx and the efflux carriers AUX1, PIN2, and PIN3 (Friml et al. 2002; Wisniewska et al. 2006; Kleine-Vehn et al. 2010). ARFs function in auxin-mediated transcriptional activation/repression, and the observation that the roots of *arf7 arf19* have reduced gravitropic curvatures when seedlings were reoriented by 90° suggests that the auxin response is also involved in root gravitropism (Okushima et al. 2005)

root cap), (3) signal transmission from the root cap to the elongation zone, and (4) differential growth in the elongation zone and the curvature response (Perrin et al. 2005). In plant roots, gravity perception occurs in root cap cells. The tip of the root consists of the root cap and the root apical meristem. In some plant species, such as maize, the root cap can be removed from the root tip without damaging root growth. Such decapped roots lose the gravitropic response, but this response is recovered following the regeneration of a new root cap (Barlow and Sargent 1978). These results suggest that the root cap is important for gravity perception in plant roots. In Arabidopsis, the root cap is comprised of four tiers of columella cells that originate from columella initial cells and lateral root cap cells, which are derived from the same source as root epidermal cells (Figs. 1 and 2) (Dolan et al. 1993). Laser ablation of specific regions of cells in the root cap showed that the inner cells of the second tier of columella cells contribute greatly to root gravitropism (Blancaflor et al. 1998). This result suggests that the central columella cells in the root cap are the most important sites for gravity sensing in root gravitropism. These cells are called statocytes and they contain plastids in which starch is highly accumulated (Haberlandt 1900; Nemec 1900; Morita 2010). Such plastids, known

as amyloplasts, are considered to act as statoliths in gravity perception. Kiss and his coworkers confirmed that starchless mutants of Arabidopsis and *Nicotiana sylvestris* show reduced gravitropic sensitivity in roots (Kiss et al. 1989, 1996, 1997; Kiss and Sack 1989). They compared the kinetics of gravitropism in this starchless mutant with wild-type Arabidopsis and showed that wild-type roots are more responsive to gravity (Kiss et al. 1989). In addition, when a starchless mutant was grown under constant 5 *g* conditions produced with a centrifuge, hypergravity was found to completely restore the gravitropic response of the mutant by causing the plastids that lacked starch to undergo sedimentation (Fitzelle and Kiss 2001). These results suggest that, although starch is necessary for the full sensitivity of gravitropism, it is not required for gravity perception itself and that the sedimentation of plastids that function as statoliths is an important step for gravity perception in roots.

Actin microfilaments were thought to play a major role in gravity perception. Sedimenting amyloplasts were hypothesized to distort the actin network and prompt the conversion from a gravity signal to a cellular signal (Sievers 1991; Perbal and Driss-Ecole 2003). In addition, the localization of endoplasmic reticulum at the periphery in columella cells and the function of the endoplasmic reticulum as an intracellular calcium reservoir were hypothesized to be important in the gravity signal-transduction mechanism in columella cells (Perbal and Driss-Ecole 2003). However, there is no direct evidence supporting roles for actin microfilaments or calcium in this process. An Arabidopsis mutant, *altered response to gravity 1* (*arg1*), shows reduced gravitropic responses in hypocotyls and roots (Boonsirichai et al. 2003). This mutation affects the early steps of gravity sensing by disrupting the interaction between plastids and the actin cytoskeleton (Guan et al. 2003; Harrison and Masson 2008; Stanga et al. 2009). ARG1, a J-domain protein localized to endomembrane organelles along with its paralog ARG1-LIKE2 (ARL2), acts in the root statocytes and the endodermis of hypocotyls to facilitate gravitropism (Fig. 1) (Boonsirichai et al. 2003; Guan et al. 2003). In the *arg1* mutant, early reactions such as changes in cytosolic pH, which occur in response to gravistimulation in columella cells, are rarely observed. Recently, two enhancers of *arg1* have been reported, called *modifier of arg1* (*mar1*) and *mar2* (Stanga et al. 2009). Both *MAR1* and *MAR2* encode different components of the translocon of the outer membrane of chloroplasts (TOC) complex (Stanga et al. 2009). These authors suggest that there is a functional interaction between plastids and the actin cytoskeleton, possibly via the activities of TOC and ARG1, in the gravity-sensing process.

Auxin (indole-3-acetic acid; IAA) signaling is crucial for many plant growth and developmental processes, from embryogenesis to senescence, and it is thought to play an important role in root gravitropism, i.e., a role in differential root growth in the elongation zone and in transmitting the gravity signal from the root cap to the elongation zone (Niihama et al. 2005). Knight (1806) reported, perhaps for the first time, the plant growth response to the acceleration of gravity. Sachs (1882) developed a clinostat and proposed the concept of plant geotropism. Darwin and Darwin (1881) studied the phototropic response of dark-grown seedlings and showed that tropic responses require signaling materials to travel from the sensing zone to the responding zone of seedlings.

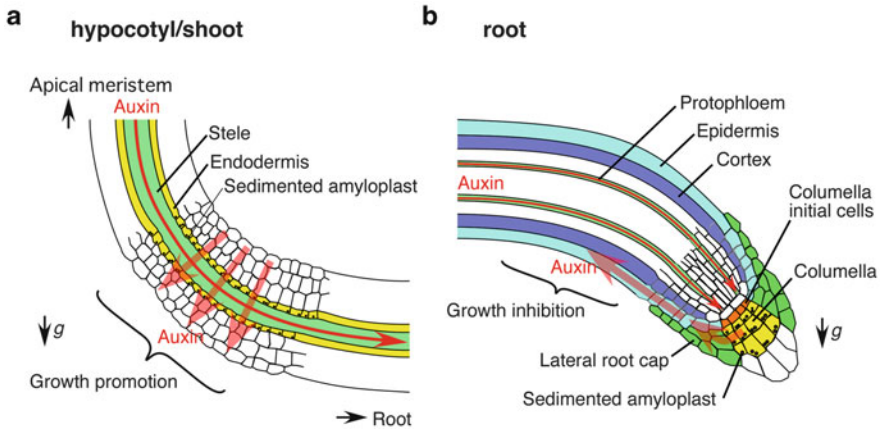

Fig. 2 Gravistimulation modulates the lateral auxin gradient in shoots and roots. Auxin synthesized in cotyledons, shoot apical meristem, and apical zone of the hypocotyl is transported in a basal direction toward the root. The lateral redistribution of auxin through the endodermis is then facilitated in the hypocotyl (**a**) and through root columella cells (**b**)

In the 1930s, Cholodny and Went clarified that the lateral relocalization of the plant hormone auxin occurred in phototropically or gravitropically bending organs (Went and Thimann 1937). This theory is known as the Cholodny–Went hypothesis. Auxin is primarily synthesized in aboveground tissues and is transported basipetally via vascular tissue. The flow of auxin then reverses in root columella cells and is transported to the elongation zone via epidermal cells and lateral root cap cells (Fig. 2) (Peer et al. 2011). Plant roots regulate this apical auxin transport via epidermal cells in a gravity-dependent manner. Auxin is distributed throughout the plant by a network of carrier proteins, and asymmetrically localized plasma membrane PIN-FORMED (PIN) transporters determine the direction of auxin flow (Zhang et al. 2010). In Arabidopsis, eight different PIN proteins were identified (Gälweiler et al. 1998; Müller et al. 1998; Friml et al. 2002, 2003). AtPIN1 is localized at the plasma membrane of the basal end of parenchymatous xylem cells in roots and shoots and regulates auxin transport from shoots to roots (Gälweiler et al. 1998). AtPIN2 is localized at the plasma membrane on the apical side of epidermal cells and plays a role in the transport of auxin from columella cells to the elongation zone (Fig. 1) (Wiśniewska et al. 2006). AtPIN3 is expressed in gravity-sensing tissues within the columella and endodermis and relocalized in the cells after 2 min of gravistimulation (Friml et al. 2002). Thirty minutes of gravistimulation is sufficient to completely relocalize AtPIN3 in the majority of the columella cells (Fig. 1) (Kleine-Vehn et al. 2010). These results suggest that a change in the gravity vector promotes AtPIN3 relocalization to one side of the columella cells and determines the direction of auxin flux, which leads to asymmetric auxin accumulation and differential growth in the elongation zone (Friml et al. 2002).

Most auxin signaling processes are mediated through auxin-regulated gene expression, and transcription of the *AUX/IAA* family of genes was found to be rapidly induced by auxin (Rouse et al. 1998). The expression of the *AUX/IAA* gene family is induced within 10 min after the application of exogenous auxin but is not affected by cycloheximide, an inhibitor of protein synthesis (Hagen and Guilfoyle 2002). Thus, AUX/IAAs are direct targets of auxin-dependent transcription (Hagen and Guilfoyle 2002). Auxin response factors (ARF) are transcriptional factors that bind auxin response element sequences in promoter regions of auxin response genes and regulate the expression of these genes (Guilfoyle and Hagen 2007). In Arabidopsis, there are 23 *ARF* genes. ARFs function in combination with *AUX/IAA* repressors, which dimerize with ARF activators in an auxin-regulated manner (Ulmasov et al. 1997; Tiwari et al. 2004). TRANSPORT INHIBITOR RESPONSE 1 (TIR1) was found to be an auxin receptor that is localized in the nucleus (Gray et al. 2001). Analysis of the crystal structure of TIR1 and pull-down assays demonstrated a direct interaction between TIR1, auxin, and AUX/IAA (Gray et al. 2001; Tan et al. 2007). SCF^{TIR1} is an E3 ubiquitin ligase that ubiquitinates target proteins, marking them for degradation in a 26S proteasome-dependent manner (Gray et al. 1999). From these studies, a model for the mechanisms of cellular auxin signaling was proposed. When the auxin concentration is relatively low, AUX/IAA interacts with the ARF transcription factor and suppresses the expression of auxin response genes. In contrast, when the auxin concentration becomes high, auxin directly binds to the auxin receptor TIR1 and mediates the binding of AUX/IAA to TIR1. This facilitates an interaction between AUX/IAA and TIR1 and enables the degradation of AUX/IAA in a 26S proteasome-dependent manner. Disinhibition of the ARF transcription factor then occurs, which activates the transcription of auxin response genes (Guilfoyle and Hagen 2007). This auxin signaling occurs inside the nucleus. Thus, the nuclear concentration of auxin is thought to play an important role in this process. Many auxin response mutants in Arabidopsis have been reported (Lincoln et al. 1990; Leyser et al. 1993). For example, *auxin resistant1* (*axr1*) transports auxin normally, but the auxin and gravitropic responses in the roots are aberrant (Lincoln et al. 1990; Leyser et al. 1993). *AXR1* encodes E1 ubiquitin ligase, which is required to connect ubiquitin properly to target proteins. Thus, the *AXR1* mutation is thought to lead to abnormal AUX/IAA degradation, which results in atypical continuous auxin responses (Leyser et al. 1993). In addition, the Arabidopsis mutant *auxin resistant3* (*axr3*), a semidominant mutation of *AUX/IAA17*, also shows an auxin-resistant phenotype and aberrant gravitropic responses in roots and shoots. The roots of *arf7 arf19* showed reduced gravitropic curvatures, compared with the wild type, when seedlings were reoriented by 90°, which suggests that auxin-inducible transcription factors are also involved in root gravitropism (Okushima et al. 2005). Collectively, the TIR1- and AUX/IAA-dependent auxin responses in the elongation zone are thought to be necessary for normal gravitropic responses in roots.

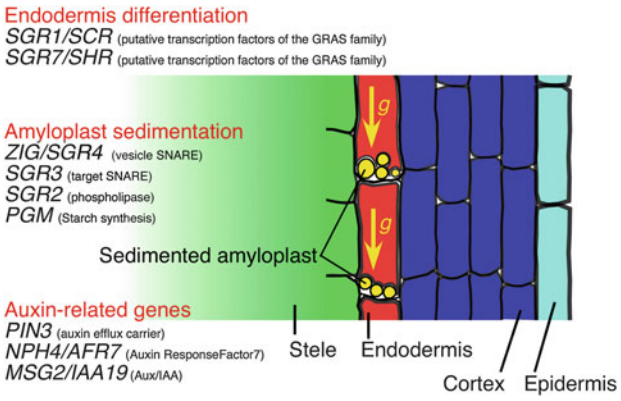

Fig. 3 The molecular mechanisms involved in gravitropism in Arabidopsis shoots/hypocotyls. In Arabidopsis hypocotyls, amyloplast sedimentation occurs in the endodermis, making this an important site for gravity perception in hypocotyls (Fukaki et al. 1998). *PGM* is also involved in starch biosynthesis in a manner similar to that of root columella cells. *SGR3* and *SGR4* encode Qa-SNARE and Qb-SNARE VTIII, respectively, and these proteins act as factors for membrane trafficking to vacuoles via late-stage endosomes (Sanderfoot and Raikhel 1999; Zheng et al. 1999). The *SGR2* gene encodes a novel protein. This gene may be involved in a vacuolar membrane system that affects shoot gravitropism. In endodermal and pericycle cells, AtPIN3 was found to be localized to the vascular bundle side at the plasma membrane (Friml et al. 2002). After gravistimulation, a strong AtPIN3 signal was found to persist in the outer regions of endodermal cells in the lower hypocotyl, whereas in the outer regions of endodermal cells in the upper hypocotyl, the signal gradually weakened (Rakusová et al. 2011)

2.2 *Shoot Gravitropism*

Compared to the studies of the cellular and molecular mechanisms involved in gravitropic responses in roots, information concerning the mechanism underlying the gravity response in hypocotyls and shoots is limited. Studies with a variety of gravitropic mutants confirmed that the molecular mechanisms of the gravitropic responses in roots, hypocotyls, and inflorescence stems are different (Kiss et al. 1989; Leyser et al. 1993; Fukaki et al. 1997; Müller et al. 1998; Harper et al. 2000; Tatematsu et al. 2004). The endodermis is thought to be an important tissue for gravitropic responses of the hypocotyl and shoot because it contains sedimentable amyloplasts (Fig. 3) (Fukaki et al. 1998). The *Arabidopsis* mutants *scarcrow* (*scr*) and *short-root* (*shr*) have completely defective gravitropic responses in hypocotyls and stems. These mutations result in an aberrant endodermal layer and consequently an absence of sedimentable amyloplasts in the endodermis (Fig. 3) (Fukaki et al. 1998). The vacuole has been shown to play an important role in sensing gravitropic stimulation in endodermal cells in aboveground tissues (Morita et al. 2002; Yano et al. 2003). The mutants *shoot gravitropism3* (*sgr3*) and *sgr4* encode soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor Qa-SNARE and Qb-SNARE VTIII, respectively, which act as factors for membrane trafficking to vacuoles via late-stage endosomes (Fig. 3) (Sanderfoot and Raikhel

1999; Zheng et al. 1999). Normally, the vacuolar membrane surrounds the amyloplast in endodermal cells, and the direction of sedimentation depends on the gravity vector, but in *sgr3* and *sgr4* mutants, some amyloplasts in the endodermis fail to sediment in the direction of gravity (Morita et al. 2002). These results suggest that vesicle transport to the prevacuolar compartment in the endodermal cells, which is mediated by a specific SNARE complex, plays an important role in shoot gravitropism (Morita et al. 2002). Recently, AtPIN3, which is strongly expressed in the hypocotyl endodermis, was found to play an important role in asymmetric auxin distribution in hypocotyl gravitropism (Friml et al. 2002; Harrison and Masson 2008; Rakusová et al. 2011). In endodermal and pericycle cells, AtPIN3 is localized in the vascular bundle side of the plasma membrane (Fig. 3) (Friml et al. 2002). After gravistimulation, a strong AtPIN3 signal was found to persist in the outer regions of endodermal cells in the lower hypocotyl, whereas in the outer regions of endodermal cells in the upper hypocotyl, the signal gradually weakened (Rakusová et al. 2011). Therefore, lateral auxin transport depends on the direction of the gravity vector. Furthermore, *massugu1* (*msg1*)/*non phototropic hypocotyl 4* (*nph4*) and *massugu2* (*msg2*) encode *ARF7* and *AUX/IAA19*, respectively, and show abnormal phenotypes in the region of Arabidopsis hypocotyls that bends (Fig. 3) (Watahiki and Yamamoto 1997; Stowe-Evans et al. 1998; Tatematsu et al. 2004). These results suggest that auxin responses that involve the differential distribution of auxin in both sides of the hypocotyl via the ARF-AUX/IAA pathway are required for normal gravitropic responses in Arabidopsis in a manner similar to that of roots.

3 Gravimorphogenesis in Cucurbitaceous Plants: Gravity-Regulated Peg Formation

As described above, the molecular mechanisms involved in gravitropism are gradually being revealed in Arabidopsis. However, the molecular mechanisms underlying gravity-dependent morphogenesis in other plant species are not well understood. Recently, we clarified the mechanisms underlying auxin transport during gravimorphogenesis in cucumber seedlings. We will therefore compare the mechanisms used by these two plants during gravimorphogenesis.

Peg formation in the Cucurbitaceae family has been shown to be a unique form of gravimorphogenesis (Trewavas 1992a, b; Takahashi 1997). A peg protuberance is formed in the transition zone between the hypocotyl and the root (Takahashi 1997). The number of pegs formed in the transition zone depends on the conditions under which the seedlings were germinated. When cucumber (*Cucumis sativus* L.) seeds germinate in a horizontal position, a peg forms on the lower side of the transition zone (Witztum and Gersani 1975). The peg anchors the seed coat to allow the elongation of the hypocotyl necessary to pull the cotyledons out of the seed coat. When cucumber seeds germinate in a vertical position with the radicle pointing down, the seedlings develop a peg on each side of the transition zone (Takahashi et al. 2000). To elucidate the role of gravity in this process, a spaceflight experiment

was conducted in which cucumber seedlings were germinated under microgravity conditions in space (Takahashi et al. 2000). The cucumber seedlings grown under microgravity also formed a peg on each side of the transition zone, indicating that peg formation itself does not require gravity and that cucumber seedlings have the potential ability to form two pegs on the transition zone. This experiment also suggests that peg formation on the upper side of the transition zone is suppressed in response to gravity when seedlings are grown horizontally on the ground (Takahashi et al. 2000).

Auxin plays an important role in gravimorphogenesis by determining the lateral placement of peg formation in the transition zone (Witztum and Gersani 1975; Takahashi and Suge 1988; Takahashi 1997). Both endogenous IAA concentration and auxin-inducible gene expression were found to be significantly reduced on the peg-suppressed side (the upper side) of the transition zone (Fujii et al. 2000; Kamada et al. 2000). The application of exogenous auxin induced the development of a peg on the upper side of the transition zone in a horizontal position (Kamada et al. 2000). Furthermore, the application of an inhibitor of auxin, *p*-chlorophenoxyisobutyric acid, inhibited peg formation (Shimizu et al. 2008). These results indicate that gravity-induced differential auxin-inducible gene expression and suppression of peg development result from a decrease in auxin levels on the upper side of the transition zone. In contrast, the lower side of the transition zone was found to maintain the auxin levels required for peg formation (Kamada et al. 2000, 2003; Shimizu et al. 2008). Treatment of seedlings with the auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) or 9-hydroxyfluorene-9-carbomylic acid (HFCA) blocked the suppression of peg formation on the upper side and induced the development of a peg on each side of the transition zone, even if the seedlings germinated in a horizontal position (Kamada et al. 2003). In addition, differential accumulation of auxin-inducible *CsIAA1* mRNA in the gravistimulated transition zone was inhibited by TIBA treatment (Kamada et al. 2003). According to the Cholodny–Went theory, lateral transport of auxin across gravity-stimulated organs may elicit differential gravitropic growth (Went and Thimann 1937; Trewavas 1992a; Iino 1995). These results suggest that lateral auxin transport, which is modified by gravity, is required for the decrease in the auxin level on the upper side of cucumber seedlings grown in a horizontal position. Kamada et al. (2003) cloned *CsPIN1* cDNA, which encodes a PIN auxin efflux facilitator in cucumber. Watanabe et al. (2012) showed that CsPIN1 is expressed in the endodermal cells of the transition zone at both the mRNA and protein levels and that the localization of the signal pattern of CsPIN1 in horizontally grown seedlings differed from the pattern observed during vertical growth (Fig. 4). When seedlings were grown in a vertical position, the CsPIN1 localization signals were detected on the lateral inner side of the endodermal cells, and signal intensities were almost equal between the two sides of the transition zone, resulting in an equal transport of auxin through the endodermis to the vascular bundles. When cucumber seedlings were grown horizontally, stronger CsPIN1 localization signals were detected on the lateral inner side of the endodermal cells on the upper side of the transition zone compared to the endodermal cells on the lower side (Watanabe et al. 2012), resulting in a decrease in

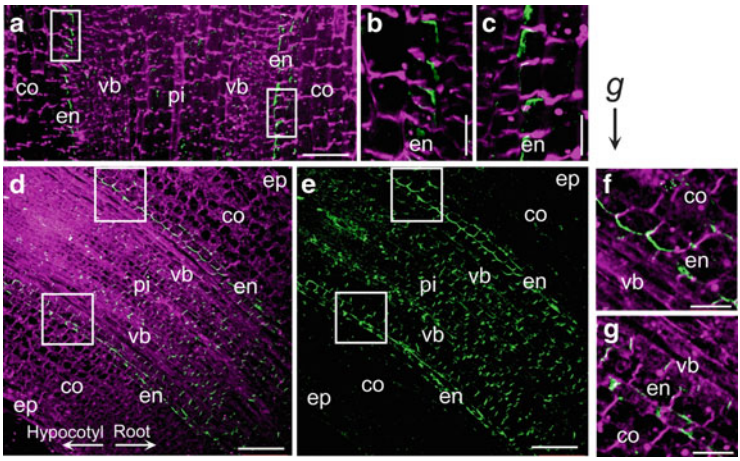

Fig. 4 The immunohistochemical analysis of CsPIN1 of the longitudinal section of the transition zone of cucumber seedlings. The signals for anti-CsPIN1 antibody appeared green in color. The cell walls stained by Fluorescent Brightener 28 appeared magenta in color. Cucumber seedlings were grown in a vertical position (**a**–**c**) or a horizontal position (**d**–**g**) for 24 h. The *left* and *right boxes* drawn by *white lines* in **a** correspond to **d** and **c**, respectively. **e** represents image **d** from which the *magenta color* was removed. The *upper* and *lower boxes* drawn by *white lines* in **d** and **e** correspond to **f** and **g**, respectively. *vb* vascular bundle, *en* endodermis, *pi* pith, *co* cortex, *ep* epidermis, and *g* direction of gravitational force. Bars = 100 μm in **a**, **d**, **e** and 30 μm in **b**, **c**, **f**, **g**. Figure was modified from Watanabe et al. (2012) that belonged to Copyright American Society of Plant Biologists (http://www.plantphysiol.org or http://www.plantcell.org)

auxin concentration in the upper side. Kamada et al. (2003) showed that the free IAA content in the upper peg-suppressed side was significantly less (69.0 ± 11.1 ng/gFW) than that in the lower peg-developed side (94.3 ± 15.6 ng/gFW) in the gravistimulated transition zone of 24-h-old seedlings. The higher level of free IAA in the peg-developed side of the horizontally oriented transition zone was similar to the level in the vertically oriented transition zone; the left side contained 89.2 ± 4.7 ng/gFW free IAA, and the right side contained 91.2 ± 3.6 ng/gFW free IAA. Thus, a decrease in the auxin level on the upper side, and the maintenance of the auxin level on the lower side of the transition zone, is thought to be important for the differences in auxin-inducible gene expression observed between the upper and the lower sides and for the suppression of peg development on the upper side.

From these results, a model of CsPIN1-dependent regulation of lateral auxin transport in the transition zone was proposed (Fig. 5) (Watanabe et al. 2012). CsPIN1 in the vascular bundle facilitates polar auxin transport through the vascular bundle from the apical portion of the seedlings to the root tip. When gravistimulated, the increase in CsPIN1 on the lateral inner side of endodermal cells might act as a barrier to inhibit auxin transport from the vascular bundle to the cortex on the upper side of the transition zone of cucumber seedlings grown horizontally (Watanabe et al. 2012). In contrast, endodermal cells that do not accumulate much CsPIN1 in the lateral inner side might diffusely move auxin from the vascular

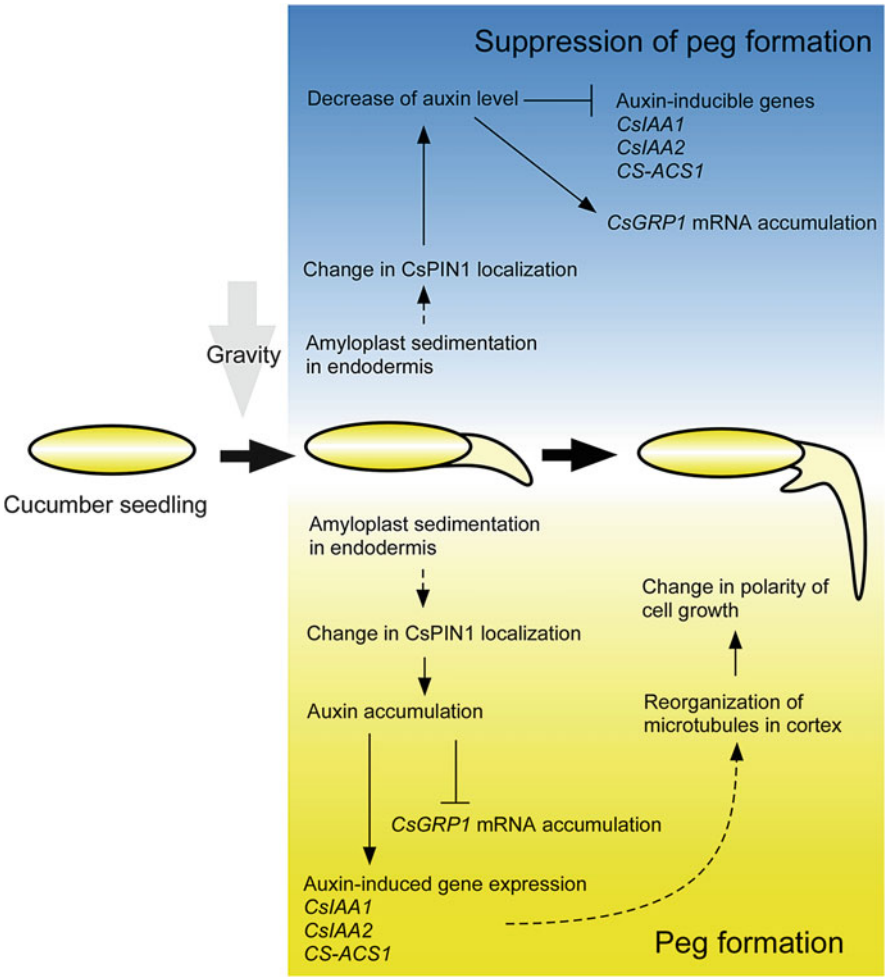

Fig. 5 A model for gravimorphogenesis in cucumber seedlings. When cucumber seedlings are grown horizontally or gravistimulated by reorientation, sedimentation of starch-containing amyloplasts occurs in the gravity-sensing endodermal cells. In the upper sides of the endodermal cells, the localization pattern of CsPIN1 (an auxin efflux facilitator protein) changes and this facilitates lateral transport of auxin from the upper side to the lower side of the transition zone. Consequently, the concentration of auxin decreases in the upper side, whereas it increases in the lower side. The upper side of the transition zone suppresses the expression of the auxin-inducible genes *CsIAA1*, *CsIAA2*, and *CS-ACS1* and induces the auxin-repressed gene *CsGRP1*, thereby suppressing peg formation on the upper side. In contrast, the lower side activates the expression of *CsIAA1*, *CsIAA2*, and *CS-ACS1* and represses the expression of *GRP1*. The orientation of cortical microtubules at the cortex of the lower side continuously changes, resulting in elongation of cortical cells in a perpendicular direction and outgrowth of a peg

bundle to the cortical and epidermal cells in the peg-forming side of the transition zone in cucumber seedlings grown in either horizontal or vertical positions (Watanabe et al. 2012). Other possibilities cannot be excluded at this stage; for an

example, auxin efflux facilitator(s) other than CsPIN1 might be responsible for auxin transfer to the cortex/epidermis in the transition zone of cucumber seedlings. It has been indicated that mRNAs of *CsPIN4* and *CsPIN6* also accumulate abundantly in the transition zone, suggesting that CsPIN4 and CsPIN6 can also be responsible for lateral auxin transport in the transition zones of cucumber seedlings (Watanabe et al. 2012). The Cholodny–Went theory proposes that plants respond to gravity by altering lateral auxin transport, which induces an asymmetric distribution of auxin (Went and Thimann 1937; Evans 1991; Trewavas 1992a; Iino 1995). The gravity responses of CsPIN1 accumulation in endodermal cells may be involved in regulating the responses of endodermal cells to gravity by altering lateral auxin transport, as postulated by the Cholodny–Went theory.

After auxin was transported laterally through the endodermis, the auxin gradient in the transition zone becomes a trigger of differential gene expression in both sides of the transition zone. To investigate the dynamics of auxin distribution in cucumber seedlings, *CsIAA1* auxin-inducible gene were identified (Fujii et al. 2000), and *CsIAA1* mRNA accumulation was analyzed by *in situ* hybridization using horizontally grown seedlings and microgravity-grown seedlings (Kamada et al. 2000). *CsIAA1* expression was higher on the lower side of the transition zone, when the seedlings were grown horizontally (Kamada et al. 2000). On the other hand, when the seedlings were grown vertically or grown under microgravity in space, the differential expression of *CsIAA1* was not observed between each side of the transition zone (Kamada et al. 2000). These results suggest that auxin in the transition zone is asymmetrically distributed in cucumber seedlings grown in a horizontal position, although that is symmetrically distributed in cucumber seedlings grown in a vertical position or under microgravity. *CsIAA2* and *CS-ACS1* (*1-aminocyclopropane-1-carboxylic acid synthase*) are also reported to be auxin-inducible genes and these genes are more expressed in the lower side than the upper side in the transition zone (Saito et al. 2004, 2005). ARFs are transcription factors, which are found to promote early/primary auxin-inducible genes, and transient expression assays demonstrated that some ARFs are activators and other ARFs are repressors of auxin-inducible genes (Ulmasov et al. 1999). Saito et al. (2004) revealed that cucumber CsARF2 is an activator and CsARF5 is a repressor of transcription of auxin-inducible gene, but neither gravity nor auxin affected mRNA accumulation (Saito et al. 2004). This result suggests that asymmetric expression of *CsIAA1* or *CS-ACS1* genes stimulated by gravity are regulating posttranscriptional level of *CsARF*s (Saito et al. 2004).

Shimizu et al. (2006) identified *Cucumis sativus glycine-rich protein1* (*CsGRP1*) as a gene whose mRNA accumulated more abundantly on the upper side than on the lower side of the gravistimulated transition zone, using fluorescent differential display methods. Auxin starvation increased *CsGRP1* mRNA in segments of the transition zone, and inhibition of polar auxin transport with TIBA prevented the asymmetric accumulation of *CsGRP1* mRNA. These results suggest that *CsGRP1* expression is auxin concentration-dependent and gravistimulation increases the

CsGRP1 gene expression on the upper side after gravistimulation and decrement of auxin on the upper side of the transition zone (Shimizu et al. 2006).

Peg develops due to a change in growth polarity of cortical cells (Kobayashi et al. 1999). When colchicine, an inhibitor of tubulin polymerization, was applied to horizontally grown seedlings, the shape of the peg in the lower side of the transition zone became aberrant, demonstrating that microtubules have a role for peg development. When the organization of cortical microtubules were investigated by using 20-h-old horizontally grown seedlings, the microtubules in the lower cortical cells that would develop peg tissue were randomly organized, whereas microtubules in the upper cortical cells were organized transversely to the cell axis. In 24-h-old seedlings, the orientation of microtubules on the upper cortical cells was not changed, but in the lower side, the orientation was arranged perpendicular to the longitudinal axis of the elongating cells. These results suggest that orientation of microtubes is important for peg development after the placement of peg is determined (Fig. 5).

4 Possible Cellular Mechanisms Underlying the Gravity-Regulated Polarization of CsPIN1 in Cucumber Seedlings

The transition zone of cucumber seedlings contains sedimentable amyloplasts in the endodermal cells (Takahashi and Scott 1994). Therefore, the mechanism underlying gravisensing and the subsequent graviresponse in the cucumber transition zone is expected to be similar to the one responsible for Arabidopsis gravitropism in hypocotyls and stems. In the transition zone of cucumber, there is an abundant accumulation of *CsPIN1* mRNA in the endodermis, which is similar to the localization of *AtPIN3* mRNA (Friml et al. 2002). AtPIN3 is strongly expressed in the hypocotyl endodermis and is known to play an important role in asymmetric auxin distribution and hypocotyl gravitropism (Friml et al. 2002; Harrison and Masson 2008; Rakusová et al. 2011). Thus, it is worthwhile to compare the cellular mechanisms underlying AtPIN3 and CsPIN1 protein relocalization in response to gravity (Fig. 6). In hypocotyls of transgenic Arabidopsis plants expressing the yellow fluorescent reporter *AtPIN3-YFP* driven by the endodermis-specific *SCR* promoter, a strong AtPIN3-YFP signal was detected at both the inner and outer lateral sides of endodermal cells (Ding et al. 2011). When plants were gravistimulated by placing the hypocotyl horizontally, asymmetry in the localization of AtPIN3-YFP gradually developed in the endodermal cells, i.e., an initially strong AtPIN3-YFP signal in the lower and the upper hypocotyl sides persisted in the lower sides but not the upper sides of endodermal cells.

Pharmacological studies using the protein biosynthesis inhibitor cycloheximide and the protein degradation inhibitor MG132 showed that *de novo* protein synthesis and proteolytic protein degradation are not necessary for gravity-induced AtPIN3 polarization. PIN proteins are known to undergo constitutive cycling between the

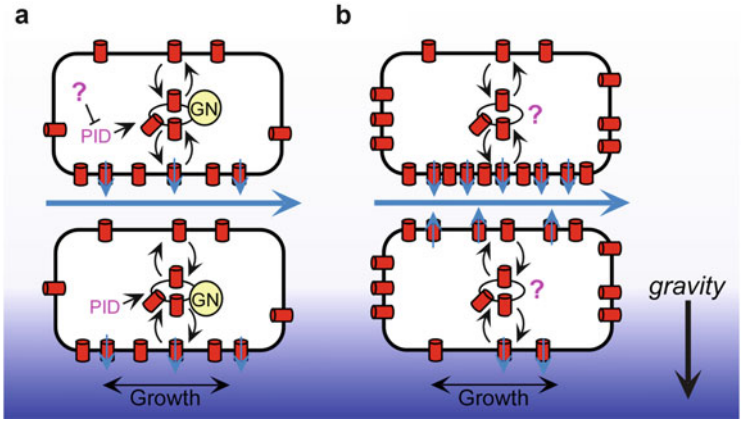

Fig. 6 Schematic representation of models for the gravity-regulated polarization of AtPIN3 and CsPIN1. (**a**) Hypocotyl AtPIN3 polarization due to gravistimulation. After gravistimulation, AtPIN3 moves to the lower side of endodermal cells within both the upper and lower sides of the hypocotyl (Rakusová et al. 2011). (**b**) CsPIN1 polarization due to gravistimulation in the transition zone of the cucumber seedlings. After gravistimulation, a CsPIN1 signal persists in the inner sides of endodermal cells on the lower side of the transition zone, whereas in the inner sides of the cell on the upper transition zone side, the signal becomes considerably stronger after gravistimulation for 30 min (Watanabe et al. 2012). *Red tube*, AtPIN3 (**a**) and CsPIN1 (**b**); *blue arrows*, auxin flux. The auxin gradient is depicted by a *blue-colored gradient* in the background

plasma membrane and endosomes. It was suggested that, in addition to the plasma membrane localization of auxin transport components, vesicle-mediated endocytic cycling is an integral component of the auxin transport machinery (Geldner et al. 2001; Kleine-Vehn et al. 2006; Dhonukshe et al. 2007). One of these processes, GNOM-dependent vesicle trafficking, is inhibited by brefeldin A (BFA) (Geldner et al. 2001). GNOM is an ADP-ribosylation factor-guanosine nucleotide exchange factor (ARG-GEF). BFA inhibits the ARF-GEF activity that converts inactive GDP-bound ARF to an active GTP-bound form (Peyroche et al. 1999). Whether ARF-GEF is BFA-resistant or BFA-sensitive is determined by the sequence of amino acid residues conserved in the exchange domain of ARF-GEF. Some of these residues were shown to be critical for the BFA resistance of ARF-GEFs (Geldner et al. 2003). Substitution of amino acids from the BFA-sensitive type to the BFA-resistant type can confer BFA resistance to a GNOM that is BFA-sensitive. BFA treatment of Arabidopsis expressing BFA-sensitive GNOM inhibited not only the polarization of AtPIN3 but also the asymmetric expression of an auxin-reporter gene (*DR5*::*GFP*) and the bending response of hypocotyls in response to gravity. The expression of the BFA-resistant GNOM reestablished the polarization of AtPIN3 and the bending response of hypocotyls in response to gravity. These results suggest that gravity-induced AtPIN3 polarization and sequential asymmetric auxin distribution and gravitropic responses in the hypocotyl require BFA-sensitive, GNOM-dependent vesicle trafficking (Fig. 6a) (Rakusová et al. 2011). In contrast, after gravistimulation in the transition zone via the reorientation of

cucumber seedlings, CsPIN1 signals persisted in the inner sides of endodermal cells on the lower sides of the transition zone, whereas in the inner cell side on the upper side of the transition zone, the signals became considerably stronger after gravistimulation for 30 min. This response more closely resembled accumulation toward one side in response to gravity rather than polarization (Fig. 6b). As previously discussed, the responses of AtPIN3 and CsPIN1 to gravistimulation were apparently different; however, the change of PIN localization in the upper side of the tissues, an event that occurred in both of these plants, was the same.

In contrast to AtPIN3, which requires GNOM ARF-GEF, the molecular mechanisms of CsPIN1 accumulation are still an open question. One possibility is that auxin flow is diverted in response to the differential targeting of CsPIN1 to the plasma membranes of endodermal cells in the upper and the lower sides, resulting in asymmetric auxin distribution in response to gravity. Watanabe et al. (2012) also employed the auxin transport inhibitors TIBA and 2-(1-pyrenoyl) benzoic acid, which induces peg formation on the upper side of the transition zone of cucumber seedlings grown horizontally. TIBA functions as an actin stabilizer and inhibits several actin-based vesicle trafficking processes, including auxin transport-dependent processes in plants (Dhonukshe et al. 2008). Immunohistochemical studies showed that treatment with 10^{-4} M TIBA effectively inhibited the accumulation of CsPIN1 that occurs after its relocalization to the lower side of the upper endodermal cells after 1 h of gravistimulation (Watanabe et al. 2012). In addition, the auxin content did not increase in the lower side of the transition zone. These results helped confirm the theory that CsPIN1 is a regulator of lateral auxin transport in the transition zone and that an actin-dependent vesicle trafficking process is involved in the relocalization of CsPIN1 to the endodermal cells during the gravity response in the transition zones of cucumber seedlings.

Auxin flow also depends on the polar subcellular localization of PIN auxin efflux regulators. In Arabidopsis, many mutants have been identified, which alter the auxin transport rate, polarity, or the abundance of PIN proteins. Genetic and pharmacological studies have indicated that phosphorylation is involved in the regulation of PIN-dependent auxin transport (Benjamins et al. 2001). PINOID encodes a protein-serine/threonine kinase and controls auxin distribution through the positive control of the subcellular localization of PIN auxin efflux facilitators (Friml et al. 2004). Overexpression of the PINOID protein kinase induces a basal-to-apical shift in PIN localization, resulting in the loss of auxin gradients and defects in embryo and seedling roots. Conversely, the *pinoid* mutation induces an apical-to-basal shift in PIN1 polar targeting at the inflorescence apex, which is accompanied by defective organogenesis. These results indicate that PINOID-dependent phosphorylation leads to apical PIN localization, whereas reduced phosphorylation leads to basal PIN targeting and mediates changes in auxin flow responsible for patterning processes (Friml et al. 2004). Michniewicz et al. (2007) identified PP2A phosphatase as a regulator of PIN apical-basal targeting and auxin distribution. This group demonstrated that PP2A and PINOID partially co-localize with PINs and work as antagonists of the phosphorylation of the central hydrophilic loops of these proteins, thereby mediating PIN apical-basal polar targeting (Michniewicz et al. 2007; Li et al.

2011). PINOID-dependent phosphorylation of PIN protein was also found to occur in rice and maize (Morita and Kyozuka 2007; Skirpan et al. 2009). PINOID is also known to affect gravitropic responses in Arabidopsis roots (Sukumar et al. 2009). Basipetal auxin transport and gravitropism were shown to be reduced in *pid-9* seedlings in Arabidopsis, although the membrane asymmetries of PIN proteins were unchanged (Sukumar et al. 2009).

5 Concluding Remarks

Here, we have made an attempt to compare the mechanisms involved in gravimorphogenesis of cucumber and the mechanisms of gravity response in Arabidopsis. From our results, common mechanisms that exist in some gravimorphogenesis have begun to appear. Namely, auxin is used as signaling molecules for gravity signal transduction, the gradient of auxin concentration is formed between two sides of the gravistimulated organs, and auxin efflux carrier PIN proteins are used for differential auxin distribution after gravistimulation. In addition, gravity-dependent PIN relocalization inside the gravisensing columella or endodermal cells is also the common response both in cucumber and Arabidopsis. This relocation of PIN proteins is an early response; thus, it is considered to be helpful to change the direction of auxin transport rapidly after gravity sensing occurs. It is quite interesting that peg formation is species-specific morphogenesis of cucurbit but would have the same kinds of mechanisms in gravity responses. The knowledge underlying the mechanism of how the relocalization of AtPIN3 or CsPIN1 in response to gravity induces asymmetric redistribution of auxin is still limited, but we believe that these mechanisms should be conserved.

Gravity also influences various aspects of growth and development in addition to gravitropism. For example, plants synthesize tough cell walls to withstand gravitational forces (Hoson and Soga 2003), and the graviresponse is involved in apical dominance, in which the shoot apical meristem rather than the axillary buds dominates shoot growth (Cline 1997; Kitazawa et al. 2008). Oscillatory movements (circumnutation or winding), which help plant organs grow upward toward suitable environmental cues, have also been hypothesized to be gravity-dependent (Hatakeda et al. 2003; Kitazawa et al. 2005, 2008). To date, there are few reports that polar auxin transport is involved in these gravimorphogenetic processes. Further studies are needed to elucidate the intimate system of gravimorphogenesis and the role of polar auxin transport in gravistimulated plant organs.

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Polar Auxin Transport Regulation in Plant–Microbe Interactions

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Abstract Both symbiotic and pathogenic microorganisms can alter the growth and development of plant hosts. The phytohormone auxin controls cell division, cell enlargement, and organogenesis and is thus a likely target for microorganisms that manipulate plants. Some microorganisms can synthesize auxin in the rhizosphere. Others synthesize specific signals that indirectly alter the plant auxin accumulation by altering auxin transport. This chapter highlights those plant–microorganism interactions in which auxin transport is targeted by symbionts and pathogens to manipulate the development of their plant host. The mechanism of auxin transport regulation by microorganisms is largely unknown, but possible mechanisms that have been studied in model organisms include the induction of plant flavonoids that indirectly alter auxin transport during nodulation and the direct targeting of auxin transporters by nematode effectors.

1 Introduction

1.1 Root–Microbe Interactions

Soil-grown plants are surrounded by a complex mixture of microorganisms that can live on plant surfaces or inside plant tissues as endophytes. Some of these microorganisms can alter the plant architecture, for example, by inhibiting or stimulating plant growth or by inducing the development of specific structures on roots and stems. For instance, symbiotic nitrogen-fixing bacteria called rhizobia cause nodulation on legume roots, which requires the initiation of cell cycle activity in root pericycle and cortical cells (Foucher and Kondorosi 2000); plant-growth-promoting

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rhizobacteria (PGPR) and mycorrhizal fungi can stimulate root growth or root branching (Gianinazzi-Pearson 1996; Persello-Cartieaux et al. 2003); plant-parasitic nematodes (PPN) can cause the development of root galls on a wide range of hosts, which involves extensive changes in cell division and differentiation leading to feeding structure formation (Goverse et al. 2000a); pathogenic *Agrobacterium* species trigger tumor formation in stems and roots of host plants (Escobar and Dandekar 2003); and certain pathogenic fungi and bacteria can lead to root and shoot deformation through galling and fasciation (Jameson 2000).

It has been suggested that in many cases these plant developmental changes are mediated by the manipulation of the phytohormone balance by microorganisms (Hirsch et al. 1997). Several hormones have been implicated with plant developmental changes caused by microorganisms. Auxin is crucial for regulating plant organogenesis (Kepinski and Leyser 2003) and for cell cycle regulation (Vanneste et al. 2005) and is therefore a likely intermediate signal to be involved in developmental changes caused by microorganisms. The formation of plant organs requires the correct localization, transport, and concentration of auxin, and alteration of auxin gradients leads to changes in plant growth and organ formation (Benkova et al. 2003; Friml 2003). There is evidence that the auxin transport machinery is one of the main targets of microorganisms infecting plants. This chapter highlights some well-studied plant–microorganism interactions and examines possible mechanisms of auxin transport manipulation by microorganisms that are involved in developmental responses in their hosts.

1.2 Regulation of Auxin Transport in Plants

All higher plants synthesize the plant hormone auxin. Indole-3-acetic acid (IAA), the most abundant form of auxin in most plants, can be synthesized from a tryptophan-dependent or a tryptophan-independent pathway (Woodward and Bartel 2005). Some auxin is stored in a conjugated, inactive form, but this pool can be reactivated through hydrolysis (Ljung et al. 2002). Auxin can be permanently inactivated by oxidation. IAA synthesis takes place mainly in young shoot tissues, from which it is transported to other parts of the plant, although other tissues, including the root, also have the capacity to synthesize auxin (Ljung et al. 2001, 2005). Auxin is transported from the shoot to the root tip through the vascular tissue and from the root tip to the elongation zone through epidermal and root cap cells (Mitchell and Davies 1975). Polar and local transport of auxin along and across plant tissues is important for auxin localization. At least two mechanisms of auxin transport have been demonstrated in plants, first via the phloem from source to sink tissues and second by active polar auxin transport (PAT) through auxin transport proteins.

IAA is a weak acid and as such occurs mainly in the protonated form (IAAH) in the acidic cell wall, from which it can diffuse into cells. In addition, auxin is also actively transported into cells by auxin importers of the amino acid permease

families AUX1 (AUXIN RESISTANT1), [LIKE-AUX1], and PGP4, a member of the MDR/PGP (multidrug resistance/P-glycoprotein) families (Terasaka et al. 2005; Yang et al. 2006). Because the pH is higher inside the cell than in the cell wall, auxin deprotonates (IAA^-) and does not diffuse out of the cell. Hence, auxin requires active export by transporters, which are encoded by members of the PIN-FORMED and PGP families (Geisler et al. 2005; Petrasek et al. 2006). The polar localization of PIN proteins on either the basal or apical side of the cell determines the polarity of auxin transport (Wisniewska et al. 2006). It has been shown that mutations or mis-expression of *PIN* genes alters auxin accumulation and subsequently organ development (Benkova et al. 2003; Friml 2003; Vieten et al. 2007).

Regulating the activity, localization, and internalization of auxin transport proteins can alter auxin transport. The synthetic auxin transport inhibitors NPA (1-*N*-naphthylphthalamic acid) and TIBA (2,3,5-triiodobenzoic acid) have been suggested to interfere with PIN activity by binding to MDR and PGP proteins and by altering intracellular cycling of PIN proteins between endosomal vesicles and the plasma membrane (Dhonukshe et al. 2008; Geisler et al. 2005; Muday and DeLong 2001; Murphy et al. 2002; Noh et al. 2001). The intracellular compartmentalization of auxin by endosomal trafficking is a necessary and sufficient mechanism that can explain the creation of local auxin gradients that regulate plant organogenesis (Kleine-Vehn et al. 2011; Wabnik et al. 2011). The trafficking of PIN proteins can also be modulated by protein modification. For example, ubiquitination of PIN2 leads to its degradation in the vacuole (Leitner et al. 2012), while PIN phosphorylation can change its polarity in the cell (Dai et al. 2012; Kleine-Vehn et al. 2009). In addition, plant mutants affected in calcium signaling were shown to have defects in polar auxin transport regulation (Zhang et al. 2011).

Auxin transport mediated by PIN and PGP proteins can also be modulated by secondary compounds in plants. Certain metabolites of the flavonoid biosynthetic pathway have been shown to act as natural auxin transport modulators through the regulation of PIN activity and localization (Peer and Murphy 2007). Flavonoids are phenylpropanoid metabolites of higher plants with a range of functions (Winkel-Shirley 2001). Specific flavonoids that inhibit auxin transport were shown to compete with synthetic auxin transport inhibitors (i.e., NPA and TIBA) for microsomal and plasma membrane and binding sites (Bernasconi 1996; Jacobs and Rubery 1988; Stenlid 1976). Flavonoids can also affect polar auxin transport by interacting with PGP proteins as well as with an aminopeptidase (Bernasconi 1996; Murphy and Taiz 1999). Arabidopsis mutants lacking flavonoids showed altered expression and localization of certain PIN proteins, suggesting that flavonoids could act by targeting PIN intracellular cycling (Peer et al. 2004). Flavonoid-deficient *Arabidopsis* and *Medicago* sp. plants show higher rates of auxin transport, whereas mutants overaccumulating flavonols are characterized by decreased auxin transport rates (Brown et al. 2001; Murphy et al. 2000; Peer et al. 2004; Wasson et al. 2006). However, the localization of PIN proteins may not be directly regulated by flavonoids but by auxin localization itself in a positive feedback loop (Peer et al. 2004).

Flavonoids are possible targets for the regulation of auxin transport by microorganisms because bacteria, nematodes, and fungi have all been shown to trigger flavonoid accumulation in plants (Dakora and Phillips 1996; Harrison and Dixon 1993; Hutangura et al. 1999; Mathesius et al. 1998a; Stafford 1997).

Other plant hormones have been shown to interact with auxin transport regulation. For example, ethylene can inhibit auxin transport (Burg and Burg 1966; Morgan and Gausman 1966; Prayitno et al. 2006), although its mechanism of action remains unclear. In *Medicago truncatula* mutants, altered ethylene signaling was shown to alter the expression of PIN genes (Prayitno et al. 2006). Ethylene could also act via the induction of flavonoids, which could indirectly affect auxin transport (Buer et al. 2006). Ethylene synthesis is targeted by many plant-interacting bacteria and fungi (Gamalero et al. 2008; Guinel and Geil 2002) and could thus mediate changes in auxin transport or signaling. In addition to ethylene, cytokinin signaling was shown to interfere with polar auxin transport by targeting the endosomal cycling of PIN proteins, leading to degradation of PIN proteins in the vacuole (Marhavy et al. 2011).

Auxin has many effects in plants, and these are mediated through the action of auxin-regulated genes. Auxin response genes contain characteristic promoter elements (auxin-responsive elements, AuxRE) that are regulated by transcription factors of the ARF (auxin response factor) family (Guilfoyle and Hagen 2001). In the absence of auxin, auxin-regulated genes are repressed by a protein complex of ARF and Aux/IAA binding to the AuxRE. When auxin binds to one of its receptors TIR1 (transport inhibitor response 1), AFB (auxin signaling F-box) 1, 2, or 3 (Dharmasiri et al. 2005a, b; Kepinski and Leyser 2005), Aux/IAA proteins are degraded by ubiquitination. Degradation of Aux/IAA proteins occurs through the SCF^{TIR1} (SKP1, cullin, and F-box protein, in this case TIR1) complex and leads to auxin-induced gene expression (Badescu and Napier 2006; Tan et al. 2007; Woodward and Bartel 2005). Different combinations of AUX/IAA and ARF proteins can lead to the triggering of a large range of auxin responses related to plant developmental changes (Vanneste and Friml 2012).

2 Microorganisms Alter Auxin Transport in Host Plants

2.1 Root Nodule Development by Rhizobia Involves Local and Long-Distance Changes in Auxin Transport

2.1.1 Evidence for Local Auxin Transport Inhibition by Rhizobia

Nitrogen-fixing bacteria called rhizobia cause the formation of nodules on many legume hosts. The exudation of specific flavonoids from host roots transcriptionally activates the synthesis of rhizobial signal molecules called Nod factors (lipochitin oligosaccharides), which are required for successful infection and nodule induction

(Oldroyd and Downie 2008). The organogenesis of nodules varies in different legume hosts. Nodules can either develop from modified lateral roots or can initiate de novo in pericycle and cortical cells in host roots (Hirsch 1992). Indeterminate nodules are formed in temperate legumes like pea and alfalfa and originate from pericycle and inner cortical cell divisions, resulting in an elongated nodule with a persistent meristem. Determinate nodules are typically formed on (sub)tropical legumes like beans and soybeans; they originate from outer cortical cell divisions and enlargements, which later fuse with dividing pericycle cells. The stimulation of cell divisions in pericycle and cortical cells can be mimicked by alterations of the auxin and cytokinin ratio (Libbenga et al. 1973). In addition, the formation of (uninfected) nodule-like structures can be phenocopied by external application of synthetic auxin transport inhibitors to roots, suggesting that rhizobia induce nodules by interfering with polar auxin transport (Hirsch et al. 1989; Wu et al. 1996).

Multiple lines of evidence have suggested transient inhibition of auxin transport as a prerequisite for nodule organogenesis in indeterminate legumes. Radiolabeled auxin is found at lower levels below the site of rhizobia inoculation in vetch (*Vicia sativa*) and *M. truncatula* (Boot et al. 1999; van Noorden et al. 2006; Wasson et al. 2006). Furthermore, expression of GUS under the control of the *GH3* promoter (an auxin-responsive element) was downregulated following rhizobia inoculation and application of purified Nod factors, in agreement with results obtained from application of synthetic auxin transport inhibitors (Mathesius et al. 1998b). However, the temporary inhibition of auxin transport has so far been only found for indeterminate nodule formation, as determinate nodule formation, such as in *Lotus japonicus* and soybean, did not show such a response following inoculation with their respective symbionts (Pacios-Bras et al. 2003; Subramanian et al. 2006), suggesting different auxin transport requirements in separate nodulation programs.

How is auxin transport inhibition regulated? Experiments using the *GH3*:*GUS* reporter suggested that certain flavonoids mimic the pattern of auxin depletion below the site of Rhizobium infection (Mathesius et al. 1998b). Flavonoids are induced locally by rhizobia (Mathesius et al. 1998a) and could potentially act as auxin transport inhibitors during nodule initiation. This was substantiated by the finding that flavonoid-deficient *M. truncatula* plants were unable to nodulate, and this was accompanied by the inability of infecting rhizobia to inhibit auxin transport (Wasson et al. 2006). In indeterminate legumes such as *M. truncatula*, the flavonols are likely to be the flavonoid subclass which regulates auxin transport (Zhang et al. 2009). Kaempferol was found to be upregulated following rhizobia inoculation and exogenous application of kaempferol was able to restore auxin transport control capability in flavonoid-deficient roots and subsequently reinstated nodulation ability (Zhang et al. 2009). Unlike indeterminate legumes, auxin transport inhibition seems not to be a requirement during nodule development in soybean, a determinate legume (Subramanian et al. 2006). The difference in auxin transport regulation between these two nodulation programs might be reflected in the different locations in which cells initially divide to form a nodule primordium (Mathesius 2008).

Ethylene may also be involved in auxin transport regulation during nodulation because an ethylene-insensitive *M. truncatula* mutant, *skl*, showed increased *PIN1*

and *PIN2* expression after inoculation with rhizobia, and auxin accumulation above the infection site was exaggerated (Prayitno et al. 2006). It is unknown whether ethylene signaling is linked to the regulation of flavonoids or whether it might affect auxin transport and *PIN* expression directly.

Auxin transport inhibition is likely to act downstream of early Nod factor signaling, because synthetic auxin transport inhibitors were able to induce pseudonodules in a range of early nodulation mutants including *nfp*, *lyk3*, *dmi1*, *dmi2*, *dmi3*, *nin*, and *rit1*, but not in the ethylene-insensitive mutant *skl* (Rightmyer and Long 2011). Nodulation in the *skl* mutant was also insensitive to NPA in the presence of rhizobia (Prayitno et al. 2006), suggesting that auxin transport control during nodulation requires ethylene signaling. Nod factor treatment on the roots of WT and the cytokinin-insensitive mutant *cre1* of *M. truncatula*, which is unable to nodulate, revealed that changes in the expressions of certain PIN genes are responsible for PAT regulation in WT but not the *cre1* mutant. This suggests that PIN proteins are required for auxin transport inhibition by rhizobia and that this is partially regulated by cytokinin signaling (Plet et al. 2011).

2.1.2 Auxin Transport Regulation in Developing Nodules

It is likely that the temporary inhibition of polar auxin transport leads to accumulation of auxin at the site of nodule initiation. In *M. truncatula* and white clover, auxin response increased in the earliest dividing pericycle and inner cortical cells of developing nodule primordia (Huo et al. 2006; Mathesius et al. 1998b; van Noorden et al. 2007). In a mature indeterminate nodule, the highest auxin response shifted towards the peripheral cells of the nodule. Unlike indeterminate nodules (which form on roots of *M. truncatula* and white clover), auxin response is localized to the outer cortical cells of the early primordium of determinate nodules, such as those found in *L. japonicus* (Pacios-Bras et al. 2003). Some auxin is also localized to the base of an early forming nodule near the vasculature, but not in the dividing cortical cells (Takanashi et al. 2011). The difference in location of high auxin response might be due to the higher local auxin concentrations contributed by asymmetrical auxin transport within the host.

Cellular auxin exporters of the PIN family have been identified based on sequence similarities with the orthologs in Arabidopsis (ten in *M. truncatula* and two in *L. japonicus*) (Schnabel and Frugoli 2004). Using reporter analysis, Huo et al. (2006) showed the similarity in expression patterns of *MtPIN2* and *AtPIN2* in the roots of the respective plants. Changes in expression patterns of *MtPIN2* during nodule initiation strongly mirror that of early lateral root development in *M. truncatula*. Furthermore, silencing of several *PIN* genes in *M. truncatula* reduced nodulation (Huo et al. 2006).

Apart from auxin exporters, there are five *AUX1-like* (*LAX*) genes, which were also identified in *M. truncatula* and at least one in *L. japonicus* (de Billy et al. 2001; Schnabel and Frugoli 2004). De Billy et al. (2001) reported that expression of three *MtLAX* genes was localized to the early primordia of lateral roots and nodules.

Subsequent maturation of the organs shifted the expression of these genes to the peripheral tissue of nodules and central tissue of lateral roots. The authors concluded that MtLAX-mediated auxin import is critical for both nodule initiation and vasculature differentiation (Fig. 1).

Computer simulations have been used to find out if the observed pattern of auxin accumulation in the cortex of legumes is most likely the result of local auxin synthesis, increased auxin influx, or decreased auxin efflux. The diffuse and broad pattern of auxin responses in the cortex of indeterminate nodules was found to be most likely the result of reduced auxin export. In addition, the changes in auxin accumulation in the inner and outer cortex, observed in indeterminate and determinate nodulation programs, respectively, could be explained by slight lateral shifts in auxin exporter localization (Deinum et al. 2012).

Actinorhizal nitrogen-fixing symbioses are formed between *Casuarina glauca* and the actinomycete symbiont *Frankia*. In *C. glauca*, the ortholog of the Arabidopsis AUX1 was found to be expressed in root cells colonized by the symbiont (Peret et al. 2007). Later, it was shown that a *PIN1*-like gene is specifically expressed in noninfected cells of the actinorhizal nodule (Perrine-Walker et al. 2010). Coupled with computational modeling data, the authors suggested that a PIN1-like and AUX1 protein in *C. glauca* act in concert to actively restrict auxin accumulation in *Frankia*-infected cells. Although isoflavones are found to play a role during *Frankia*-induced actinorhizal symbiosis in *C. glauca*, whether they are necessary for auxin transport regulation remains a question (Auguy et al. 2011).

2.1.3 Long-Distance Auxin Transport Control by Rhizobia

Rhizobia affect not only the local auxin accumulation and transport at the site of nodule development, but they also interfere with shoot-to-root auxin transport via systemic signals. Inoculation of legume roots with rhizobia triggers "autoregulation," a systemic control mechanism that limits the numbers of nodules on a root system (Caetano-Anolles and Gresshoff 1991). Autoregulation mutants are defective in a leucine-rich repeat receptor-like kinase and supernodulate as they are unable to downregulate nodule numbers (Stacey et al. 2006). The *M. truncatula* autoregulation mutant *sunn1* (*super numeric nodules*) is characterized by increased auxin transport from the shoot to the root under uninfected conditions (van Noorden et al. 2006). Whereas inoculation of wild-type plants with rhizobia inhibits shoot-to-root auxin transport, auxin transport in the *sunn* mutant remains unaffected, suggesting that inhibition of systemic auxin transport is part of the autoregulation control (van Noorden et al. 2006). So far, none of the signals regulating the systemic control of auxin transport by rhizobia have been identified. The supernodulation phenotype of the *sunn1* mutant can be rescued by NPA, suggesting that it involves PIN-mediated auxin transport (van Noorden et al. 2006). It is possible that auxin transport control by SUNN is a more general mechanism to control root architecture in response to nitrogen, because the *sunn1* mutant is unable to control lateral root density in response to nitrate through the modulation of auxin transport (Jin et al. 2012).

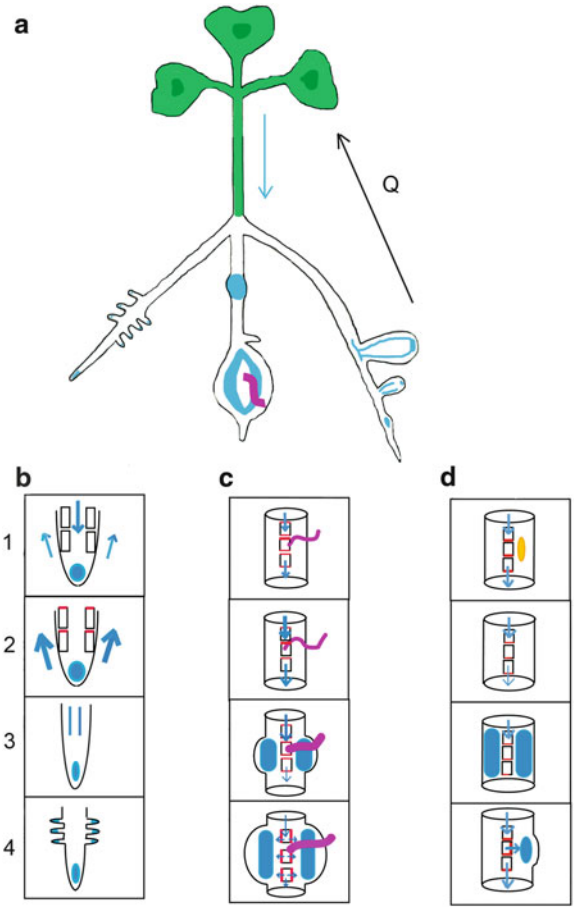

Fig. 1 Models for the targeting of the auxin transport machinery by soil microorganisms. In all figures, auxin responses as determined by *GH3*:*GUS* or *DR5*:*GUS* expression studies are shown in *blue*. Auxin transport is shown as *blue arrows*, with the arrow thickness indicating the amount of auxin transported. (**a**) A plant infected by ectomycorrhizal fungi that cause extensive lateral root formation (*left* root), parasitic nematodes (*pink*) causing the formation of feeding structures (*middle*), and rhizobia inducing development of root nodules (*right*). During nodulation, an unidentified signal is sent to the shoot (Q), which triggers autoregulation, restricting further nodule formation. This is accompanied by reduced auxin transport from the shoot to the root. *Blue shading* indicates sites of auxin response. (**b**) Proposed mechanism of auxin transport regulation by ectomycorrhizal fungi: Auxin accumulates at the root apex (1) and this triggers enhanced expression of PIN2 at the basipetal side of epidermal cells (2) leading to increased auxin accumulation in vascular cells behind the root tip from where lateral roots are initiated (3). This causes increased root branching (4). See Felten et al. (2009). (**c**) Proposed mechanism of auxin transport regulation by cyst nematodes: A nematode-injected effector protein, 19C07, interacts with AUX1 (*red*) to enhance auxin import into a forming feeding structure (1). This is accompanied by reduced auxin export via PIN proteins (2). Auxin accumulates in the forming syncytium (3). Lateral redistribution of PIN proteins then channels auxin towards the edge of the expanding feeding structure (4). See Grunewald et al. (2009) and Lee et al. (2011). (**d**) Proposed mechanism of auxin transport

Ethylene signaling might also play a role in long-distance auxin transport regulation as shoot-to-root auxin transport was insensitive to rhizobia in the *skl* mutant, in contrast to wild-type plants (Prayitno et al. 2006). The mechanism of action of ethylene in shoot-to-root auxin transport is currently not known.

2.2 *Mycorrhizal Fungi Affect the Root Auxin Balance*

Symbiotic mycorrhizal fungi invade and colonize the roots of a large range of host plants, with which they exchange phosphorus and other nutrients for host-derived carbon. Arbuscular mycorrhizal (AM) fungi colonize plants intra- and intercellularly and induce the formation of branched structures called arbuscules in the root cortex, whereas ectomycorrhizal fungi produce extracellular mycelia that form a sheath around root tips and usually lead to root tip bifurcation and arrest of root growth (Barker et al. 1998; Gianinazzi-Pearson 1996; Harrison 2005). Both types of symbiotic fungi can affect root development, usually by increasing the number of lateral roots, probably as a strategy to increase the number of infectable roots. Because auxin is an important regulator of lateral root formation (Fukaki et al. 2007; Peret et al. 2009), it is likely that increased root branching is caused by enhanced auxin synthesis or signaling during mycorrhizal symbiosis. This could be a result of auxin synthesis by the fungal partner. Auxin is synthesized by both AM and ectomycorrhizal fungi and could have direct effects on hosts (Rudawska and Kieliszewska-Rokicka 1997; Sirrenberg et al. 2007). Moreover, mycorrhizal fungi affect the auxin synthesis (Jentschel et al. 2007; Kaldorf and Ludwig-Muller 2000) and the hydrolysis of auxin conjugates in the host (Campanella et al. 2008; Fritze et al. 2005). Root branching can be caused by a diffusible signal exuded from the AM hyphae and requires some of the same signal transduction genes necessary for the successful infection of legumes by rhizobia (Olah et al. 2005). The root branching could not be phenocopied by external application of auxin, suggesting that auxin is not, or not the only, signal involved in root responses to AM fungi (Olah et al. 2005).

However, a specific pattern of auxin localization inside roots might be required for developmental responses, and this is unlikely to be achieved by external auxin treatment. Localization of auxin responses using the auxin sensitive reporter genes showed uneven auxin accumulation in AM-infected roots (Jentschel et al. 2007). Interestingly, mycorrhization can be stimulated by treatment of roots with Nod

Fig. 1 (continued) regulation during indeterminate nodule development: Rhizobia cause flavonoid accumulation (*orange*) and cytokinin signaling at the site of infection in the inner cortex (1). This leads to polar auxin transport inhibition via an unknown mechanism probably involving PIN expression or activity (*red*) (2). Auxin accumulates at the site of nodule initiation (3). Following cell division, auxin is likely to be redirected into the forming nodule via AUX/LAX and PIN proteins (*red*) to regulate nodule organogenesis (4)

factors (which can inhibit auxin transport in legumes, see Sect. 2.1) and the synthetic auxin transport inhibitor TIBA, suggesting that changes in auxin transport might be part of AM formation (Xie et al. 1997). While the signals used by mycorrhizal fungi to potentially affect the auxin transport are unknown, it is unlikely that flavonoids play a role in auxin transport regulation in AM associations because flavonoid-deficient plants still form mycorrhizae (Becard et al. 1995).

In ecomycorrhizal associations of *Laccaria bicolor* with poplar and Arabidopsis, polar auxin transport via PIN proteins is likely to be required for auxin accumulation at root tips and subsequent lateral root initiation by the fungal partner (Felten et al. 2009). The authors of this chapter showed that auxin transport inhibitors prevented *PIN* expression, auxin accumulation, and lateral root initiation by the fungal partner. In addition, Arabidopsis *pin2* mutants showed reduced ability to initiate lateral roots in the presence of the fungus. The most likely model suggested by this chapter is that auxin accumulation at the root apex (by an unknown mechanism) induces expression of the At*PIN2* homolog in epidermal cells, leading to auxin accumulation further basipetal in provascular cells (Fig. 1). This would then increase auxin concentration in pericycle cells and stimulation of lateral root initiation (Felten et al. 2009).

2.3 *Plant-Parasitic Nematodes Alter Auxin Transport to Form Feeding Structures*

Plant-parasitic nematodes induce the formation of feeding sites on many plant hosts. Cyst nematodes (*Globodera* spp. and *Heterodera* spp.) cause the development of cysts on host roots, which are usually initiated in the pericycle, cortex, or procambium, and can later incorporate xylem parenchyma cells that form a syncytium following cell wall dissolution and fusion of multiple cells (Goverse et al. 2000a). Root-knot nematodes (*Meloidogyne* spp.) stimulate vascular parenchyma cells to endoreduplicate, resulting in the formation of multinucleate giant cells (Goverse et al. 2000a). This is usually accompanied by extensive divisions of nearby pericycle and cortical cells. In many cases, the initiation of pericycle cell divisions leads to the development of lateral roots at the base of root galls (Goverse et al. 2000b; Karczmarek et al. 2004). Gall formation is initiated by esophageal gland cell secretions of nematodes, although it is not known which effectors contained in the secretions cause feeding structure formation.

Since auxin was detected in root galls (Balasubramanian and Rangaswami 1962; Yu and Viglierchio 1964), it has been suggested that auxin is involved in the regulation of cell division and differentiation of root galls (Gheysen and Fenoll 2002; Goverse et al. 2000a). It is possible that some auxin is synthesized by the nematodes, although this has been questioned (Goverse et al. 2000b). Alternatively, the infecting nematode secretions could cause changes in auxin metabolism or transport in the host.

Microarray experiments have shown that the expression of a number of genes encoding auxin response and auxin transport proteins is upregulated in nematode galls (Gheysen and Fenoll 2002; Ithal et al. 2007a, b; Klink et al. 2007), although some auxin response genes are also downregulated (Ithal et al. 2007b). In addition, auxin-inducible cell cycle genes were found to be induced (de Almeida-Engler et al. 1999) and auxin-repressed genes were downregulated in cyst nematode-infected roots (Hermsmeier et al. 1998). Increased expression of auxin-responsive promoter: *GUS* and *DR5*:*GUS* fusions was demonstrated in early dividing cells, including feeding cells of galls induced by root-knot (Hutangura et al. 1999; Karczmarek et al. 2004; Wang et al. 2007) and cyst nematodes (Grunewald et al. 2009; Karczmarek et al. 2004; Wang et al. 2007). At later stages of gall development, these auxin responses were downregulated (Fig. 1). The requirement for auxin was confirmed by the fact that auxin-insensitive tomato and Arabidopsis mutants are defective in feeding cell establishment (Goverse et al. 2000b). Interestingly, Arabidopsis mutants defective in auxin transporter-encoding genes were also impaired in syncytium development, and treatment of plants with the synthetic auxin transport inhibitor, NPA, caused severely distorted syncytium development and abnormal cell divisions, suggesting that nematodes could cause auxin accumulation by inhibiting auxin efflux (Goverse et al. 2000b). Similar to nodule development, this was suggested to be mediated by flavonoids, which accumulate in young galls (Hutangura et al. 1999). However, flavonoid-deficient mutants of Arabidopsis were still able to form feeding sites when infected with cyst nematodes (Jones et al. 2007). Similarly, *M. truncatula* hairy roots in which the flavonoid pathway was inhibited by RNA interference against chalcone synthase, the first enzyme of the flavonoid pathway, were capable of forming giant cells after infection with root-knot nematodes, although the galls were found to be smaller (Wasson et al. 2009). Alternatively to the action of flavonoids, auxin could be directed into developing galls and cysts through increased activity of auxin import proteins, and this is supported by strong expression of the gene encoding the auxin importer AUX1 in developing feeding structures of both cyst and root-knot nematodes (Mazarei et al. 2003). Interestingly, the cyst nematode effector HS19C07 from *Heterodera schachtii* was found to interact directly with the LAX3 auxin importer from Arabidopsis (Lee et al. 2011), and both *aux1*/*lax3* and *aux1*/*lax1*/*lax2*/*lax3* mutants showed reduced infectivity with *H. schachtii*. A study utilizing *PIN* mutants and *PIN* reporter genes in Arabidopsis showed that cyst formation requires downregulation of *PIN1* at the initiation stage, probably to cause auxin accumulation. This is followed by lateral auxin transport by PIN3 to redirect auxin flow into a developing feeding site (Grunewald et al. 2009). These studies suggest that the nematodes target both auxin import into the growing feeding structure as well as regulating auxin export laterally to expand cell divisions and fusions.

While most of the active compounds in nematode secretions involved in gall formation have not been identified, apart from the effector 19C07 mentioned above, recent reports suggest that some of the effectors might have been "hijacked" from rhizobia or plants. For example, NodL, an *N*-acetyltransferase required for Nod factors synthesis in rhizobia, is likely to have been acquired by *Meloidogyne* sp.

through horizontal gene transfer (Scholl et al. 2003). Secretions from nematodes also appear to act through some of the same plant receptors as those required for Nod factor perception in legumes (Weerasinghe et al. 2005), and similar expression of meristem-specific genes is induced in developing galls and nodules, suggesting an overlap in signaling required for both developmental programs (Koltai et al. 2001). The presence of a peptide of the plant CLE [CLAVATA3/ENDOSPERM SURROUNDING REGION] family, which control meristem differentiation in plants, was identified from *Heterodera glycines* (Wang et al. 2005), suggesting that nematode CLE peptides mimic their plant counterparts and thus interfere with cell division and differentiation (Mitchum et al. 2008). It would be interesting to test whether nematode CLE peptides interfere with auxin transport or signaling in order to induce feeding site development (Friml 2003).

2.4 Auxin Transport Control by Pathogenic Fungi

While most studies on the modification of auxin transport by microorganisms have focused on biotrophic interactions leading to changes in host development, necrotrophic and hemibiotrophic fungi have recently also been shown to alter auxin transport, and this has been implicated in host resistance to these pathogens. For example, infection of Arabidopsis with *Alternaria brassicola* leads to increased auxin accumulation and response, and this is accompanied by downregulation of auxin transport capacity, most likely mediated through jasmonic acid (Qi et al. 2012). Consistent with this, infection with *A. brassicola* is impaired in the Arabidopsis *doc1* auxin transport mutant (Qi et al. 2012). Similarly, auxin signaling mutants and Arabidopsis plants with altered auxin transport capacity, including the *doc1*, *aux1*, and *pin2* mutants, showed increased infection with the hemibiotroph *Fusarium oxysporum*, while treatment with the auxin transport inhibitor TIBA reduced infection (Kidd et al. 2011). In contrast, auxin synthesis mutants were unimpaired in infection, suggesting a specific requirement for targeting the auxin transport machinery for infectivity. The purpose of the alteration of auxin transport in these pathogenic interactions remains unclear, but it is likely that auxin plays a role in defense responses in these interactions. For example, external application of auxin on tomato plants enhanced pathogenicity symptoms caused by *Pythium ultimatum* (Gravel et al. 2007). Concomitant application of auxin and the pathogen *Pseudomonas syringae* caused enhanced disease symptoms in *Arabidopsis* plants, whereas the auxin-insensitive *axr2-2* mutant was more resistant to *P. syringae* (Wang et al. 2007). Similarly, an auxin-insensitive mutant of *Arabidopsis* was found to be resistant to *P. thivervalensis* infection (Persello-Cartieaux et al. 2001).

Two R-gene products from Arabidopsis, SGT1b (Austin et al. 2002) and its interactor RAR1 (Azevedo et al. 2002), were reported to be required for resistance to downy mildew. The SGT1b protein was subsequently characterized in a screen for factors required for activation of the SCF^{TIR1} complex (Gray et al. 2003), which

is involved in the proteolytic degradation of auxin-regulated proteins through ubiquitination (Gray et al. 2001). These studies suggest that auxin signaling is required for (certain) R-gene-mediated resistance mechanisms and that auxin transport regulation could be one mechanism that microorganisms use to increase auxin in the host.

3 Conclusions and Perspectives

The auxin transport machinery appears to be the target of several microorganisms that infect plants, both symbionts and pathogens. In cases where infecting organisms cause changes to the host root development, like rhizobia and certain plant-parasitic nematodes, auxin export is targeted so that auxin accumulates at sites of new organ formation. The mechanisms used by different organisms vary (Fig. 1). For example, while flavonoids seem to be required for auxin transport regulation by rhizobia causing indeterminate nodule formation, flavonoids are dispensable for determinate nodule formation as well as for nematode feeding site development. Instead, nematodes seem to involve a more direct mechanism of protein–protein interaction between effectors secreted into the host and auxin transport proteins from the plant. Many questions remain in this research area. For example, it is unknown how flavonoids interact with the auxin transport machinery during nodule formation, where flavonoids act, and how they are transported from the site of induction to the site of auxin transport. It is also not known how nematodes cause very specific lateral relocalization of PIN proteins for lateral auxin transport into a forming cyst or gall. In addition, future studies could be aimed at finding out whether changes in auxin accumulation in symbiotic interactions also affect host defense responses, as they appear to do in some pathogenic interactions.

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Auxin Biosynthesis and Polar Auxin Transport During Tropisms in Maize Coleoptiles

Takeshi Nishimura and Tomokazu Koshiba

Abstract In 1880, Charles Darwin and his son published a book, *The Power of Movement in Plants*, in which they described plant tropic behavior. This observation was the first suggestion of the importance of some influence transmitted from the tip to the basal growing parts. Following their suggestion, much research was conducted on plant tropic curvature, which indicated that the influence was a substance, auxin (indole-3-acetic acid; IAA), the first plant hormone to be identified. Tropic responses are generally explained by the Cholodny–Went hypothesis, that is, they occur via differential growth on the two sides of the elongating shoot that results from asymmetrical IAA distribution. In this mini-review, we summarize classic and modern research as the story of tip-specific IAA biosynthesis and its essential role on gravitropic and phototropic curvatures in maize (*Zea mays*) coleoptiles.

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1 IAA Biosynthesis in Maize Coleoptiles

1.1 The Coleoptile Tip Region Is the Site of Vigorous IAA Biosynthesis

Gravitropism and phototropism have fascinated plant biologists since the pioneering work of Charles and Francis Darwin (Darwin and Darwin 1880) more than 130 years ago. These tropic responses of monocot plants, as explained by the Cholodny–Went Hypothesis, occur via differential growth on the two sides of the elongating shoot, which results from the asymmetrical distribution of indole-3-acetic acid (IAA) (Went and Thimann 1937). Thus, it is generally accepted that the apical tip region of monocot coleoptiles is the site of IAA production. Maize coleoptiles in particular have been recognized as an ideal model tissue for research into IAA biosynthesis (Fig. 1). Nevertheless, mainly because of the limitations in accurate IAA quantification from small pieces of plant tissue, only a few works have directly determined IAA levels in maize coleoptiles in detail (Iino and Carr 1982; Koshiba et al. 1995). Recent improvements in the methods of determining IAA and its conjugate or precursor levels have provided many insights into IAA production in the tip. Using a rapid, microscale method for IAA determination by gas chromatography–mass spectrometry, the free and conjugated IAA in maize (*Zea mays* L.) coleoptiles was investigated in detail (Mori et al. 2005). The highest concentrations of IAA (approximately 250 ng/g FW) were found within 0–0.5 mm of the apical tip. These amounts decreased steeply until 2 mm from the top and then gradually decreased further down the shoot, reaching about 13 ng/g FW in the basal region. It was also revealed that about 500 pg/h of IAA was diffused from a 3 mm tip section into an agar block. The total amount of free IAA accumulated in an agar block after 3.5 h incubation was more than six times higher than the free IAA present in the 2 mm tip before incubation. These results provided definite evidence that the apical 2 mm region of maize coleoptiles has the potential to produce IAA de novo, which may lead to a constant flow of IAA toward the base. The fact that decapitation of the coleoptile tip region causes inhibition of gravitropic and phototropic curvature has also supported the coleoptile tip being a source of IAA. Recently, IAA quantification coupled with pharmacological experiments using an IAA transport inhibitor such as 1-*N*-naphthylphthalamic acid (NPA) has provided more definite evidence that the coleoptile tip produces IAA and that IAA is necessary for cell elongation or tropic responses in coleoptiles (Mori et al. 2005; Nishimura et al. 2009). Treatment with NPA caused a large amount of free IAA to accumulate in the 2 mm tip. The accumulation began within 30 min of treatment specifically in the 2 mm tip region. In contrast, in the 2–4 and 4–6 mm regions of the coleoptiles, the IAA levels were not affected by NPA treatment. These results suggested that the inhibition of IAA transport by NPA caused accumulation of IAA only in the IAA synthetic tip tissue. Interestingly, when NPA was applied to only the 0–2 mm tip region, gravitropism and phototropism were completely inhibited (Nishimura et al. 2009).

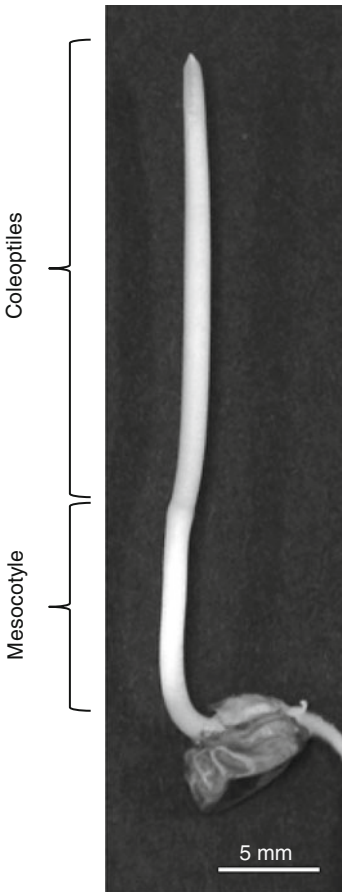

Fig. 1 Four-day-old seedling of maize (*Zea mays* L. cv. Golden Cross Bantam 70)

Although the distribution of putative IAA synthetic enzymes has remained unknown, an immunohistochemical approach was developed to investigate the distribution of IAA synthetic cells in maize coleoptiles using an anti-IAA-C monoclonal antibody (Nishimura et al. 2011). The antibody was raised against carboxyl-linked IAA; therefore, to conserve the antigenicity of IAA, 1-ethyl-3-(dimethyl-aminopropyl)-carbodiimide (EDAC) treatment was necessary as the fixative. EDAC cross-links the carboxyl group of IAA to proteins in tissues and preserves the antigenicity of IAA to this IAA-C antibody. The specificity of the antibody was confirmed by comparing the amounts of endogenous free and conjugated IAA to the signal obtained from the IAA antibody. Depletion of endogenous IAA caused a corresponding decrease in immuno-signal intensity, and negligible cross-reactivity against IAA-related compounds, including tryptophan, indole-3-acetamide, and conjugated IAA was observed. Immunolocalization showed that the IAA signal was intense in the approximately 1 mm tip region and the outer epidermis in the region approximately 0.5 mm from the top of coleoptiles

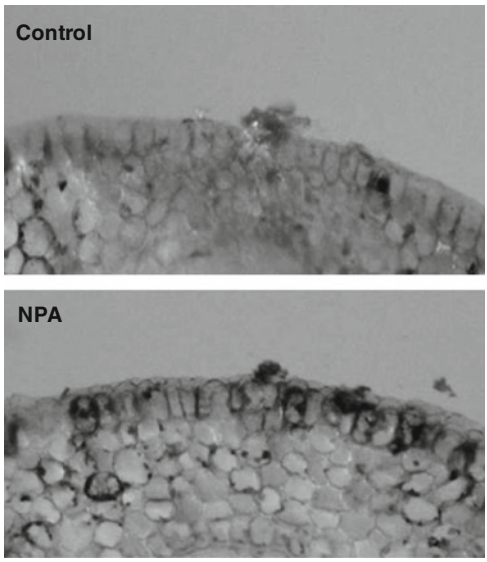

Fig. 2 Immunolocalization of IAA in the outer epidermis of the coleoptile tip. The IAA signal in the outer epidermal cells of NPA-treated coleoptile tips becomes clearly intense compared to the cells in the control. This indicates that the outer epidermis of the tip comprises active IAA synthetic cells

treated with NPA (Fig. 2). Although the distribution of putative IAA synthetic enzymes is unknown, it was proposed that the outer epidermis at the very tip of the coleoptile comprises active IAA synthetic cells. Furthermore, determination of the cellular localization of IAA was attempted using immunogold labeling of IAA with an anti-IAA-N-polyclonal antibody (Schlicht et al. 2006) in the outer epidermal cells preceded by high-pressure freezing/freeze-substitution (HPF/FS). In this experiment, a rabbit IAA-*N*-polyclonal antibody designed to recognize IAA fixed with aldehyde-type fixatives was used to visualize IAA to avoid the disruption of cell structure caused by EDAC. The results showed cytoplasmic localization of free IAA, but none in the cell walls or vacuoles. Therefore, at least in maize coleoptiles, IAA synthesis is restricted to the 0–2 mm tip region, and free IAA in the lower regions has been transported from the tip. Higher contents of IAA in the coleoptile tips were also reported in rice and *Avena*, but the production of IAA in these plants has not been studied in detail.

1.2 IAA Is Synthesized from Tryptophan

Whether IAA biosynthesis in maize coleoptiles occurs via a Trp-dependent or Trp-independent pathway has been discussed for many years, and many experimental results have been obtained in support of each hypothesis. Developing kernels are an active IAA biosynthetic tissue in maize, and relatively large quantities of IAA esters are stored in the endosperm (Epstein et al. 1980). It was reported that transport of such conjugated IAA from seed kernels participates in the production of free IAA at the tip (Cohen and Bandurski 1982). In 1991, the maize mutant *orange pericarp* was identified, a tryptophan auxotroph deficient in detectable

tryptophan synthase beta activity (Wright et al. 1991). As tryptophan biosynthesis is feedback controlled, the *orange pericarp* kernels accumulated large amounts of the tryptophan precursors indole and anthranilate. Because *orange pericarp* seedlings had elevated IAA concentrations in comparison to the wild type, tryptophan-independent IAA biosynthesis was inferred.

In contrast, there has been much experimental evidence indicating that IAA is synthesized via a Trp-dependent pathway. In a modified in vitro kernel culture, [$^{13}C_2$]-tryptophan was added to monitor its incorporation into IAA. Tryptophan was efficiently incorporated into IAA with retention of the 3,3′ bond (Glawischnig et al. 2000), which excludes the possibility that proffered tryptophan is degraded to indole and then serves as an IAA precursor. This tryptophan labeling experiment provided evidence for tryptophan-dependent IAA biosynthesis in maize kernels. In the coleoptiles, tracer experiments with radiolabeled Trp indicated that IAA was synthesized from Trp in the tip both in vivo (Koshiba et al. 1995) and in vitro (Koshiba and Matsuyama 1993). Recent work using stable isotope-labeled Trp showed in more detail the incorporation site of the isotope label in IAA (Nishimura et al. 2006, 2009). When varying concentrations of [$^{13}C_{11}{}^{15}N_2$]-Trp were applied to the tip region, the effective incorporation of the labeled Trp into free IAA in the 3 mm tip and the IAA transported from the tip were observed after incubation with no significant changes in IAA amounts of the buffer control. [$^{13}C_{10}{}^{15}N_1$]-IAA was detected in the experiment, indicating that the IAA production from applied Trp does not occur via indole (Fig. 3). The proportion of labeled molecules in the free IAA increased until 60 min after [$^{13}C_{11}{}^{15}N_2$]-Trp application, when the ratio observed was about 49 %. The ratio continued to increase up to 120 min after application. Dose dependency was observed from concentrations of 50, 200, and 500 ng/μL. The ratio of labeled molecules in transported IAA from the tip was relatively similar to that of free IAA. These results indicated that the system was operating under natural in vivo conditions for IAA biosynthesis. The efficiency of label incorporation at different regions within the tip was also determined by applying labeled Trp directly to the different inner regions of the coleoptile dome. The results showed that the apical region located approximately 0–1 mm from the tip was the site where labeled Trp was incorporated most effectively into IAA. This indicates that the very tip region is the primary site of IAA synthesis from Trp. Higher contents of IAA in the coleoptile tips were also reported in rice and *Avena*, but the pathways of IAA production in these plants have not been studied in detail.

1.3 The IAA Biosynthetic Pathway in Maize

Several pathways from tryptophan to IAA have been suggested after incorporation experiments with specific precursors and the identification of enzymatic steps in vitro. A pathway from tryptophan via indole-3-pyruvate (IPyA) and indole-3-acetaldehyde (IAAld) was characterized in bacteria (Cooney and Nonhebel 1989).

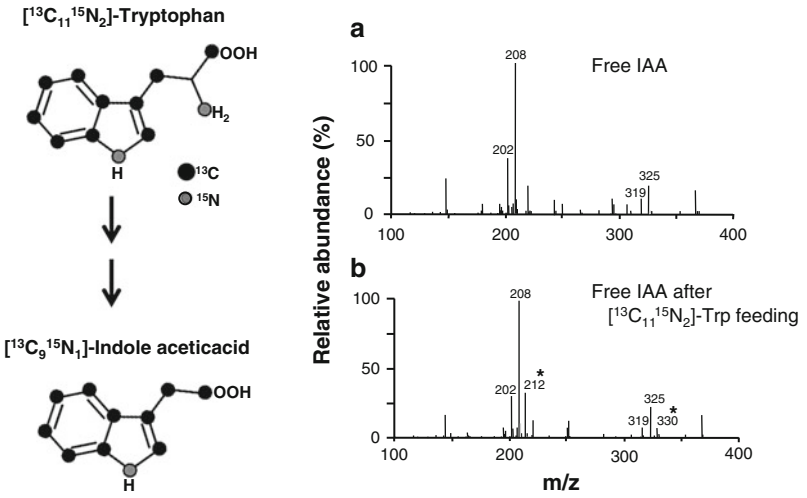

Fig. 3 Mass spectra of free IAA after the application of [$^{13}C_{11}{}^{15}N_2$]-Trp Free IAA from the coleoptile tip 2 h after application of labeled Trp was analyzed by full-scan GC-MS. (**a**) Free IAA without [$^{13}C_{11}{}^{15}N_2$]-Trp application and (**b**) from [$^{13}C_{11}{}^{15}N_2$]-Trp-treated coleoptiles. The *asterisks* indicate the position of fragments (*m/z* 212 and 330) labeled with stable isotopes from [$^{13}C_{11}{}^{15}N_2$]-Trp. Fragments such as *m/z* 208 and 325 are from the [$^{13}C_6$]-IAA standard for the IAA quantification

Based on the results of recent genetic research, it is now widely accepted that IAA is synthesized from Trp in plants (Fig. 4, also in Woodward and Bartel 2005; Gallavotti et al. 2008b; Sugawara et al. 2009; Mashiguchi et al. 2011). Pathways via the intermediates indole-3-acetaldoxime (IAOx), indole-3-acetonitrile (IAN), IPyA, IAAld, and indole-3-acetamide (IAM) have been mainly investigated in the model system *Arabidopsis thaliana* (Woodward and Bartel 2005; Sugawara et al. 2009; Mashiguchi et al. 2011). Among the putative IAA biosynthetic pathways, the IPyA route of Trp-dependent auxin biosynthesis is believed to be common to all plants, based on the ubiquitous distribution of at least some of the genes implicated in these pathways. Arabidopsis *TAA1*, which encodes a Trp aminotransferase responsible for the conversion of Trp into IPyA, has homologous sequences across a wide array of evolutionarily diverse plant species (Tao et al. 2008; Stepanova et al. 2008; Yamada et al. 2009). Conversely, *YUCCA*, which encodes a flavin monooxygenase, was initially proposed to catalyze the conversion of tryptamine to *N*-hydroxy-tryptamine (*N*-hydroxy-TAM), a rate-limiting step in the YUCCA pathway (Zhao et al. 2001). However, later research showed that most, if not all, detectable IAOx in Arabidopsis is likely produced via CYP79B2/B3, suggesting that the YUCCA pathway does not converge on IAOx as previously believed. In addition, serious doubts about the involvement of YUCCA1 in the production

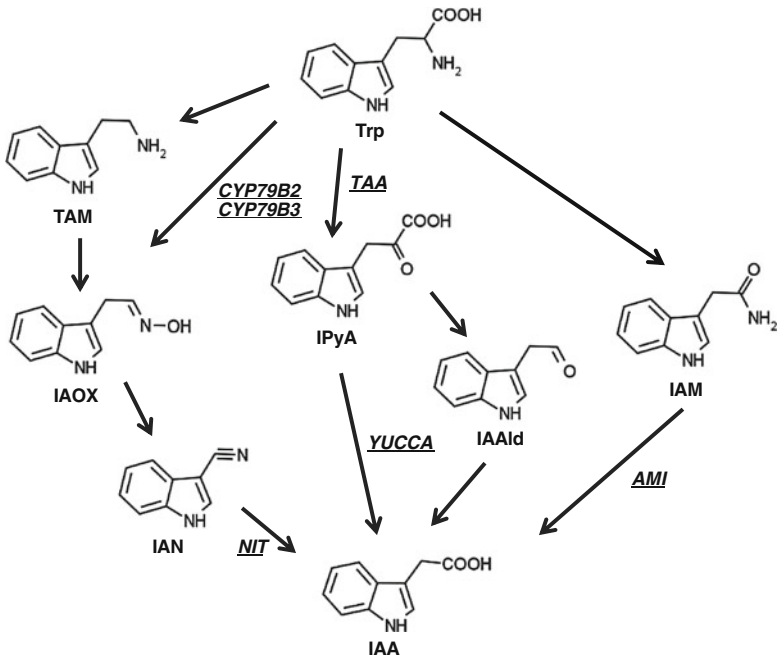

Fig. 4 Putative IAA biosynthetic pathways in the Trp-dependent pathway, Trp is considered to be the IAA precursor because of its structural similarity to IAA. At present, the IPyA pathway catalyzed by TAA and YUCCA is the most reliable candidate pathway in a wide range of plant species

of *N*-hydroxy-TAM and the nature of its in vivo substrate have recently been raised (Tivendale et al. 2010). More recently, it was revealed that recombinant YUCCA protein converts Trp to IPyA via enzymatic reaction (Mashiguchi et al. 2011; Won et al. 2011). Therefore, at present, YUCCA- and TAA-family proteins are the strongest candidates for key enzymes in IAA biosynthesis in plants.

In maize coleoptiles, aldehyde oxidase (AO) and cytosolic ascorbate peroxidase (Koshiba 1993) were shown to be involved in the synthesis of IAA in vitro, and several further studies on aldehyde oxidase showed its ability to catalyze the conversion of IAAld to IAA (Koshiba et al. 1996; Seo et al. 1998; Sekimoto et al. 1998). However, genetic and reverse genetic analyses with Arabidopsis suggest that AO is not the main enzyme in IAA production, because AO knockout lines (*aao1*, *aao2*, *aao3*) and a Mo-cofactor (which is necessary for AO activity)-deficient mutant (*aba3*) did not show IAA-defective phenotypes. Also, two forms of L-Trp aminotransferases (L-TAT1 and L-TAT2) were separated from maize coleoptiles (Koshiba et al. 1993). L-TAT1 and L-TAT2 had similar properties, with an optimum pH of 8–9, a high optimum temperature of 50–60 °C and a requirement for

pyridoxal-5-phosphate for catalytic activity. As the amino group acceptor for these two enzymes, α-ketoglutaric acid was more effective than pyruvic, oxaloacetic, and glyoxylic acids. The molecular masses of L-TAT-1 and L-TAT-2 estimated by gel filtration were approximately 80 kDa and 45 kDa, respectively. However, the involvement of these enzymes in the IAA synthetic pathway has not been elucidated. In contrast, recent genetic research in rice and maize has provided other candidates for IAA synthetic genes. *ZmNIT1* and *ZmNIT2*, which encode enzymes catalyzing the conversion of IAN to IAA, are two such candidates in maize (Park et al. 2003; Kriechbaumer et al. 2007). Despite its relatively high homology to Arabidopsis NIT4, ZmNIT2 shows no activity toward β-cyano-alanine, the substrate of Arabidopsis NIT4, but instead hydrolyzes IAN to IAA, which it does more efficiently than AtNIT1/2/3. *Zmnit2* transposon insertion mutants were compromised in root growth in young seedlings and sensitivity to IAN and accumulated lower quantities of IAA conjugates in their kernels and root tips, suggesting a substantial contribution of ZmNIT2 to total IAA biosynthesis in maize. However, the free IAA and total IAA levels in coleoptiles were unchanged in *Zmnit2* plants. In addition, IAOx and IAN were not detected in the coleoptile tip by LC-ESI-MS/MS analysis (Sugawara et al. 2009).

Seven *YUCCA*-like genes were identified in rice, and it was shown that overexpressing *OsYUCCA1* resulted in increased IAA levels and a characteristic auxin-overproduction phenotype (Yamamoto et al. 2007). Soon after that report, the rice mutant *Osyucca8*/*Oscow1*/*Osnal7* was shown to have a narrow-leaf phenotype, and it was confirmed that *YUCCA* was involved in auxin biosynthesis (Fujino et al. 2008). *OsYUCCA8*/*OsCOW1*/*OsNAL7* belongs to the same group as *OsYUCCA1* and *YUCCA1* from Arabidopsis. In maize, *sparse inflorescence1* (*spi1*) was identified as a YUCCA-like gene that causes defects in the initiation of axillary meristems and lateral organs during vegetative and inflorescence development. In Arabidopsis, loss of function of single members of the *YUC* family has no obvious effect, but in maize the mutation of a single *yuc* locus causes severe developmental defects (Gallavotti et al. 2008a, b). *VT2* was identified as a maize homolog of Arabidopsis *TAA1* (Phillips et al. 2011). Unlike single mutations in Arabidopsis *TAA1*, which cause subtle morphological phenotypes in Arabidopsis, mutations in *VT2* have dramatic effects on vegetative and reproductive development. Furthermore, *vt2* mutants share many similarities with sparse inflorescence1 (*spi1*) mutants in maize. Interestingly, *vt2 spi1* plants did not show a further reduction of IAA level compared with *vt2* single mutant plants. Therefore, it was suggested that *spi1* and *vt2* function in the same IAA synthetic pathway, which raised the possibility that the substrate of YUCCA is IPyA synthesized by TAAs (Phillips et al. 2011). Although recent work has indicated that a Trp-dependent IPyA pathway plays the main role in IAA biosynthesis in maize, the pathway and the mechanisms that control the biosynthetic rate of IAA in the coleoptile tip remain obscure.

2 Basipetal Polar IAA Transport and Lateral IAA Transport During Tropic Responses in Maize Coleoptiles

2.1 Basipetal Polar IAA Transport in Maize Coleoptiles

Polar IAA transport, carrying IAA from its source, is one of the important mechanisms for creating a dynamic gradient of IAA (Benkova et al. 2003; De Smet and Jurges 2007). In monocot plants, coleoptiles have long been a good system for investigating IAA biosynthesis as well as its polar and lateral transport (Went and Thimann 1937; Goldsmith 1967a, b; Parker and Briggs 1990a; Nishimura et al. 2009). In previous works, the IAA transport rate was examined in maize coleoptiles using [^{14}C]-IAA to demonstrate the transport of IAA in coleoptile sections (Goldsmith 1967a, b). Coleoptile sections were supplied with an exogenous source of [^{14}C]-IAA for a limited time, and after the source was removed, a pulse of [^{14}C]-IAA moved downward at 12–15 mm/h. When sections with a moving pulse were transferred to nitrogen, the rate of transport fell below 2 mm/h. Goldsmith also showed that anaerobic conditions inhibited polar IAA transport in the coleoptile sections. Most investigators have had to study auxin transport in decapitated or cut segments of stem tissues to be able to get enough tracer, either tritiated or [^{14}C]-labeled auxin, into the plant to measure transport. The amounts of tracer added to these wounded tissues were large, almost certainly perturbing to the plant's growth physiology as the rate of auxin transport seems to be different in cut and intact tissues. Parker and Briggs demonstrated that [^{3}C]-IAA applied to intact maize coleoptile tips moved downward at a velocity of 20 mm/h around the tip and 12 mm/h around the base of coleoptiles (Parker and Briggs 1990a). Recently, the transport rate of IAA synthesized at the tip was investigated in a [$^{13}C_{11}{}^{15}N_2$]-Trp tracer experiment (Nishimura et al. 2009). Because the labeled IAA from [$^{13}C_{11}{}^{15}N_2$]-Trp was observed by GC-MS analysis, it was excluded that the exogenously applied IAA had artificial effect on IAA transport itself or other physiological events. To investigate labeled-IAA movement from the tip region, labeled Trp was pulse fed for 15 min at the top of intact seedlings. The amount of IAA and the proportion of labeled IAA/total free IAA were quantified in each 1 mm section from the tip to 13 mm below the tip. No significant change was observed in the endogenous IAA levels before and after Trp feeding. The peak of labeled IAA moved down the tip after pulse feeding at approximately 7 mm/h, indicating that the labeled IAA was transported at exactly the same rate as the native IAA. These results clearly indicated that IAA is constantly synthesized from Trp in a rate-limited manner in the tip region and that the synthesized IAA is immediately transported to the lower tissues in a polar manner. Molecular genetic studies in the model system Arabidopsis and other species have shown that a combination of transport systems comprising the PINFORMED (PIN) efflux carriers, ATP-binding

cassette group B (ABCB) auxin transporters, and AUX1/LAX uptake permeases coordinately mobilize auxin transport streams (Titapiwatanakun and Murphy 2009; Friml 2010). In maize, *ZmPIN1a*, *1b*, and *1c*, putative orthologs of *AtPIN1*, were recently identified (Carraro et al. 2006; Gallavotti et al. 2008a). A highly conserved 16 amino acid peptide sequence in the large intracytosolic loop of AtPIN1 was used to raise rabbit antiserum as described in Boutte et al. (2006). The antibody recognized at least the ZmPIN1a and ZmPIN1b proteins in maize. Immunohistochemical studies revealed that the ZmPIN1 proteins were localized in the leaves, the primary root, and the shoot apical meristem (Boutte et al. 2006; Carraro et al. 2006; Gallavotti et al. 2008a; Forestan et al. 2010). Analysis of *ZmPIN1a*::*ZmPIN1a*:*YFP* expression patterns showed that ZmPIN1a:YFP expression was upregulated in axial meristems and lateral organ primordia. These data suggested that directional IAA transport is necessary to form axial meristems and lateral primordia in maize, as in Arabidopsis. The expression of *ZmPIN1a*, *ZmPIN1b*, and *ZmPIN1c* mRNAs were analyzed by RT-PCR using RNA extracted from coleoptiles (Nishimura et al. 2009). The *ZmPIN1a* and *ZmPIN1b* transcripts were expressed at almost the same levels, while *ZmPIN1c* expression was much lower. Thus, it was postulated that both ZmPIN1a and ZmPIN1b proteins are present in maize coleoptiles. Furthermore, Western blotting analysis showed that the affinity-column-purified serum recognized a 65 kDa polypeptide. The molecular size was consistent with the putative ZmPIN1a (65.2 kDa) and ZmPIN1b (64.5 kDa) polypeptides. Immunohistochemical analyses revealed that ZmPIN1(s) was present in almost all cells. ZmPIN1 showed a relatively nonpolar distribution at the tip but a basal cellular localization in the lower regions of the coleoptile (Fig. 5). These results indicate that IAA movement at the IAA synthetic region in the tip is nondirectional, whereas it is transported in a polar manner in the subapical region toward the lower parts of the plant. At present, there is little information about maize auxin-influx carrier, AUX1, and MDR/PGP genes, although it is known that the MDR/PGP gene encoding ABCB1/BR2 has a role in shoot growth, as revealed from analyses of the maize dwarf mutant *br2* (Multani et al. 2003; Knöller et al. 2010).

2.2 Gravitropism and Phototropism in the Coleoptiles

In general, whole seedlings have been used in a number of studies on the tropic curvature of monocot coleoptiles. Iino and coworkers conducted detailed observations on tropic curvatures by measuring the degree of curvature or growth increment of coleoptiles (Iino and Briggs 1984; Iino 1991). Their results showed that the subapical region was the main area of bending in photo-stimulated coleoptiles, while in gravity-stimulated coleoptiles, bending occurred in both the subapical and

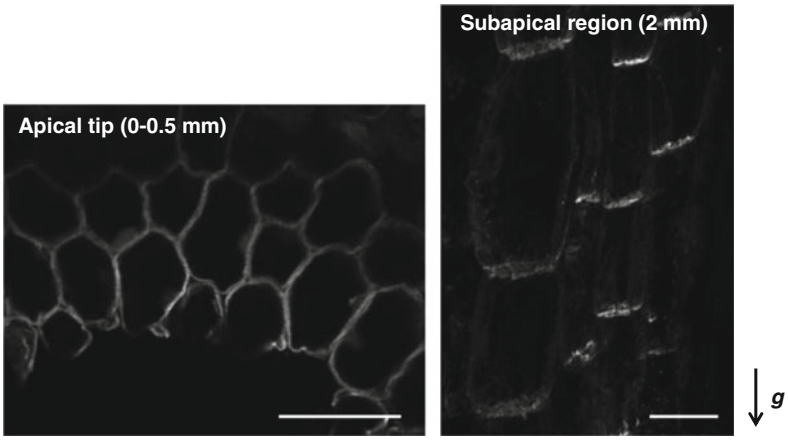

Fig. 5 Cellular localization of ZmPIN1 in the coleoptiles. Immunolocalization of PIN1 showed that it is localized to the basal membrane of cells in the subapical region of coleoptiles, but not in the apical tip

basal regions (Iino 1991). These results indicated that the mechanisms that form an asymmetric IAA distribution pattern differ between gravitropism and phototropism; an asymmetric IAA distribution is formed at the tip and migrates to the basal region in the phototropic response but forms throughout the length of the coleoptile in the gravitropic response.

More recently, a simpler system using 2 cm detached coleoptile segments from maize seedlings was developed to investigate gravi- and phototropic curvatures (Nishimura et al. 2009; Matsuda et al. 2011). In the case of phototropic curvature (first positive curvature), bending was visible within 30 min and increased until 60 min before gradually decreasing (Matsuda et al. 2011). This pattern of movement appeared to show the effects of gravity; phototropic stimulus makes the segments bend away from the vertical axis (Nick and Schafer 1988). Furthermore, in microgravity conditions the curvature continued after 60 min and persisted until at least 120 min. On the other hand, the gravitropic response was also observed using a similar system to the one for phototropism (Nishimura et al. 2009), in which bending occurred after 60 min of gravity stimulus. Because decapitation of coleoptiles strongly inhibits tropic curvature (Iino 1995), treatments with the IAA transport inhibitor NPA almost completely eliminated the gravitropic curvature of maize coleoptiles (Fig. 6). Particularly in the gravitropism, because NPA specifically inhibits IAA transport, the results indicate that the IAA supplied from the tip region is a key factor in tropic curvature. Therefore, a specific transport system that relocates IAA and forms an asymmetric IAA distribution pattern is important in tropic curvature, especially at the tip region. This result also clearly confirms that polar-transported IAA from the tip region is responsive to gravi- and phototropic curvature of coleoptiles.

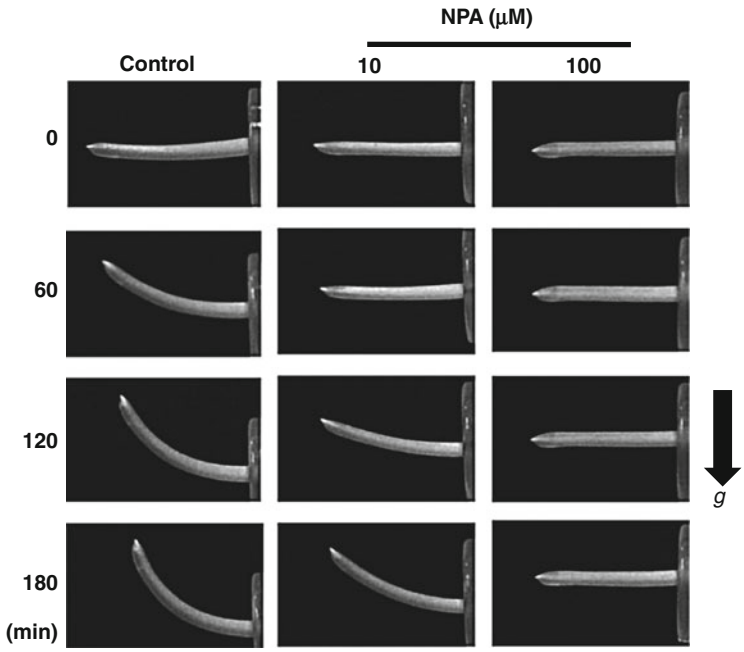

Fig. 6 Gravitropic curvature of maize coleoptiles and the effect of NPA on the response. When NPA is applied to top 0–2 mm of coleoptiles, gravitropic curvature is inhibited in dose-dependent manner

2.3 Lateral IAA Transport and Asymmetric IAA Distribution During Tropic Responses

The IAA produced specifically at the tip region of grass coleoptiles has been shown to participate in gravi- and phototropic curvature. It is believed that the distribution of IAA in the tissues is altered by lateral IAA transport after the perception of gravity and light stimuli. In gravitropism, the lateral IAA gradient throughout the length of the maize coleoptile is important (Parker and Briggs 1990b; Iino 1995). In phototropism, it is generally accepted that the tip region detects the direction of the photo-stimulus, and lateral IAA transport from the tip establishes an asymmetric IAA distribution that moves basipetally, causing the lower region to bend (Briggs et al. 1957; Iino 1991, 1995; Fuchs et al. 2003). However, there have been only a few reports in which asymmetric IAA distribution was determined with GC-MS. In their report, Fuchs et al. (2003) used GC-MS to determine IAA levels in 0–5 mm and 0–20 mm maize coleoptile sections separated into shaded and irradiated sides under second positive light conditions. Recently, accurate IAA localization in 0–3, 3–6, 6–9, and 9–12 mm sections of maize coleoptiles was determined by comparing the irradiated and shaded sides after 8 s of blue light at 0.33 μmol/(m^2 s) (first pulse-induced positive phototropism) (Matsuda et al. 2011). The results showed that IAA

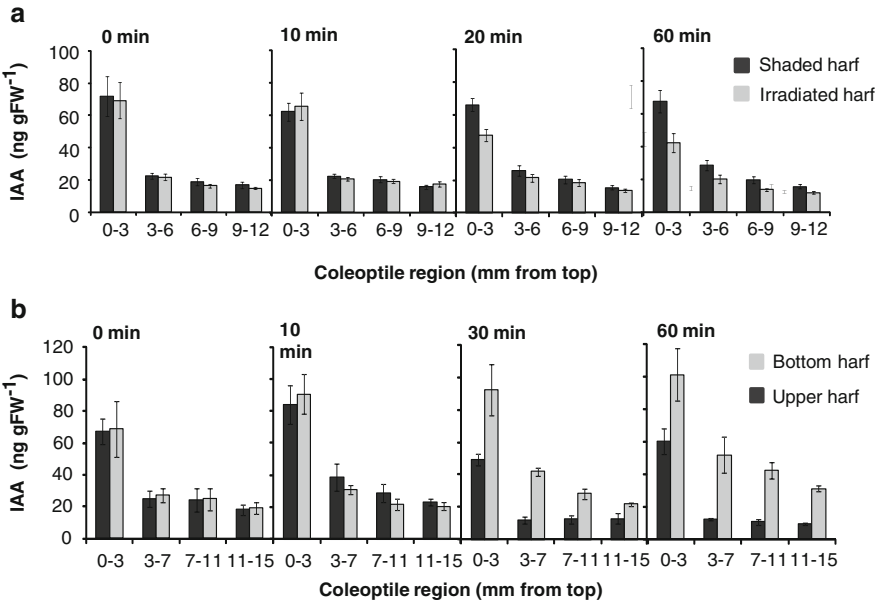

Fig. 7 Observation of asymmetric IAA distribution during phototropism and gravitropism. Changes in IAA levels in both (**a**) the irradiated and shaded halves after light stimulus along the top 0–12 mm of the coleoptiles and (**b**) the upper and lower halves after gravity stimulus along the top 0–15 mm of the coleoptiles

is first redistributed asymmetrically in the 0–3 mm tip region between 10 and 20 min after light stimulation, and then the asymmetric IAA distribution moves into the lower parts of the coleoptiles (Fig. 7a). This result also indicates that light-dependent IAA lateral movement occurs mainly within the top 3 mm region. However, in gravitropism, the lateral IAA gradient throughout the length of the coleoptile is important (Parker and Briggs 1990b; Iino 1991, 1995). In a [^{3}H]-IAA tracer experiment, Parker and Briggs (1990b) showed that IAA had been redistributed in the coleoptiles by 40 min after a gravity stimulus. Philippar et al. (1999) used GC-MS analyses to examine the asymmetric IAA distribution in the 0.5–1.5 cm regions of maize coleoptiles. Their results showed that the 2 cm coleoptile segments exhibited the same gravitropic curvature that occurs in intact etiolated maize seedlings. Detailed IAA levels in both the upper and lower halves of 0–3, 3–7, 7–11, and 11–15 mm sections of the coleoptile were determined after gravi-stimulus by Nishimura et al. (2009). After 30 min of gravity stimulus, IAA was clearly redistributed throughout the coleoptile, and IAA levels on the lower side were greater than those on the upper side (Fig. 7b). This result indicated that lateral movement occurred between 10 and 30 min after the gravity stimulus throughout the length of the coleoptiles but mainly at the region of IAA biosynthesis at the tip (Nishimura et al. 2009). These physiological studies suggest that there is a specific transport system that relocates IAA and that the asymmetric IAA distribution is a key event in tropic curvature.

2.4 Candidate Genes Involved in Tropic Responses in Monocot Coleoptiles

Recent powerful advances in molecular genetic analysis have led to the isolation of several candidates for key components of the tropic signal transduction pathway. In Arabidopsis, AtPIN2 localized on the upper membrane of epidermal and lateral root cap cells mediates basipetal IAA transport in the root tip. *Atpin2* roots show an agravitropic phenotype, indicating that AtPIN2 has a role in creating the lateral distribution of IAA for root gravitropism (Abas et al. 2006; Jaillais et al. 2006). AtPIN3 is also a good candidate for having a role in IAA redistribution in root tip cells in response to gravity stimuli, although *pin3* mutants exhibit only a weak agravitropic phenotype (Friml et al. 2002). Recently, redirection of PIN3 protein generated asymmetric IAA distribution during tropic responses in the hypocotyl (Ding et al. 2011; Rakusová et al. 2011). However, molecular components of the lateral IAA transport system, such as the IAA transporter PIN protein(s), have not been reported for monocot coleoptiles. The NPH3 protein, which directly interacts with phot1, was originally identified as a signaling component essential for the phototropic response in Arabidopsis (Inada et al. 2004). *CPT1*, a rice ortholog of Arabidopsis *NPH3*, was identified from the *cpt1* mutant, which exhibited a non-phototropic coleoptile phenotype. CPT1 may act upstream of the auxin redistribution induced by unilateral illumination (Haga et al. 2005). In maize, our microarray and qRT-PCR experiments showed that *NPH3*-like and *PGP*-like genes are expressed specifically in the 0–2 mm tip region of the coleoptile (Matsuda et al. 2011), indicating the possible role of an *NPH3*-like gene in phototropic signal transduction in this region. The uppermost tip region is not only the site of IAA biosynthesis but also the site of lateral IAA movement; therefore, the product of a *PGP*-like gene might have some role in regulating the lateral translocation of IAA. NPH3- and PGP-like proteins have emerged as useful tools for understanding the molecular and cellular mechanisms not only in phototropic but also in gravitropic responses of maize coleoptiles. However, neither the molecular components of the lateral IAA transport system, such as the IAA transporter PIN protein(s), nor the cellular mechanisms of IAA redistribution have been reported for monocot coleoptiles.

3 Conclusions

In maize coleoptiles, a number of reports have shown that IAA is synthesized from Trp in the very tip region. Recently, candidate genes that are involved in the synthesis of IAA have been identified. In particular, an IPyA-mediated IAA synthetic pathway catalyzed by TAA(s) and YUCCA(s) proteins is the most reliable candidate in Arabidopsis and also in maize. However, the involvement of such proteins in IAA biosynthesis in coleoptiles has not yet been reported. Therefore,

more research is required to fully elucidate the details of IAA biosynthesis, including the complete pathway(s) and related enzymes/genes, in the coleoptile. In both gravitropism and phototropism in maize coleoptiles, it has been shown that the IAA synthesized at the tip is necessary for inducing the tropic responses. Recent accurate and precise IAA determination methods with GC-MS or LC-MS analysis have revealed the formation of asymmetric IAA distributions during tropisms and indicated that the mechanisms differ between gravitropism and phototropism; an asymmetric IAA distribution is formed at the tip and migrates to the base in phototropism and forms throughout the length of the coleoptiles in gravitropism. However, the IAA transporter involved in lateral IAA transport has not been reported. Research is now underway to clarify the direction of IAA transport and how these factors differ in other physiological phenomena, such as gravitropic curvature responses.

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Signaling and Transport of Auxin and Plant Development

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Abstract The past decades have seen tremendous progress in our understanding of the role of polar auxin transport (PAT) in diverse plant developmental processes and growth responses to biotic and abiotic signals. These studies have been made possible by the advanced molecular and genetic tools developed in the model plant, *Arabidopsis thaliana*. As other model genetic organisms have been developed, the regulatory mechanisms of polar auxin transport in plant development have been investigated in other plant lineages with interesting biological processes. This chapter reviews the current progress in understanding of the role and the mechanisms of PAT in several model species.

1 Introduction

Auxin, coined from the Greek word, auxein, meaning "to increase" or "to grow," was the first plant hormone discovered. It is well known that auxin is involved in diverse plant growth and developmental processes, including morphogenesis, organogenesis, apical dominance, vascular differentiation, tropic responses to environmental cues including light and gravity, and biotic interactions with microbes (Woodward and Bartel 2005; Leyser 2006; Teale et al. 2006; Guilfoyle 2007). The role of auxin is mainly mediated by its regulation of cell division, expansion, and differentiation. At the molecular level, auxin functions as molecular "glue," promoting interaction between Aux/IAA proteins, negative regulators of auxin response, and auxin receptor proteins belonging to the Transport Inhibitor Response1 (TIR1) family of F-box proteins (Dharmasiri et al. 2005a, b; Kepinski and Leyser 2005a, b; Tan et al. 2007). This leads to polyubiquitination and subsequent degradation of the

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Aux/IAA proteins by the ubiquitin 26S proteasomes. The proteolysis of Aux/IAA proteins in the presence of a high level of auxin removes the inhibitory effect of Aux/IAA proteins on activities of transcription factors, auxin response factors (ARFs), leading to upregulation of Aux/IAA genes (Kepinski and Leyser 2005a, b). This negative regulatory loop ensures that auxin homeostasis is properly maintained and changes in the intracellular auxin level are sensed and linked to changes at the transcriptional and cellular levels. In *Arabidopsis thaliana*, there are 29 Aux/IAA and 23 ARF proteins. The antagonistic interactions between Aux/IAA and ARF proteins are astonishing due to their specific and sometimes overlapping spatiotemporal expression patterns, explaining why loss-of-function mutations in individual family members do not often result in drastic phenotypic changes.

The predominant form of auxin in higher plants is indole-3-acetic acid or IAA. IAA is primarily synthesized in actively dividing cells, including shoot and root apical meristems, young leaves, and developing seeds. IAA biosynthesis uses both tryptophan-dependent and tryptophan-independent pathways. Once synthesized in plant cells, IAA exists in both free or active and conjugated or inactive forms. Importantly, before auxin influx and efflux transport proteins were discovered in the late 1990s, it was well established that IAA is transported directionally in plants. The Cholodny–Went hypothesis postulates that a redistribution of spatial information (a hormone) basipetally from coleoptiles results in a concentration gradient necessary for gravi- and phototropic bending. Later, this hormone was characterized as indole-3-acetic acid (IAA). The chemiosmotic model of auxin transport explains that, in the apoplast where pH ~ 5.5, IAA is primarily protonated and can freely diffuse or be taken up by auxin influx carriers into cells. Once inside of cells, where pH is neutral, IAA becomes anionic and can only be taken out of cells by auxin efflux carriers. The identification of auxin influx and efflux transport proteins and subsequent demonstration of asymmetric plasma membrane localization of PIN-FORMED (PIN) auxin efflux proteins support the previous model and further extend our understanding of the polar auxin transport process in plants. Eight *PIN* genes are identified and found to be expressed in distinct and in some instances partially overlapping cells and tissues in *A. thaliana*. At present, four PIN proteins, PIN1, PIN2, PIN4, and PIN7, have been shown to exhibit asymmetric plasma membrane localization. PIN3 exhibits predominantly diffused cytoplasmic localization in root columella cells. Upon gravistimulation, some PIN3 proteins are localized asymmetrically to the plasma membrane facing the new bottom (Friml et al. 2002; Kleine-Vehn et al. 2010). On the other hand, PIN5, PIN6, and PIN8 are localized to the endoplasmic reticulum (ER) membrane, consistent with a role in intracellular auxin distribution (Mravec et al. 2009; Ding et al. 2012). *PIN1* was first discovered through genetic analysis of loss-of-function mutants with pin-like inflorescences without lateral organs (Okada et al. 1991; Galweiler et al. 1998). *PIN2* was independently identified through genetic studies of different alleles, including *ethylene insensitive root1* (*eir1*), *agravitropic1* (*agr1*), and *wavy6* (*wav6*), all of which exhibit pronounced root agravitropic phenotype and insensitivity to ethylene and auxin transport inhibitors (Chen et al. 1998; Luschnig et al. 1998; Muller et al. 1998; Utsuno et al. 1998).

PIN proteins have characteristic N- and C-terminal hydrophobic domains consisted of about five transmembrane spans. Most of them including PIN1–PIN4 and PIN7 also have a long divergent central hydrophilic loop, with PIN6 having a shorter loop. These canonical proteins are called "long" PINs. On the other hand, PIN5 and PIN8, together with a group of related proteins named PIN-like proteins (PILs), have the conserved N- and C-terminal hydrophobic domains but lack the central hydrophilic loop. Studies of these so-called "short" PINs indicate that they likely play a role in intracellular auxin distribution from cytosol to ER lumen where auxin is conjugated or metabolized (Barbez et al. 2012).

2 Root Tropic Responses

Plant growth is highly dynamic in response to environmental cues. In soil, the growth of plant roots responds to gravity, light, water, mechanical hindrance, and many other cues to supply plants with water and nutrients for shoot growth. Root columella cells are sites of gravity sensing. Upon gravistimulation, starch-containing statoliths sediment to the bottom of columella cells and trigger a downstream signaling process, although the mechanism is not yet known. This results in a lateral auxin gradient in the root tip region presumably mediated by columella cell-expressed PIN3, PIN7, and possibly PIN4 (Kleine-Vehn et al. 2010). This lateral auxin gradient is further transported to the root elongation zone by epidermal and cortical cell-expressed PIN2, where a high level of auxin inhibits cellular elongation and causes a downward curvature response of roots toward the gravity (Chen et al. 1998; Luschnig et al. 1998; Muller et al. 1998; Utsuno et al. 1998; Paciorek et al. 2005). The root lateral cap and epidermal cell-expressed auxin influx carrier, AUX1, also plays a role in facilitating transport of auxin and root gravitropic response (Pickett et al. 1990; Bennett et al. 1996; Marchant et al. 1999).

The majority of PIN3 proteins in root columella cells in non-stimulated vertically grown roots exhibit diffused cytoplasmic expression pattern. Upon a short gravistimulation, some existing PIN3 proteins are targeted to plasma membrane at the new bottom side and presumably mediate auxin transport in the bottom flank of roots and thus generate a lateral auxin gradient in the root tip region (Kleine-Vehn et al. 2010). Interestingly, targeting of PIN3 proteins to the plasma membrane has been shown to involve clathrin-coated vesicles and ARF-GEF-dependent recycling of endosomes.

PIN2, also called EIR1, AGR1, or WAV6, is a key regulator of root gravitropic response. The classical *eir1*, *agr1*, or *wav6* mutants all exhibit pronounced defects in root gravitropic response. PIN2 proteins asymmetrically localize to the plasma membrane toward the shoot–root junction in root epidermal and some cortical cells in the distal elongation zone, consistent with its role in basipetal transporting auxin toward the shoot. Loss-of-function mutations of *PIN2* reduce auxin transport to the elongation zone and cause auxin accumulation at the bottom flank in the root tip. In some cortical cells toward the root tip, PIN2 exhibit asymmetric rootward plasma

membrane localization. This localization is to channel some auxin back to the root tip, which appears to be essential for maintaining root meristematic activity because without this loop, root meristems collapse (Friml et al. 2004).

3 Transport Inhibitor Response1/Arabidopsis F-Box Proteins (AFBs) and Membrane Sterol Regulation of Auxin Transport

After de novo synthesis, PIN proteins are targeted to the plasma membrane domain either toward the shoot or the root tip via different mechanisms. The asymmetric plasma membrane targeting of PIN1 proteins toward the root tip utilizes the brefeldin A (BFA)-sensitive ARF-GEF-mediated vesicle trafficking process (Geldner et al. 2001, 2003). On the other hand, the plasma membrane targeting of PIN2 proteins toward the shoot utilizes a BFA-insensitive process (Jaillais et al. 2006). PIN protein phosphorylation by the protein kinase, PINOID, which is counterbalanced by the protein phosphatase 2A (PP2A), is required for the proper targeting of PINs to the shootward plasma membrane domain (Friml et al. 2004; Michniewicz et al. 2007). Plasma membrane-localized PIN proteins undergo endocytosis via clathrin-coated vesicles and recycling between plasma membrane and endosomes (Dhonukshe et al. 2007; Kitakura et al. 2011). Auxin has been shown to inhibit PIN2 endocytosis and promote its plasma membrane accumulation in root epidermal and cortical cells (Paciorek et al. 2005; Pan et al. 2009). Shortly after gravistimulation, a lateral auxin gradient developed at the bottom flank of the root tip reaches the distal elongation zone and increases PIN2 plasma membrane accumulation, which in turn increases basipetal auxin transport. This positive effect of auxin on its own transport allows plant's roots to quickly reorient their direction of growth with the new gravity vector.

To elucidate the regulatory mechanism, Pan et al. (2009) investigated the requirement of the auxin receptor TIR1/AFB-dependent signaling processes in auxin inhibition of PIN endocytosis, using a well-established endocytic assay in which short-term treatments with the fungal toxin brefeldin A at a low concentration (25 μM) results in the accumulation of PIN proteins in FM4-64-positive endosomes in root epidermal and cortical cells. In *tir1* single mutant, the inhibitory effect of auxin on PIN2 endocytosis is marginally affected, consistent with functional redundancies of the TIR1/AFB proteins in auxin signaling (Dharmasiri et al. 2005a, b; Kepinski and Leyser 2005a, b). However, in *tir1 afb2* double mutants, the auxin effect was greatly compromised, whereas in *tir1 afb1*, *2* triple and *tir1 afb1*, *2*, *3* quadruple mutants, the negative effect of auxin was lost. The same results were observed in other auxin signaling mutants, including *axr6-3* (Quint et al. 2005) and *axr1-12 tir1* mutants (Timpte et al. 1995; Pan et al. 2009). Using the protein synthesis inhibitor, cycloheximide, Pan et al. (2009) showed that the auxin inhibitory effect on PIN2 endocytosis does not require de novo protein synthesis. Similarly, de novo protein synthesis is not involved in the compromised auxin regulation

of endocytosis in auxin signaling mutants, indicating that posttranscriptional processes are required for the auxin regulation of PIN endocytosis. Taken together, these results clearly indicate that the inhibitory effect of auxin on PIN protein endocytosis requires the TIR1/AFB-dependent posttranscriptional processes.

Pan et al. (2009) showed that auxin does not significantly inhibit general endocytosis as indicated by the endocytic marker FM4-64 dye. Consistent with this, auxin does not inhibit endocytosis of some plasma membrane proteins, including the auxin influx carrier AUX1. Rather, auxin inhibits endocytosis of a specific subset of plasma membrane cargo proteins including PIN1 and PIN2 and, interestingly, other unrelated proteins such as RCI2A (Cutler et al. 2000). And as discussed earlier, the auxin effect does not require de novo protein synthesis. To further elucidate the regulatory mechanism, Pan et al. (2009) investigated the involvement of membrane sterols in auxin regulation of PIN endocytosis. Surprisingly, a short-term treatment of wild-type roots with fenpropimorph, a sterol biosynthesis inhibitor targeting the C14 sterol reductase, FACKLE (FK) (He et al. 2003), effectively reversed the inhibitory effect of auxin on PIN2 endocytosis, similar to that found in auxin signaling mutants. Similarly, the inhibitory effect of auxin on PIN endocytosis was compromised in sterol biosynthetic mutants, *cvp1-3* and *fk-J79* (Jang et al. 2000; Schrick et al. 2000; Carland et al. 2002). These results clearly indicate that membrane sterols play an important role in endocytic regulation of PIN proteins by auxin. Gas chromatography–mass spectrometry (GC–MS) measurements indicated that levels of the four major plant sterols, sitosterol, stigmasterol, campesterol, and cholesterol, were all significantly reduced in the root membrane fraction of the strong auxin signaling mutant, *axr1-12*, compared with the wild-type plants. These results suggest that the compromised auxin regulation of PIN endocytosis in auxin signaling mutants is likely due to altered membrane sterol profiles in the mutant. These results collectively support a posttranscriptional membrane sterol-dependent regulation of PIN endocytosis by auxin. Thus, the TIR1/AFB-dependent signaling processes may directly or indirectly regulate the membrane sterol composition, which is known to play a role in various endocytic processes in plant, animal, and yeast cells (Willemsen et al. 2003; Pichler and Riezman 2004; Souza and Pichler 2007; Men et al. 2008). It is highly plausible that the membrane sterol-dependent PIN endocytic processes targeted by auxin are evolutionarily conserved with animal and yeast cells. However, it remains to investigate how the specificity of auxin regulation of PIN endocytosis is determined.

4 Auxin Binding Protein1 and Clathrin-Dependent Regulation of Auxin Transport

AUXIN-BINDING-PROTEIN 1 (ABP1) has long been postulated to be an auxin receptor since its identification (Palme et al. 1992; Jones et al. 1998; Timpte 2001). However, its role has not been unambiguously confirmed due to the observation that

loss-of-function mutations are embryonic lethal. Recently, the role of ABP1 in PIN endocytosis has been evaluated using *abp1* knockdown and gain-of-function lines. In wild-type plants, most of ABP1 proteins are localized to the endoplasmic reticulum (ER) due to the presence of a KDEL-ER retention peptide with a small portion being secreted to the extracellular space, where it has been shown to play a functional role (Jones and Herman 1993). Deletion of the KDEL-ER retention peptide results in ABP1$^{\Delta KDEL}$-GFP fusion protein being localized largely to the plasma membrane in tobacco suspension cultured cells and in transgenic *Arabidopsis* roots (Robert et al. 2010). In *abp1* knockdown lines, using antisense or immunomodulation, PIN1 internalization was decreased in response to 25 μM BFA treatments. By comparison, in tobacco suspension cultured cells or transgenic *Arabidopsis* lines ectopically expressed ABP1$^{\Delta KDEL}$-GFP fusion proteins, using the constitutive CaMV 35S promoter, internalization of PIN1-RFP fusion proteins or endogenous PIN1 proteins was increased in response to BFA treatments.

In *abp1-5* mutant, a point mutation changing histidine to tyrosine (H94Y) in the conserved auxin-binding pocket of ABP1 is predicted to reduce the auxin-binding affinity of the ABP1-5 protein. Overexpression of *ABP1-5*$^{\Delta KDEL}$ induced the PIN1-RFP internalization in tobacco BY-2 cells. In contrast to the inhibitory effect of auxin on PIN1-RFP internalization in *ABP1*$^{\Delta KDEL}$*-GFP* overexpressed tobacco cells, auxin was not inhibitory to PIN1-RFP internalization promoted by overexpression of ABP1-5$^{\Delta KDEL}$ protein, presumably due to a decreased auxin binding to the modified ABP1-5$^{\Delta KDEL}$ protein. These results suggest that auxin binding to the plasma membrane-localized ABP1 inhibits the promotive action of ABP1 on PIN endocytosis.

PIN endocytosis utilizes the clathrin-dependent mechanism (Dhonukshe et al. 2007; Kitakura et al. 2011). The inhibitory effect of auxin on endocytosis of PIN proteins and the human transferrin receptor (hTfR) in plant cells has been linked to its effect to decrease the dynamic association of clathrin-coated vesicles, as indicated by the clathrin light chain GFP fusion protein (CLC-GFP), with the plasma membrane, whereas the association of CLC-GFP with intracellular compartments was not affected by auxin in *A. thaliana*. Similarly, the association of clathrin-coated vesicles with the plasma membrane was also strongly reduced in *abp1* knockdown lines. Interestingly, in *abp1-5* line, in which a point mutation in the auxin-binding pocket reduces auxin-binding activity of ABP1-5, the auxin effect on clathrin abundances at the plasma membrane was significantly lower than in wild-type seedlings. Authors of this work concluded that auxin binding to ABP1 inhibits the positive action of ABP1 on clathrin-mediated endocytosis. Some questions remain to be investigated to further understand the mechanism of ABP1 and auxin regulation of clathrin-dependent endocytosis (1) Is plasma membrane-localized ABP1 itself endocytosed by the clathrin-dependent mechanism? (2) Does ABP1 physically interact with components of the clathrin complex? (3) Does the majority of ER-localized ABP1 play a role in inter- and intracellular auxin transport mediated by “long” and “short” PIN proteins? And (4) does overexpression of ABP1 and its modified versions alter gene expression in tobacco cultured cells and

transgenic *Arabidopsis* lines? A discrepancy in the involvement of the TIR1/AFB-dependent processes in auxin regulation of PIN endocytosis exists (Pan et al. 2009; Robert et al. 2010). However, the reason for this discrepancy is not clear.

5 Vacuolar Targeting of PIN Proteins by Light Signaling

Light plays an essential role in diverse developmental processes, including seed germination, seedling de-etiolation, leaf expansion, stem and root elongation, phototropism, and flowering (Von Arnim and Deng 1996; Correll and Kiss 2005). Without light, plants initially develop shade avoidance syndrome, including elongation of hypocotyls with unexpanded cotyledons and exaggerated apical hooks and greatly reduced root growth and with extended darkness plants eventually senesce (Morelli and Ruberti 2002). It has been shown that light signaling plays a key role in root growth partially through signaling cross talks with the growth regulator auxin (Jensen et al. 1998; Ruppel et al. 2001; Tian and Reed 2001; Correll et al. 2003; Salisbury et al. 2007). Remarkably, in etiolated seedlings, PIN2-GFP signals were greatly reduced from the plasma membrane and accumulated in vacuolar compartments in root epidermal and cortical cells (Laxmi et al. 2008). Similar to PIN2-GFP, PIN1-GFP, and PIN7-GFP proteins were also accumulated in vacuolar compartments in etiolated root cells, indicating that plasma membrane-localized PIN proteins are targeted to lytic vacuoles for turnover. The vacuolar targeting of PIN proteins can be observed in light-grown seedlings after transition to dark for a few hours or in light-grown *det3-1* mutant defective in the vacuolar H^+-ATPase (Schumacher et al. 1999; Laxmi et al. 2008). The vacuolar targeting of PIN proteins does not require de novo protein synthesis and is largely mediated by endocytosis of plasma membrane proteins via FM4-64-labeled vesicles (Laxmi et al. 2008).

Using monochromatic lights, Laxmi et al. (2008) showed that after transition from white light to continuous blue light (475 nm) for various lengths of time, PIN2-GFP remained at the PM, indicating that blue light signaling is sufficient to maintain the plasma membrane localization of PIN2-GFP. By contrast, after white light to continuous red (660 nm) or far red (730 nm) light transition, a detectable level of PIN2-GFP accumulated in vacuoles, supporting the notion that phytochrome-dependent red/far red signaling is not sufficient to maintain PM localization of PIN2-GFP.

Light signaling involves nucleocytoplasmic partitioning of photoreceptors and the negative regulator, CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), an E3 ubiquitin ligase involved in 26S proteasome-mediated proteolysis (von Arnim and Deng 1994; Yi and Deng 2005). In darkness, COP1 accumulates in the nucleus to degrade transcription factors including HY5, HYH, HFR, and LAF, thereby suppressing light-regulated gene expression (Ang et al. 1998; Holm et al. 2002; Seo et al. 2003; Duek et al. 2004; Jang et al. 2005; Yang et al. 2005). By contrast, light activates the expression of light-regulated genes through degradation and

exclusion of COP1 from the nucleus by the multi-subunit COP9 complex (COP9 signalosome or CSN) (Chamovitz et al. 1996). Consistent with a role of light signaling in PIN2 vacuolar targeting, in light-grown *hy5-1* mutant, PIN2-GFP plasma membrane localization was greatly reduced compared with the wild-type control. On the other hand, in the dark-grown *cop9-1* mutant, PIN2-GFP vacuolar accumulation was reduced with concomitant increase in plasma membrane localization. Interestingly, in the presence of the 26S proteasome inhibitor, MG132, PIN2-GFP vacuolar accumulation was greatly reduced and its plasma membrane localization was increased after light-to-dark transition, suggesting a role of the 26S proteasome-related function in PIN2 intracellular distribution.

Auxin efflux PIN proteins undergo constitutive recycling between endosomal compartments and the plasma membrane, which is dependent on brefeldin A (BFA)-sensitive ADP-ribosylation factor GTP-exchange factors (ARF-GEFs), such as GNOM (Geldner et al. 2001, 2003; Kleine-Vehn et al. 2008a, b; Kleine-Vehn and Friml 2008). GNOM appears to regulate PIN recycling to the plasma membrane facing the root tip. On the other hand, the retromer complex components, SORTING NEXIN1 (SNX1) and VACUOLAR PROTEIN SORTING29 (VPS29), have been shown to play a role in endocytic translocation of PIN2 to lytic vacuoles in the dark and retrieval of PIN2 from vacuoles back to the recycling pathway under the light condition (Jaillais et al. 2006, 2007; Kleine-Vehn et al. 2008a, b). Vacuolar targeting of PIN2 has been shown to be sensitive to the fungal toxin BFA but independent from GNOM. Consistent with these, SNX1 resides in intracellular compartments distinct from GNOM and involves in recycling of PIN2 but not PIN1 to the plasma membrane facing the shoot-root junction, and both SNX1 and VPS29 are involved in mediating steady-state levels of PIN2 in root epidermal and cortical cells important for root gravitropic response (Kleine-Vehn et al. 2008a, b).

6 Auxin Transport Regulation of Legume–Rhizobia Symbiotic Interactions

Legumes (Fabaceae) including many important grain and forage crops such as soybean (*Glycine max*) and alfalfa (*Medicago sativa*) are the third largest family of flowering plants. Legumes are capable of symbiotic nitrogen fixation in specialized organs, root nodules. Extensive signaling between the host and compatible soil bacteria, *Rhizobia*, culminates in the release of lipo-chitin oligosaccharides called Nod factors from the bacteria and subsequent root hair deformation and curling and entrapment of bacteria in the curls. Bacteria enter root epidermal and outer cortical cells in a tubular structure called the infection thread. A subset of cortical cells is mitotically activated and forms nodule primordia. Once the infection thread reaches nodule primordia, bacteria are released into the cytoplasm in membrane bound structures called "symbiosomes" in endocytic processes. Nodules are classified as "indeterminate" or "determinate." Determinate nodules such as those of

soybean initiate from outer cortical cells, but cell division stops soon after infection. Determinate nodules are spherical without meristematic cells. On the contrary, indeterminate nodules such as those of alfalfa (*M. sativa*) and its diploid cousin, *Medicago truncatula*, initiate from inner cortical cells and have a persistent apical meristem that typically gives rise to a cylindrical or branched structure with easily distinguished zones.

It has been speculated that auxin plays a role in nodule development in peas (Thimann 1936). Exogenous application of N-1-naphthylphthalamic acid (NPA), a polar auxin transport inhibitor, results in formation of pseudonodules on roots of alfalfa and some non-nodulating white clover (*Trifolium repens*) mutants and expression of early nodulation genes (Allen et al. 1953; Hirsch et al. 1989; Van De Wiel et al. 1990; Scheres et al. 1992; Wu et al. 1996). These results support a role of auxin in early nodule primordia development (Hirsch and LaRue 1997). Local application of rhizobia inhibits acropetal auxin transport in vetch (*Vicia sativa*) roots within 24 h (Boot et al. 1999). Consistent with this, the expression of an auxin-responsive promoter (GH3) was reduced acropetally from the site of inoculation within 24 h (Mathesius et al. 1998). This was followed by an increase in auxin accumulation at the site of nodule initiation in the root inner cortex. Interestingly, in *Lotus japonicus* forming determinate nodules, no auxin transport inhibition was detected after rhizobia inoculation (Pacios-Bras et al. 2003). However, an increase of GH3 expression was detected in nodule initials at the root outer cortex (Takanashi et al. 2011). These observations suggest that a high level of auxin is required for the initiation of both determinate and indeterminate nodules, a process also observed in lateral root development.

Symbiotic nitrogen fixation in nodules requires large energy input from the host. The number of nodules is tightly controlled by the host. The mechanism of restricting nodule numbers is termed autoregulation of nodulation (AON). In indeterminate legumes, such as *M. truncatula*, autoregulation blocks the formation of new nodule primordia. On the other hand, autoregulation in determinate legumes including soybean (*G. max*) blocks initiated primordia from further development (Caetano-Anolles and Gresshoff 1991). Mutations in a *Leu-Rich Repeat Receptor-Like Kinase* (*LRR-RLK*) gene impair the autoregulation and result in supper numeric nodules on roots of soybean (*G. max*), *M. truncatula*, *L. japonicus*, and pea (*Pisum sativum*) (Carroll et al. 1985; Krusell et al. 2002; Penmetsa et al. 2003). Grafting experiments between shoots and roots of wild-type and supernodulating mutants indicate that a shoot-derived signal of unknown nature inhibits nodule formation in the root. It has been shown that *Rhizobia* inoculation of wild-type roots reduces auxin loading from the shoot to the root (van Noorden et al. 2006). The reduction in auxin loading results in a low level of auxin in the root, which is not sufficient to stimulate or sustain the development of new nodules in the younger part of the root. However, in *Medicago sunn* mutants, auxin loading at the shoot-root junction is not affected by rhizobia inoculation due to lack of the LRR-RLK activity; thus, high amounts of auxin continue to be transported to the root and stimulate development of additional nodules. It has been hypothesized that an unknown signal produced in roots upon rhizobia infection is perceived directly or

indirectly by the LRR-RLK protein encoded by *SUNN* in the shoot, resulting in inhibition of auxin loading from shoot to the root. Thus, inhibition of long-distance auxin transport from shoot to root may be part of the autoregulation of nodulation.

7 Auxin Transport Regulation of Compound Leaf Development

Leaves initiate from leaf founder cells developed at the periphery of the shoot apical meristem (SAM) in specific spatiotemporal patterns. The development of leaf primordia requires downregulation at incipient sites of the class I KNATTED homeobox (KNOX1) transcription factors, whose function is to promote and/or maintain the meristematic activity of SAM (Smith et al. 1992; Sinha et al. 1993; Jackson et al. 1994; Long et al. 1996), and auxin convergent points formed by the auxin efflux protein PIN1 with asymmetric plasma membrane localization in epidermal cells (Benkova et al. 2003; Reinhardt et al. 2003; Heisler et al. 2005; Hay et al. 2006; Hay and Tsiantis 2006). In plants with simple leaves, such as *A. thaliana*, *KNOX1* genes are permanently downregulated in leaf primordia (Long et al. 1996; Bharathan et al. 2002). In tomato (*Solanum lycopersicum*) and many other eudicot species including *Cardamine hirsuta* with compound leaves, *KNOX1* genes are reactivated in initiating leaf primordia (Bharathan et al. 2002). This reactivation appears to be essential for compound leaf development in these species. In some legume plants that belong to the inverted repeat lacking clade (IRLC), including pea (*P. sativum*), alfalfa (*M. sativa*), and *M. truncatula*, *KNOX1* genes are not linked to compound leaf development because they are not reactivated in leaf primordia (Hofer et al. 2001; Champagne et al. 2007; Peng et al. 2011). Instead, in these compound-leafed species, the *FLORICULA* (*FLO*)/*LEAFY* (*LFY*)/*UNIFOLIATA* (*UNI*)/*SINGLE LEAFLET1* (*SGL1*) pathway is required for the development of compound leaves (Hofer et al. 1997; Wang et al. 2008), consistent with convergent evolution of compound-leafed species in different lineages.

In both simple- and compound-leafed species, the mechanism of auxin convergent points is involved in the initiation of leaf primordia and development of serrations and leaflet primordia in leaf margins. In *C. hirsuta*, a compound-leafed relative of *A. thaliana*, auxin maxima as visualized by the auxin response DR5::VENUS marker are formed at incipient sites of leaf primordia and along leaf margins preceding the development of lateral leaflets (Barkoulas et al. 2008). A loss-of-function mutation of *C. hirsuta PIN1* results in naked inflorescences and simplified leaves. Inhibition of auxin transport with NPA results in loss of auxin maxima in the leaf rachis and compromises the development of lateral leaflets. Conversely, exogenous applications of the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) results in ectopic expression of the DR5::VENUS marker and ectopic lateral leaflets. Similar results are also observed in tomato (Koenig et al. 2009).

The tomato *entire* (*e*) mutant, which carries a point mutation in the negative regulator of auxin response, *IAA9*, or a transgenic plant in which *IAA9* is downregulated, has simplified leaves (Wang et al. 2005; Zhang et al. 2007; Koenig

et al. 2009; Ben-Gera et al. 2012). Early development of compound leaf primordia appears to be similar in the *e* mutant as in the wild type, although the initiation is delayed and the number of leaf primordia is reduced. In contrast to auxin convergent points in wild-type plants, *PIN1*::*PIN1-GFP* is also expressed in the intercalary region between terminal and lateral leaflets, in agreement with diffused auxin response observed in the intercalary region of the mutant. Thus, E functions to inhibit auxin response and blade outgrowth between initiating leaflets (Ben-Gera et al. 2012). The *No Apical Meristem* (*NAM*)/*Cup-Shaped Cotyledon* (*CUC*) gene is expressed at boundaries of leaves, leaflets, and serrations and plays an important role in the development of simple and compound leaves in diverse species (Blein et al. 2008). Its role in compound leaf development in tomato is likely mediated by modulating auxin response (Koenig et al. 2009; Ben-Gera et al. 2012). Loss-of-function mutants of the *Medicago NAM/CUC* gene have defective flower and compound leaf phenotypes, including defective shoot apical meristem, fusion of cotyledons and leaflets, reduced floral organs and whorls, and compromised floral organ identities (Cheng et al. 2012).

Loss-of-function mutants of the *Medicago PIN10* gene, which is closely related to the *Arabidopsis PIN1*, have pleiotropic phenotypes, including an increased number and fusion of cotyledons, defective compound leaves with various numbers of leaflets with smooth margins and defective floral organs (Peng and Chen 2011; Zhou et al. 2011). Most of the compound leaf defects appear to be due to fusion of compound leaf primordia at various developmental stages (Peng and Chen 2011). Fusion at an early stage, as indicated by the outgrowth of a broad or the entire periphery of the shoot apical meristem, would lead to fusion of the distal region (terminal leaflets) of compound leaves, whereas fusion at a late stage would lead to fusion of the proximal region (petioles). In addition, *Mtpin10* mutants exhibit altered phyllotaxy due to impaired timing and spacing of leaf primordia initiation.

8 Auxin Efflux PIN Proteins in *M. truncatula*

Previous studies identified ten *Medicago* auxin efflux *PIN* genes (Schnabel and Frugoli 2004). However, their chromosomal locations were not identified. GenBank searches and analyses of the recent release of *M. truncatula* and soybean (*G. max*) genome sequences identified 11 *Medicago PIN* genes, seven of which have chromosomal locations, and 21 soybean *PIN* genes (Table 1). Phylogenetic relationship analysis of *Arabidopsis*, *Medicago*, and soybean PIN sequences places MtPIN1 and MtPIN3 in a clade with AtPIN3, AtPIN4, and AtPIN7 and four soybean sequence; MtPIN8 and MtPIN11 in a clade with AtPIN8 and four soybean PIN sequences; MtPIN6 with AtPIN6 and two soybean PINs; MtPIN4, MtPIN5, and MtPIN10 with AtPIN1 and five soybean PINs; MtPIN2 and MtPIN7 with AtPIN2 and two soybean sequences; and MtPIN9 with AtPIN5 and four soybean PINs (Fig. 1).

MtPIN2 and *MtPIN7* are located in tandem on chromosome 2, suggesting that they may be derived from a recent gene duplication event (data not shown). In silico

Table 1 *Medicago truncatula*, soybean (*Glycine max*), and *Arabidopsis* auxin efflux *PIN* genes

Gene ID	Locus	*Arabidopsis* close homolog(s)	*Medicago* close homolog(s)
MtPIN1	Contig_107783_1.1	*AtPIN3*, *AtPIN4*, *AtPIN7*	
MtPIN2	Medtr4g127100.1	*AtPIN2*	
MtPIN3	contig_76586_1.1	*AtPIN3*, *AtPIN4*, *AtPIN7*	
MtPIN4	Medtr6g069510.1	*AtPIN1*	
MtPIN5	Medtr8g107360.1	*AtPIN1*	
MtPIN6	Contig_8722_1.1	*AtPIN6*	
MtPIN7	Medtr4g127090.1	*AtPIN2*	
MtPIN8	Medtr7g009370.1	*AtPIN8*	
MtPIN9	Medtr7g079720.1	*AtPIN5*	
MtPIN10	Medtr7g089360.1	*AtPIN1*	
MtPIN11	Contig_5277_1.1	*AtPIN8*	
GmPIN1a	Glyma07g22340.1	*AtPIN3*, *AtPIN4*, *AtPIN7*	*MtPIN1*, *MtPIN3*
GmPIN1b	Glyma09g20580.1	*AtPIN3*, *AtPIN4*, *AtPIN7*	*MtPIN1*, *MtPIN3*
GmPIN2a	Glyma13g00390.1	*AtPIN2*	*MtPIN2*
GmPIN2b	Glyma17g06460.1	*AtPIN2*	*MtPIN2*
GmPIN3a	Glyma07g34190.1	*AtPIN3*, *AtPIN4*, *AtPIN7*	*MtPIN1*, *MtPIN3*
GmPIN3b	Glyma20g01760.1	*AtPIN3*, *AtPIN4*, *AtPIN7*	*MtPIN1*, *MtPIN3*
GmPIN4a	Glyma07g11550.1	*AtPIN1*	*MtPIN4*
GmPIN4b	Glyma09g30700.1	*AtPIN1*	*MtPIN4*
GmPIN5	Glyma08g05900.1	*AtPIN1*	*MtPIN5*
GmPIN6a	Glyma13g09030.1	*AtPIN6*	*MtPIN6*
GmPIN6b	Glyma14g27900.1	*AtPIN6*	*MtPIN6*
GmPIN7a	Glyma15g25690.1	*AtPIN5*	*MtPIN7*
GmPIN7b	Glyma09g06970.1	*AtPIN5*	*MtPIN7*
GmPIN8a	Glyma09g37560.1	*AtPIN8*	*MtPIN8*
GmPIN8b	Glyma18g49080.1	*AtPIN8*	*MtPIN8*
GmPIN9a	Glyma09g38700.1	*AtPIN5*	*MtPIN9*
GmPIN9b	Glyma18g47630.1	*AtPIN5*	*MtPIN9*
GmPIN10a	Glyma19g30900.1	*AtPIN1*	*MtPIN10*
GmPIN10b	Glyma03g28130.1	*AtPIN1*	*MtPIN10*
GmPIN11a	Glyma05g23180.1	*AtPIN8*	*MtPIN11*
GmPIN11b	Glyma17g16870.1	*AtPIN8*	*MtPIN11*

gene expression analysis (Benedito et al. 2008) indicates that *MtPIN7* is not expressed in most tissues and growth conditions to a detectable level by the microarray analysis (Fig. 2). The gene expression analysis also indicates that *MtPIN1*, *MtPIN3*, *MtPIN4*, *MtPIN5*, and *MtPIN10* are expressed in most tissues and growth conditions tested, whereas *MtPIN2* is specifically expressed in root tissues, consistent with its close relationship with AtPIN2, which plays a role in root gravitropic response. Interestingly, *MtPIN1*, *MtPIN2*, *MtPIN3*, *MtPIN5*, and *MtPIN10* are expressed to different degrees in roots inoculated with the soil bacteria *Rhizobia* at various developmental stages, suggesting a role of these *PIN* genes in legume–microbe symbiotic interactions. On the other hand, the gene expression analysis suggests that *MtPIN1* to *MtPIN5* may be involved in legume–arbuscular mycorrhizal fungal interactions. Consistent with the gene expression analysis, antisense downregulation of *MtPIN2*

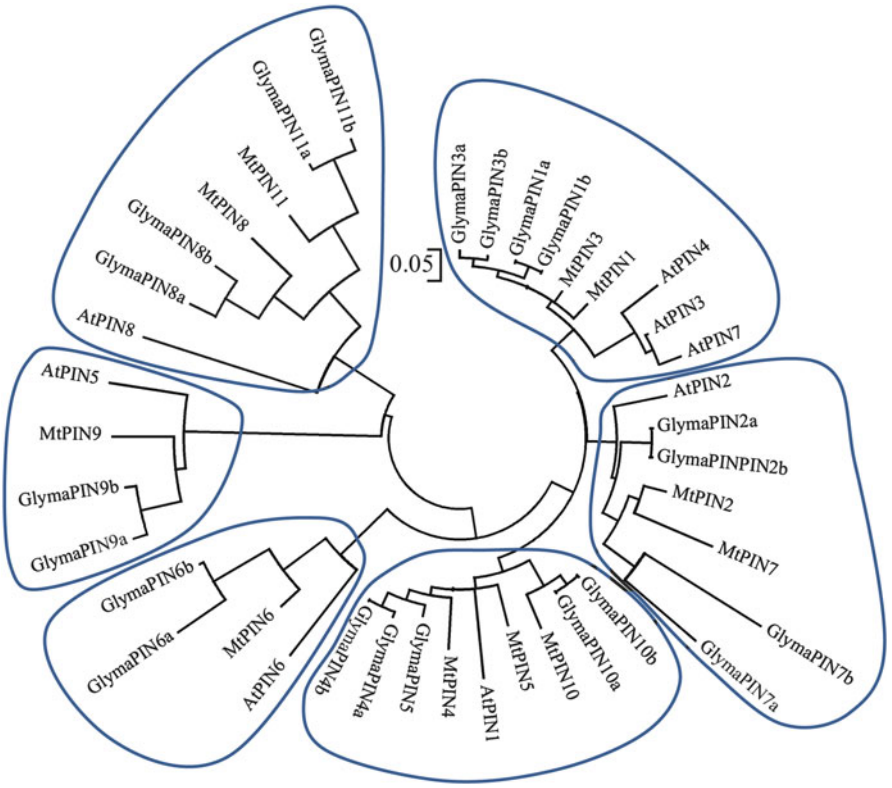

Fig. 1 Phylogenetic relationship analysis of *A. thaliana*, *M. truncatula*, and soybean (*G. max*) auxin efflux PIN proteins

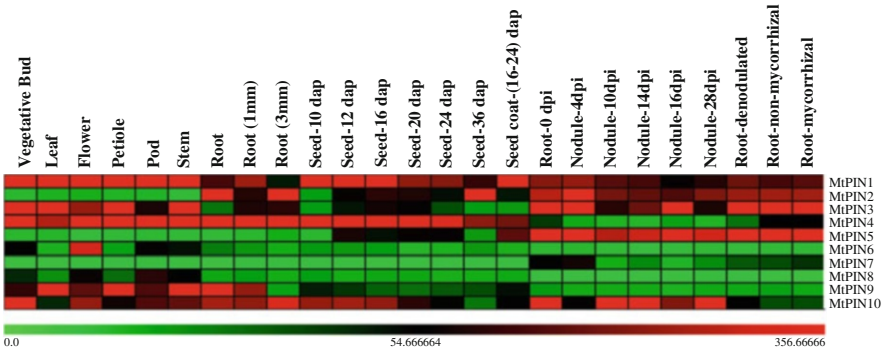

Fig. 2 A heat map of in silico gene expression analysis of *M. truncatula PIN* genes in major vegetative and reproductive tissues and in legume–rhizobia and legume–arbuscular mycorrhizal fungi symbiotic interactions

reduces the number of nodules developed in transgenic hairy roots (Huo et al. 2006). On the other hand, loss-of-function mutations in *MtPIN10* result in pleiotropic phenotypes (Peng and Chen 2011; Zhou et al. 2011).

9 Concluding Remarks

The characteristic a symmetric plasma membrane localization of auxin efflux PIN proteins determines the direction of auxin transport and local concentration gradients (maxima), which appear to be essential for diverse developmental processes. The plasma membrane targeting of PIN proteins is a dynamic process. Distinct intracellular trafficking pathways have been identified in asymmetrically targeting of PIN proteins to the plasma membrane. Both protein phosphorylation counterbalanced by dephosphorylation and the gravity stimulation have been shown to play a role in transcytosis of PIN proteins. Auxin itself plays a role in the plasma membrane accumulation of PIN proteins; therefore, feedback regulates its transport. Studies have shown that other environmental signals such as the light–dark cycle regulate PIN plasma membrane localization. In the dark, PIN proteins are targeted to lytic vacuoles for degradation via the retromer complex. On the other hand, light promotes retrieval of PIN proteins from prevascuolar compartments to the plasma membrane. In addition to the canonical plasma membrane-localized PIN proteins, a group of related "short" PINs are identified to be ER localized, consistent with a role in intracellular distribution and homeostasis of auxin. Thus, plant cells appear to have evolved with multiple regulatory processes to tightly regulate auxin levels. Most of the discoveries are made in the model plant, *A. thaliana*. Evidence obtained from studies in other model species suggests that auxin transport proteins play key roles in diverse species. However, specific roles of auxin transport proteins in complex biological processes such as plant–microbe interactions and dissected leaf morphogenesis await further investigations in diverse plant lineages.

Accession numbers Amino acid and transcript sequences discussed in this chapter can be found in the NCBI GenBank under the following accession numbers: MtPIN1 (AY115836), MtPIN2 (AY115837), MtPIN3 (AY115838), MtPIN4 (AY115839), MtPIN5 (AY115840), MtPIN6 (AY553209), MtPIN7 (AY553210), MtPIN8 (BK005119), MtPIN9 (AY553211), MtPIN10 (AY553212), MtPIN11 (KC344363), GmPIN1a (KC344367), GmPIN1b (KC344371), GmPIN2a (KC344375), GmPIN2b (KC344379), GmPIN3a (KC344368), GmPIN3b (KC344384), GmPIN4a (KC344366), GmPIN4b (KC344372), GmPIN5 (KC344369), GmPIN6a (KC344376), GmPIN6b (KC344377), GmPIN7a (KC344378), GmPIN7b (KC344370), GmPIN8a (KC344373), GmPIN8b (KC344382), GmPIN9a (KC344374), GmPIN9b (KC344381), GmPIN10a (KC344383), GmPIN10b (KC344364), GmPIN11a (KC344365), and GmPIN11b (KC344380).

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From Auxin Transport to Patterning

Marina Oliva, Teva Vernoux, and Jan Traas

Abstract The phytohormone auxin is often observed to be asymmetrically distributed across tissues and regulate crucial events of plant development. These gradients are generated by a network of polarly localized membrane-bound transporters, called PIN-FORMED (PIN) proteins. Understanding how auxin controls patterning in plants comes to define how polar auxin transport is regulated to form gradients and how these gradients will be, in turn, interpreted by cells. The long-standing belief that auxin regulates its own transport via a positive feedback loop has led to the establishment of different theoretical models for auxin-dependent patterning processes that are tested in silico. Most of these simulation models are able to reproduce patterns observed in planta and have been crucial to predict and test the behavior of the PIN network in several developmental processes. Here, we review computational approaches to describe PIN polarization together with recent experimental data on PIN polar targeting, which can explain the patterned distribution of auxin throughout tissues. We also discuss how auxin gradients are perceived by the downstream signaling pathway and transduced to generate specific cellular responses.

1 Introduction: Auxin as a Crucial Morphogenetic Regulator in Plant

How multicellular organisms are able to create de novo patterns is a central question in developmental biology. One way to achieve patterning in developing organisms is through gradients of small molecules that we call morphogens. This term was first introduced by Turing (1952) who was interested in how interacting

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molecules could lead to the formation of patterns across a group of cells in both animal and plants. Morphogens are long-range signaling molecules that induce cellular responses in a dose-dependent manner. In a given tissue, a graded distribution of a morphogen exposes cells to different concentrations of the molecule. Cells exposed to higher levels of morphogen will adopt different fates than cells exposed to lower levels, thereby subdividing tissues into distinct cell types according to their distance from the morphogen source.

In plants sciences the best potential candidates for morphogen-like regulators are phytohormones. The most studied of them is unquestionably auxin. Auxin plays a crucial role in a large range of patterning processes and dose-dependent responses such as apicobasal axis formation (De Smet et al. 2007; Friml et al. 2003), de novo organ positioning (Heisler et al. 2005; Okada et al. 1991; Reinhardt et al. 2003a; Swarup et al. 2008; Vernoux et al. 2000), vascular development (Sachs 1981, 1991; Scarpella et al. 2006), and tropisms (Abas et al. 2006; Band et al. 2012; Friml et al. 2002b; Kleine-Vehn et al. 2010).

To date, evidence shows that auxin fulfills at least some of the criteria associated with morphogens in animals. First of all, its movement and graded distribution seems to be crucial for its action. In fact, it has been shown principally in *Arabidopsis thaliana* that in different developmental contexts, auxin response maxima (visualized by auxin signaling sensors) define both growth direction and tissue polarities (Benkova et al. 2003; Friml et al. 2002a; Sabatini et al. 1999).

Are these concentration gradients important for determining cell fates? Some evidence suggests that this is the case. During vascular tissue differentiation, for instance, local application can trigger distinct development response at specific distances from the point of application (Warren-Wilson et al. 1991, 1994). In the root apex, altering auxin distribution causes pattern reestablishment, and cells close to the newly formed auxin maximum acquire new developmental features (Friml et al. 2002a; Sabatini et al. 1999). In the shoot meristem, perturbing auxin transport also results in dramatic effects on cell fate (Vernoux et al. 2000) and auxin buildup during organ initiation is correlated with patterning. However, whether auxin actually functions as a plant morphogen is still a matter of debate (Benkova et al. 2009; Bhalerao and Bennett 2003) and would require proving that cells can respond directly to this molecule in a concentration-dependent manner. Along this line, we will discuss at the end of this chapter how auxin is perceived and cells elicit specific responses.

A key question for understanding how auxin can control patterning processes in plant is to know how auxin gradients are generated and maintained. In animals it has been proposed that morphogens diffuse from a producing localized source. In this case, local production of morphogen is a key process in determining gradients. For auxin a different scenario seems to apply, as the hormone is actively transported by polarly localized membrane proteins. This is linked to the fact that auxin cannot freely diffuse throughout tissues. According to the chemiosmotic model (Goldsmith et al. 1981; Raven 1975; Rubery and Sheldrake 1974), auxin which is a weak acid is largely protonated at extracellular pH and as such can enter cells by diffusion. Once inside cells, because of the higher pH, the hormone is deprotonated and trapped.

In order for auxin to leave a cell, it requires the activity of efflux carriers located at the plasma membrane, probably asymmetrically since auxin is known for some time to be transported in a polar fashion (Goldsmith 1966; Leopold and Hall 1966). Therefore, one major difference between auxin and animal morphogens as described by Turing is that its distribution involves an active transport. As we will see, this active polar transport is essential for the control of auxin-dependent patterning processes since inactivation of auxin cellular transporters can alter patterning in plants.

In this chapter we will discuss how this active transport in combination with different feedback mechanisms could self-organize to generate patterns of auxin distribution and how they are, in turn, interpreted by the signaling network in terms of cell differentiation.

2 Auxin Transport as a Pattern-Generating Process

2.1 The Basic Cellular Machinery

The chemiosmotic model has largely been confirmed by the discovery of different families of auxin transporters that contribute to either the import or the export of the molecule. Before discussing the processes that lead to the patterned distribution of auxin throughout tissues, we will briefly consider the cellular mechanisms that regulate distribution of the main transporters at the plasma membrane.

2.1.1 Auxin Efflux Carriers

The founding members of the PIN-FORMED (PIN) family of auxin efflux carriers were identified by classical forward genetics (Galweiler et al. 1998; Luschnig et al. 1998; Muller et al. 1998; Wisniewska et al. 2006). Most PIN family members are localized at the plasma membrane and distributed in a polar fashion in cells. They play an essential role in controlling the establishment of auxin gradients throughout plant development, and strong evidence exists that their polar localization in cells determines the direction of auxin fluxes (Wisniewska et al. 2006).

In this context membrane transport plays a central role. The colocalization of PIN proteins with an endocytic tracer has shown that PINs are continuously internalized (Paciorek et al. 2005). Evidences for recycling of PINs at the plasma membrane have also been provided by drug treatments. The fungal toxin brefeldin A (BFA), known to block trafficking from the endoplasmic reticulum to the plasma membrane, causes an aggregation of endosomes and internalized endocytic cargos into the so-called BFA compartments. It has been shown that PINs are found in BFA bodies after treatment and this effect is reversible, providing indirect evidence of PIN recycling (Geldner et al. 2001). Clathrin-dependent PIN cycling between the plasma membrane and

internal compartment has been demonstrated directly later on, by using a green-to-red photo-convertible EosFP fluorescent reporter (Dhonukshe et al. 2007).

How do cells address the transporters to a restricted portion of the plasma membrane? Although the question is far from being solved, several mechanisms essential for targeting PINs to correct membranes have been identified. Both the actin and microtubular cytoskeleton play important roles in guiding trafficking vesicles within cells. By using BFA and drugs that depolymerize actin filaments, it was demonstrated that the PIN recycling back to plasma membrane is actin dependent (Geldner et al. 2001; Kleine-Vehn et al. 2006, 2008). Although PIN movements do not seem to be directly controlled by microtubules, except during cell division (Geldner et al. 2001), there is a significant correlation between their localization in various tissues. PIN proteins are predominantly localized on membranes that are parallel to the main microtubule orientation in roots, in the shoot apical meristem, and in the leaf epidermis (Heisler et al. 2010). Interestingly, PIN remains restricted to certain membranes when microtubules are depolymerized, while microtubule reorients in a coordinated manner, even when auxin transport is inhibited. This indicates that their localizations might be regulated by a common upstream factor. Good candidates for such upstream regulators that control both PIN localization and cytoskeleton organization in cells are members of the Rho-GTPase (Rho in Plants or ROP) family. Indeed, dominant-negative mutants of ROP2 show that, at least in epidermal leaf cells, PIN1 localization is dependent on ROP2 signaling, which is also crucial (together with ROP6 signaling) in controlling cytoskeleton organization in these interdigitated cells (Fu et al. 2002, 2005; Xu et al. 2010). An additional element required for PIN localization is the cell wall. Removing the cell wall by enzymatic digestion, for example, causes a loss of PIN polarity and even leads to a complete retrieval of PIN from the plasma membrane (Boutte et al. 2006), while genetic and pharmacological interference with the cellulose synthesis perturbs PIN localization (Feraru et al. 2011).

Posttranslational modifications in the PIN proteins themselves also function in their localization. In this context, a striking role for PIN1 phosphorylation has been demonstrated. Mutants of the serine/threonine kinase PINOID (PID) produce pin-like inflorescences (Christensen et al. 2000), and in this mutant, PIN1 polarization switches from apical to basal membranes (Friml et al. 2004). Further studies have shown that the kinase PID and the phosphatase PP2A act antagonistically to control PIN polarization in different cell types (Michniewicz et al. 2007). Three serine residues conserved within the PIN1 hydrophilic loop are directly phosphorylated by PID (Huang et al. 2010). Neither the loss of phosphorylation nor phosphomimicking versions of PIN1—generated by mutating the three serine residues—were able to rescue the defects of *pin1* mutant highlighting the importance of the dynamics of PINs' reversible phosphorylation for proper localization (Huang et al. 2010).

A scenario thus emerges where cytoskeleton-based membrane traffic and interactions with the cortical microtubules and cell wall lead to specific localizations of PIN at the plasma membrane (Fig. 1). This leads to another question, that is, how is this basic cellular machinery coordinated throughout

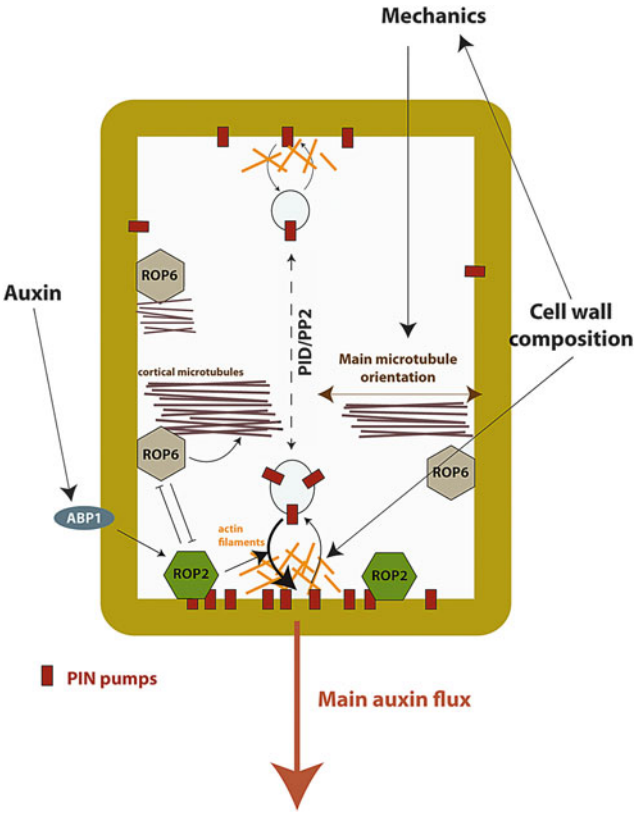

Fig. 1 Regulation of PIN distribution at the plasma membrane. Auxin can enter cells both by diffusion or auxin influx carrier. Once inside cells, auxin is trapped and needs PIN efflux transporters to leave cells. Interestingly, most of the time PIN proteins are polarized at the plasma membrane in order to direct auxin fluxes. PINs have been demonstrated to continuously cycle between internal compartments and the plasma membrane. Different factors have been demonstrated to control and influence PIN polarization at the plasma membrane. First, cytoskeleton seems to be essential to address the transporters to a restricted portion of the plasma membrane: actin is important for the recycling back to plasma membrane of PIN vesicles and PINs have been shown to be mainly addressed to the membranes parallel to the main microtubule orientation. This organization might be triggered by members of the ROP family. ROP2 would address PINs to the plasma membrane in an actin-dependent manner while ROP6 would promote microtubule attachment. Since both classes of ROP proteins mutually inhibit each other, this would lead to alternate microtubule-rich and PIN-/actin-rich domains. Others factors such as auxin or mechanical tensions would then contribute to this polar organization by promoting, respectively, PIN recycling at the plasma membrane (via an ABP1-dependent activation of ROP2) or microtubule organization. Posttranslational modifications could then help in targeting PINs to the right actin-rich portions. In fact, it has been shown that the PINOID (PID) kinase and the PP2A phosphatase act antagonistically to control PIN polarization and PID mutations can trigger switches from basal to apical membranes

tissues to generate auxin fluxes and auxin distributions. Indeed, the generation of particular patterns of auxin distribution in tissues somehow implies that cells have to coordinate PIN polarities. We will address these questions in Sect. 2.2.

2.1.2 Auxin Influx Carriers and Other Regulators of Polar Auxin Transport

Genetic studies have also led to the discovery of auxin influx carriers of the AUX1/LIKE_AUX1 (LAX) family. The *auxin resistant 1* (*aux1*) mutant was isolated from a screen for auxin-resistant and agravitropic mutants. Positional cloning demonstrated that *AUX1* encodes an H+−symport plasma membrane permease (Bennett et al. 1996). Studies on the effects of exogenous auxins on root growth in both wild-type and *aux1* seedlings have demonstrated that *aux1* mutant was much more resistant to the membrane-impermeable synthetic auxin 2,4-D than to the membrane-permeable 1-NAA (Maher and Martindale 1980). In fact, the root agravitropic phenotype could be rescued by supplying roots with 1-NAA, whereas 2,4-D did not show this effect (Marchant et al. 1999; Yamamoto and Yamamoto 1998). Besides, it has been shown that *aux1* root tips accumulate 50 % less radiolabeled IAA or 2,4-D than the wild-type root tips do (Marchant et al. 1999; Rahman et al. 2001). Taken together these data strongly supports a role for AUX1 in auxin uptake. This hypothesis was confirmed by heterologous expression of AUX1 in *Xenopus laevis* oocyte cells which demonstrated a specific, high-affinity, pH-dependent auxin uptake entirely dependent on AUX1 (Yang et al. 2006).

In addition to AUX1, three LAX genes have been identified in *Arabidopsis* (Parry et al. 2001). They function similarly as auxin influx carriers (Peret et al. 2012) and play partially redundant role with AUX1 during development (Bainbridge et al. 2008; Peret et al. 2012). The AUX/LAX proteins are mainly asymmetrically localized on plasma membrane, except for AUX1 in the phloem in *Arabidopsis* root tips (Swarup et al. 2001). AUX1 membrane-targeting mechanism is also different from the PIN mechanisms since AUX1 and PIN proteins accumulate in different subsets of vesicles and their trafficking show different sensitivities to various inhibitors (Dharmasiri et al. 2006; Kleine-Vehn et al. 2006).

Members of the P-glycoprotein subfamily of ATP-binding cassette (ABC) proteins (PGPs) are also involved in both the cellular efflux and influx of auxin (Cho et al. 2007; Geisler et al. 2005; Petrasek et al. 2006; Santelia et al. 2005). PGPs are important regulators of plant development since their mutant exhibit obvious developmental defects (Noh et al. 2001). However, PGP mutants exhibit weak auxin-related phenotypes, thereby leaving open the question of whether auxin transport represents their primary physiological function (Santelia et al. 2005; Terasaka et al. 2005).

2.2 *From Transport to Pattern: A Key Role for Modeling*

2.2.1 Auxin Distributions in Different Organs

The difficulty to obtain a detailed picture of local concentrations for small molecule-like auxin has been partly compensated by the development of auxin-inducible markers, the most widely used being the one driven by the DR5 promoter (Ulmasov et al. 1997) and, more recently, the DII-VENUS sensor (Brunoud et al. 2012). Both markers were designed from knowledge on the auxin signaling pathway but depend on different steps of this pathway that we present further in Sect. 3. DR5 reports transcriptional activation in response to auxin, whereas DII-VENUS monitors the degradation of Aux/IAAs, the first step of the auxin signaling pathway, thus relating more directly to auxin concentrations. Together with high-resolution mass spectrometry (Novak et al. 2012), these markers have provided a detailed picture of auxin distribution in different parts of the plant. In this context three organs, the root apex, the shoot apex, and the developing leaves, have received particular attention.

At the root apex, the DR5 auxin-responsive promoter is activated in a restricted group of cells comprising columella and the quiescent center. Inhibition of polar auxin transport by chemicals is able to shift and expand DR5 activity domain at the root tip, and treatment with exogenous 2,4-D auxin activates homogeneously DR5 in the tissue (Sabatini et al. 1999). Thus, in the root meristem, DR5 was suggested to report an auxin maximum in a restricted group of cells. This auxin maximum has been recently confirmed using DII-VENUS (Brunoud et al. 2012).

Analysis of PIN polarities in the root suggests the existence of three regions with specific polarities: basal polarity in vasculature and cortex, homogeneous distribution of PINs on membranes in columella cells, and apical polarity in the epidermis in addition to a lateral polarity at the transition between the meristem and the elongation zone (Blilou et al. 2005). The topography of the PIN network was suggested to create a reflux loop maintaining the auxin maximum at the root meristem, a hypothesis supported by simulations of auxin transport on a simplified root structure (Grieneisen et al. 2007). More recently, a model using this topography could also lead to auxin accumulation at the start of the elongation zone, a prediction confirmed using DII-VENUS (Brunoud et al. 2012; Santuari et al. 2011). These two studies strongly support the idea that the auxin distribution in the root apex results mostly from the polar auxin transport activity.

At the shoot apical meristem, the importance of patterned auxin distribution in organ formation became evident from experiments where the PIN machinery is impaired. Indeed, inhibition of polar auxin transport genetically (*pin1* mutant) or chemically (NPA treatment) completely abolishes organ initiation at the shoot apical meristem (Okada et al. 1991; Reinhardt et al. 2000, 2003b). In both cases, local application of exogenous auxin can restore organogenesis in a position-specific manner, suggesting that auxin is an instructive signal in this process and that local auxin maxima are required to induce the formation of a new organ. Using the

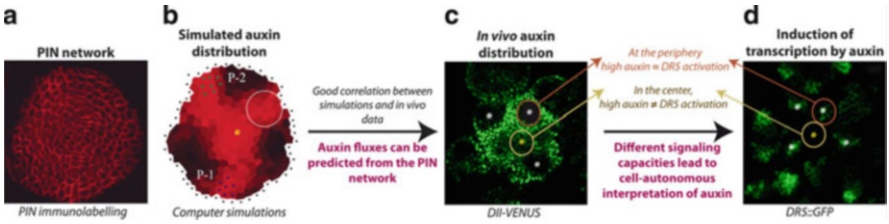

Fig. 2 From transport to patterning: the example of the shoot apical meristem. (**a**, **b**) Simulations of auxin distribution in the shoot apical meristem can be inferred from the topography of the PIN1 network in the structure (adapted from Barbier de Reuille et al. 2006). They suggest that auxin is accumulated in young primordia (visualized by *green* and *blue dots*) but also in the meristem center (*yellow asterisk*). (**c**) In order to confirm these predictions, the pattern of DII-VENUS—a sensor that monitors Aux/IAA degradation which is directly related to auxin levels (see Fig. 3)—is analyzed at the shoot apex. Auxin is shown to accumulate both in incipient organs (*white asterisks*) and at the meristem summit (*yellow asterisk*) as predicted by simulation studies, demonstrating that PIN1 proteins effectively control auxin distribution in the structure (adapted from Vernoux et al. 2011). (**d**) To test whether cells can perceive these auxin gradients and respond accordingly in order to create patterns, activation of the DR5 auxin-inducible promoter is analyzed. It has been shown that at the periphery of the meristem, DR5 is upregulated in sites where auxin is accumulated [which corresponds to young organs, visualized by *white asterisks*, also seen in (**c**)], whereas in the center of the structure it is not the case. In fact, in the center of the meristem (*yellow asterisk*), auxin levels are relatively high (**c**) but DR5 is not activated (adapted from Vernoux et al. 2011). These results suggest that the center and the periphery of the shoot apical meristem have different sensitivities to auxin and that the auxin signaling pathway limits gene activation in response to auxin in the meristem center. Thus, local differences in competence and tissue-specific response (mediated by the downstreaming signaling pathway) appear crucial to understand auxin-mediated pattern formation

synthetic auxin-responsive DR5 promoter, it has been shown that auxin effectively accumulates in young initia, forming spots that display a phyllotactic arrangement (Benkova et al. 2003; Smith et al. 2006). Based on these data and on PIN1 polarization patterns at the shoot apex, Reinhardt (2003a, b) proposed a model to explain regulation of phyllotaxis based on polar auxin transport. Auxin would be transported apically towards developing primordia that would act as auxin sinks by transporting the hormone in provascular tissues. As a result, surrounding tissues would then be depleted of auxin, preventing new organs from being formed too close to the previous ones. This hypothesis is supported both by simulations of auxin distribution based on the topography of the PIN1 network in the shoot apical meristem (Barbier de Reuille et al. 2006) and more recently by DII-VENUS pattern in this tissue (Brunoud et al. 2012) (Fig. 2). These data also demonstrated that the existence of a maximum of auxin at the center of the shoot meristem, and similarly to the root, suggests that the PIN1 network is controlling auxin distribution in the shoot.

Similar conclusions came from the analysis of auxin distribution during vein formation in young leaves. Indeed, DR5 expression marks the future site of leaf veins prior to the expression of the early pre-procambium marker (Mattsson et al. 2003; Scarpella et al. 2004, 2006). This early DR5 expression occurs concomitantly with PIN1 expression, and both are considered the earliest known markers for vascular initiation (Scarpella et al. 2006).

2.2.2 Different Feedback Models for Auxin Transport

As we have seen, auxin and its transport have been intimately linked to patterning processes. How do neighboring cells communicate to coordinate auxin transport? How are PIN transporters aligned in supracellular patterns? These questions are not new. Over the last 50 years, different concepts were proposed to explain how directed fluxes of auxin are generated. Since the precise cellular processes are only understood in a very partial manner, these concepts remain abstract, although they provide a general framework for analyzing the processes that lead to particular auxin distributions.

The Canalization Hypothesis: Transporting Auxin with the Flux

Sachs (1969, 1975, 1981) was the first to hypothesize that auxin could regulate its own transport, years before the discovery of PIN proteins. He was interested in the development of the vascular network. By performing local auxin applications on pea hypocotyls, he showed that auxin was able to induce the formation of tracheary elements from the site of auxin application to the central vein already present. The differentiation of such narrow vascular strands could not be explained by diffusion, so he proposed that an active mechanism—which he called canalization—was acting to connect auxin sources (i.e., the local applications in his experiments) to auxin sinks (the central vein). He proposed that auxin fluxes across cells are able to enhance auxin transport and that this feedback of auxin flux on transport drives the selection of strands of cells that will then differentiate into vascular tissues.

Mitchison further tested the ability of auxin flux-feedback model to generate patterns (Mitchison 1980, 1981). He proposed two variants whereby the canalization process could be realized through either facilitated diffusion or polarized auxin transport. In the first case, auxin transport is mediated via passive channels which are bidirectional, increasing the ability of auxin to move in both directions. In the second variant, the number of transporters can vary on each side of the interface. The direction of flux is then an important component of the model: auxin transport is reinforced only when there is a net efflux of auxin across the interface. Mitchison showed that both variations of the model were able to generate discrete channels of auxin flow within a uniform field of cells, similar to those observed during vascular development in leaves. One constraint for the model to work is that the relationship between the increase in transport rate and auxin flux must be nonlinear, thus resulting in amplification of the flux.

The actual discovery of the PIN transporters and their localization permitted a more precise examination of the canalization hypothesis. To further validate Mitchison's model, Rolland-Lagan and Prusinkiewicz performed simulations on the polar transport version of Mitchison's model and compared it with PIN1 expression data (Rolland-Lagan and Prusinkiewicz 2005). During midvein initiation in leaves, PIN proteins polarize away from auxin maxima at the leaf tip towards the vasculature below (Bayer et al. 2009; Reinhardt et al. 2003a; Scarpella et al.

2006). Rolland-Lagan and coworkers showed that the model was able to reproduce this behavior of PIN1. In addition, they demonstrated that canalization models were able to reproduce a large range of venation patterns that occur *in planta*, including connected veins and discontinuous strands in mutants (Bayer et al. 2009; Reinhardt et al. 2003a; Sauer et al. 2006; Scarpella et al. 2006).

However, some predictions from these classical models remain in (at least apparent) contradiction with experimental data. Sachs supposed that preexisting veins were acting like auxin sinks, with low auxin concentrations compared to surrounding tissues, thereby attracting developing veins towards them. However, as preexisting strands exhibit a high DR5 expression (Scarpella et al. 2006), it is likely that they contain high auxin concentrations. To assess these shortcomings, different hypotheses were made. A model proposed that the number of PIN proteins within a cell is fixed and that different regions of the plasma membrane compete for intracellular PINs (Feugier et al. 2005). In that case, fully developed veins exhibited high auxin concentration. In another model, the AUX/LAX influx carriers were proposed to facilitate accumulation of auxin in vascular cells (Kramer 2004).

The discovery of PIN proteins also revealed that auxin transport is not always occurring along narrow channels. This is typically the case at the shoot apical meristem, where PIN polarities adopt highly complex patterns throughout the tissue. This might seem in contradiction with the canalization hypothesis, but recent simulations showed that this is not necessarily the case. Stoma et al. (2008) demonstrated that this phenomenon could be due to a low amplification response to flux of the epidermal cells. Indeed as long as this flux-based amplification remains linear, no canalization occurs, but only diffuse patterns are obtained.

Another observation that is in apparent contradiction with canalization is that PIN1 proteins at the meristem surface tend to polarize towards high auxin concentrations that correspond to sites of organogenesis. The same is true for the root, where an auxin maximum is maintained at the root tip, with auxin fluxes going in and out. However, flux-based models do not exclude such a situation. Analogous to water flowing in and out of a basin, it is perfectly possible for canalized auxin fluxes to go against or downstream of preexisting gradients. Indeed, Stoma et al. showed that canalization could explain not only the venation patterns but also the patterns of auxin maxima observed in root and shoot. At the shoot, meristems produce leaves and flowers in highly ordered patterns. These organs can be produced in opposite, spiraled, or whorled arrangements, also called phyllotaxis. Stoma et al. tested if canalization could reproduce these patterns using a small field of virtual cells. This was indeed the case, and importantly, a flux-based model was also able to reproduce realistic patterns of PIN distribution in virtual tissues representing root and shoot meristems.

Transport Against the Gradient

The complex patterns of PIN distribution at the shoot apical meristem surface suggest fluxes of auxin towards local auxin maxima. This leads to propose an alternative scenario for auxin transport, where auxin would flow against a gradient.

In these so-called up-the-gradient models, each cell senses auxin concentrations in neighboring cells and subsequently polarizes its PINs towards neighbors with higher concentration. It assumes the existence of a short-range signaling pathway that gathers information about auxin content in surrounding tissues.

Computational models based on this concept were able to reproduce a large variety of phyllotactic patterns observed *in planta*, including spiral, distichous, decussate, and tricussate ones. Even switches between patterns could be reproduced by modifying model parameters (Jonsson et al. 2006; Smith et al. 2006). In principle, such a mechanism could also account for the formation of veins in leaves. Merks et al. (2007) showed that an up-the-gradient mechanism could in principle also lead to a moving maximum. This *traveling wave* mechanism could in principle account for the formation of veins. However, a limitation of the up-the-gradient model is that it does not easily explain, in contrast to canalization, the orientation of PIN transporters away from an auxin maximum as observed in the root and during vein development in the shoot meristem, for example.

Combined Model

As the up-the-gradient model does not provide a straightforward explanation for PIN transporters pointing away from auxin maxima, Bayer et al. (2009) proposed a hybrid model, where both canalization and up-the-gradient would coexist. In this model, cells with low auxin concentrations follow the *up-the-gradient* mode, whereas above a certain threshold cells would switch to a canalization mechanism. In this scenario, relative low auxin levels at the meristem surface would induce an *up-the-gradient* behavior, which would lead to the local accumulation of the hormone. These local maxima would then cause a switch to canalization towards the inner tissues. This combined model was able to reproduce observed PIN polarization patterns in both phyllotaxis and vein formation. Several predictions made by this model were validated. First, the hybrid model predicts high auxin concentrations in the forming vein as observed in reality. It therefore does not require extra hypotheses to explain this phenomenon, in contrast to the canalization hypothesis, which requires the presence of an auxin importer to keep auxin at sufficiently high levels (see above). Second, the model also predicted a temporary orientation of PIN transporters in inner tissues towards the auxin maximum at the surface, an event that was again observed in reality and not easily explained by canalization. However, whereas canalization and up-the-gradient only need a single mechanism, the hybrid model is not a minimal hypothesis and requires the coexistence of two different mechanisms.

2.2.3 Towards a Mechanistic View of Patterned Auxin Transport

To compare and further test the theoretical frameworks we have just discussed, more details are required on the actual cellular and molecular processes involved.

These models indeed ask the question of how incoming and outcoming fluxes or concentrations are sensed. Although our understanding remains limited, recent findings provide a number of interesting leads.

Cell–Cell Communication via the ROP Pathway

As we have seen above, both PIN localization and microtubule organization are strongly correlated in several tissues. In leaves, this coordination likely involves several ROP proteins that mutually inhibit each other (Xu et al. 2010). More specifically, ROP2 would address PIN to the plasma membrane via an actin-driven process, while ROP6 would cause the attachment and organization of microtubules. Since both ROP proteins mutually inhibit each other, it is easy to see how this would lead, through stochastic variations, to alternate microtubule-rich and actin-/PIN-rich domains (Fig. 1). Xu et al. (2010) also found that auxin itself promotes ROP activity via the auxin receptor AUXIN BINDING PROTEIN1 (ABP1), a process that does not involve transcriptional regulation. Importantly, the two proteins show a differential sensitivity to auxin as the activation of ROP2 is saturated at lower hormone concentrations than that of ROP6. Computer simulations taking into account all these properties have shown that, in a multicellular context, such a system would lead to complementary patterns of ROP2/PIN and ROP6/microtubule distributions along neighboring membranes. Although this remains to be proven, such a mechanism could explain how directed fluxes could arise in all tissues throughout the plant. It also remains to be seen whether this system would rather correspond to a canalization process, transport up the gradient, or something completely different.

A Role for Biomechanics?

Whereas the mechanism mentioned above can potentially explain how very local chemical interactions between cells generate patterns, a second line of evidence points at the importance of mechanical feedbacks. There is strong evidence that microtubules themselves are at least in part reacting to mechanical tension within tissues (Hamant et al. 2008). Such tensions can arise, for example, when cells in neighboring regions grow at different rates. In both roots and shoots, microtubules align along the predicted main force directions. Since they are also controlling the orientation of the cellulose microfibrils in the wall in the same direction, they might reinforce the cell wall to resist the forces, causing organ outgrowth and tissue folding. If microtubules respond to stresses and PIN localization is correlated with microtubule directions, it is tempting to propose that auxin transport itself is also influenced by mechanics. This hypothesis was further corroborated by experiments where mechanical tensions were perturbed. Cell ablations as well as drug treatments affecting the cell wall caused concerted changes in microtubule organization and PIN localization, and together the data suggested an interesting

explanation for the *up-the-gradient* model. In this context, Heisler et al. (2010) tested if mechanical signals would be sufficient to explain the observed patterns of PIN1 using a computer model. They investigated the hypothesis that PIN1 in each cell localizes towards the wall that is most mechanically stressed. An important postulate was the existence of stress-induced signals from the cell wall that act only locally to promote accumulation of PIN1 at the nearest membrane. The model also assumed that auxin-induced cell wall loosening in response to auxin concentrations inside a cell is limited to its own wall. In other words, the cell would sense via biomechanical signals transmitted through the cell wall, which of its neighbor has the highest concentration of auxin, and polarize its PIN proteins towards this cell.

Mechanistic Insights: Conclusions

A better understanding of the cellular mechanisms leading to polar PIN localization is currently absolutely required. The results regarding the role of ROPs and the cytoskeleton are very promising and have provided a first basis for more mechanistic models for auxin transport. In parallel, the link with the membrane traffic machinery should also be further explored. A step in this direction was taken by Wabnik et al., for example, who combined the feedback of auxin on both transcription and endocytosis in a single model (Wabnik et al. 2010). Combined with extracellular perception, this model was able to reproduce venation patterns, suggesting that it could behave like a canalization model.

3 From Auxin Distribution to Downstream Gene Expression Patterns

Once auxin gradients are established, the cells need to perceive local hormone concentrations and respond accordingly. If auxin acts as a morphogen, one should expect a dose-dependent response. The reality, however, appears more complex than that. First, there seems to be a tissue-specific difference in competence to react to auxin.

For instance, auxin causes the upregulation of a DR5 reporter at the periphery of the meristem, while it is not able to do so in the meristem center (Barbier de Reuille et al. 2006; Vernoux et al. 2011) (Fig. 2). Second, there is a tissue-specific modulation of auxin response: auxin triggers organogenesis at the periphery of the shoot apical meristem, leads to vascular strands development in young leaves, and also induces lateral root formation (Reinhardt et al. 2000; Scarpella et al. 2006). Therefore, it is not possible to understand auxin-based pattern formation without considering local differences in competence and tissue-specific responses. This, in turn, suggests that we have to consider the downstream signal transduction pathway as a plausible key regulation level of auxin responses.

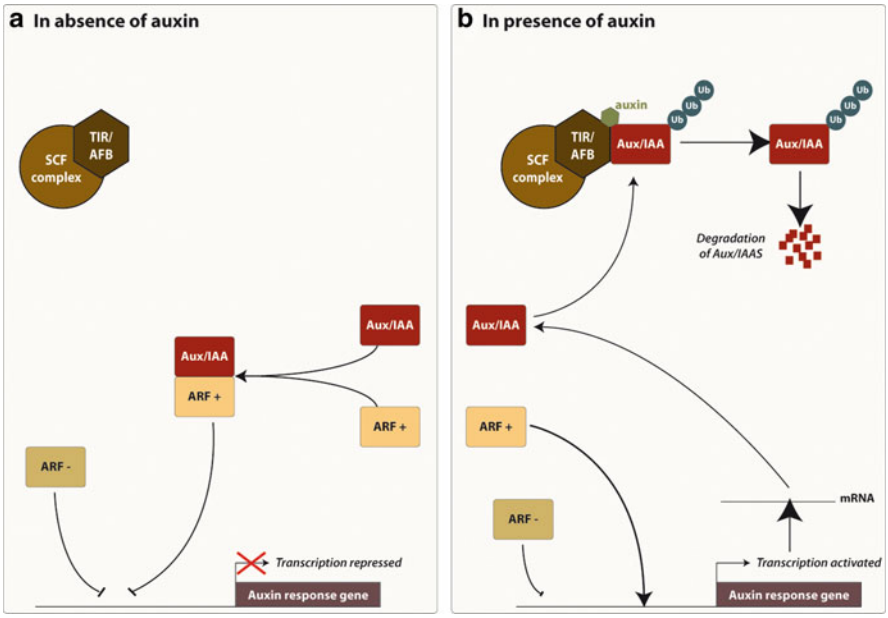

Fig. 3 Regulation of gene expression by auxin. The auxin signal transduction requires two families of transcriptional regulators: Aux/IAAs and auxin response factor (ARFs). At low auxin levels, Aux/IAAs bind to the ARFs preventing them from regulating target genes. When auxin levels rise, the F-box protein (TIR1 or its close AFB relatives) interacts with Aux/IAA repressors in an auxin-dependent manner, leading to their degradation by the 26S proteasome. Aux/IAA degradation likely dissociates corepressors from ARF proteins residing on promoters of auxin response genes, allowing them to promote transcription. This includes activation of most of the *Aux/IAA* genes themselves, thus establishing a negative feedback loop. Note that the ARF family is composed of both transcriptional repressors (ARF−) and activators (ARF+) and both of them may regulate the same target genes. The Aux/IAAs mainly interact with ARF+

The auxin signal transduction requires two families of transcriptional regulators: Aux/IAAs and auxin response factor (ARFs) which are composed, respectively, of 29 and 23 different elements. Aux/IAAs and ARFs have been shown to form homo- and heterodimers both within and between families (Chapman and Estelle 2009). In absence of auxin, Aux/IAAs bind to the ARFs and, by recruiting corepressors of the TOPLESS family, prevent them from regulating auxin target genes. In the presence of auxin, Aux/IAA proteins bind to the TIR1 F-box of SCF complex (or its AFB homologues) and act as auxin coreceptors. This interaction leads to the ubiquitination and subsequent degradation of Aux/IAAs thereby allowing the ARFs to regulate transcription (Fig. 3). This includes activation of the *Aux/IAA* genes, thus establishing a negative feedback loop. Importantly, sequence analysis and transient assays suggest that most ARFs act as transcriptional repressors, while only 5 ARFs are activators (Guilfoyle and Hagen 2007).

To understand how the auxin transduction pathway might modulate local auxin responses, not only the expression patterns of the effectors of the pathway but also

information on protein interactions and identification of ARF targets is required. Relevant information has been recently obtained in different auxin-dependent developmental contexts. During lateral root initiation, it was shown that two different sets of ARFs/IAAs (a crucial early IAA14–ARF7–ARF19-dependent auxin response module, followed by a second IAA12–ARF5 module) were expressed successively and that both were required from proper development (De Smet et al. 2010). It suggests that auxin could coordinate distinct consecutive developmental steps through the expression of different response modules over time. In *Arabidopsis* embryo, a key role for specific ARF-Aux/IAA pairs has been shown (Weijers et al. 2005, 2006) and an expression map of all 23 ARFs during embryogenesis indicates that the transcriptional regulation of ARFs generates combinatorial patterns that might create a prepattern essential in the regulation of cell fate (Rademacher et al. 2011).

In a recent study, a full ARF-Aux/IAA interactome was obtained. This work revealed three clusters corresponding to the Aux/IAAs, ARF activators, and ARF repressors, respectively, and showing stereotypical interaction capacities (Vernoux et al. 2011). Aux/IAAs interact with themselves and with the ARF activators. The ARF activators mainly interact with AUX/IAAs and poorly with themselves. Finally the ARF repressors show low or no interactions with the rest of the network or with themselves. The expression of these genes was tested at the shoot apical meristem and 25 of them were detected (among which some Aux/IAAs, ARF activators and repressors). Importantly, here the situation is different from what was observed in the embryo since most of the Aux/IAA and ARFs are co-expressed, albeit at different levels: weakly in the center of the meristem, where the stem cells are located, and much more strongly at the cell periphery where organs are initiated. Mathematical modeling of the network predicted that the differences in expression levels between the center and the periphery of the meristem result in different auxin sensitivities (high sensitivity at the periphery, low at the center). The demonstration of high level of auxin both in lateral organs and at the center of the meristem using DII-VENUS (Vernoux, Brunoud), while DR5 is activated specifically in lateral organs, confirmed this prediction (Fig. 2). In addition, this work suggests a surprisingly simple scenario for the shoot apical meristem, where auxin-activated gene expression would be controlled by a *double brake* system (Vernoux et al. 2011). In this scenario activating ARFs would be both inhibited by interactions with the AUX/IAAs and by competition with the ARF repressors for binding sites in the promoters of target genes. Increasing auxin concentrations would cause the degradation of AUX/IAAs but not influence the competition with the ARF repressors. This high level of inhibitory regulation can buffer local variations in auxin levels (Vernoux et al. 2011), thus likely contributing to the robustness of patterning in the shoot meristem.

This illustrates clearly that auxin responses do not only depend on auxin concentrations but also on the competence to react to hormone levels with different mode of regulation observed in different tissues.

Importantly, a recent report has shown yet another level of complexity in auxin signaling. Aux/IAAs and TIR/AFBs have been demonstrated to be both necessary and sufficient for auxin binding thus defining pairs of coreceptors (Calderon

Villalobos et al. 2012). As there are, as mentioned, 6 different TIR/AFBs and 29 different Aux/IAA proteins, an important combination of coreceptors can be formed. Interestingly, each of them has a different affinity to auxin, suggesting that the combination of coreceptors expressed within a cell might determine the range of auxin concentration to which the cell can react. Further work should reveal a key role for this mechanism in patterning in response to auxin.

4 Conclusions and Perspectives

Auxin is a versatile signaling molecule in plants that is involved in many developmental processes. The polar transport of auxin has been shown to play a crucial role in controlling auxin-regulated development since it is able to redistribute auxin in specific patterns, in different tissues. Most of the attention has been paid to understand how the PIN network can self-organize to generate such specific patterns. It seems now established that both chemical (auxin itself) and mechanical signals influence PIN polarization in cells, but the precise molecular mechanisms by which these different signals are integrated to precisely control PIN orientation still remain a major issue.

Once distributed in specific patterns, auxin is locally interpreted by its perception pathway. Given the abundance and structure of the downstream network, cellular responses to auxin depend not only on hormone concentrations but also on the interaction of all the effectors of the signaling pathway. Besides, auxin is able to induce a wide range of responses in different tissues by activation of specific pathways and it is yet not fully clear where the specificity lies.

Although genetic studies have made clear that polar transport is essential in controlling auxin-dependent patterning, biosynthesis might also be an important component in this process. Indeed, it has been shown in different developmental contexts that local biosynthesis of auxin is required for proper patterning (Cheng et al. 2006, 2007a, b, 2008; Stepanova et al. 2008), suggesting that it might be an important mechanism to stabilize PIN-dependent patterns. In this case, the control of patterning by auxin would not be so different from animal morphogens: both would be locally produced and distributed in a directional fashion. It will thus be really crucial to integrate biosynthesis data into current models of auxin patterning.

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Plant Competition: Light Signals Control Polar Auxin Transport

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Abstract Plants typically grow at high densities in natural vegetations and agricultural fields, where they interact with their neighbours to compete for limited resources, and respond with morphological acclimations that facilitate resource capture. A well-studied example of aboveground responses to neighbours is the shade avoidance syndrome (SAS), which is comprised of enhanced stem and petiole elongation, upward leaf movement, apical dominance and early flowering. SAS is induced upon detection of neighbouring vegetation and the earliest signals to exist are a reduction of the red:far-red light ratio (R:FR), caused by far-red light reflection by neighbouring plants. Plants sense this reduced R:FR through their phytochrome photoreceptors which regulate a signal transduction cascade that induces shade avoidance. A key target of this cascade is the biosynthesis and transport of the plant hormone auxin. It is reviewed here how photoreceptors control auxin biosynthesis and auxin transport to promote shade avoidance and fitness of plants in dense stands.

1 History

The concept of chemical messengers (hormones) in plants emerged from a series of classic experiments on how plant stems (or coleoptiles) grow towards the light, a response that is called (positive) phototropism. Phototropism directs growing plant organs towards the sunlight, for example, at the border of a dense field, or towards a gap in the canopy for maximal light capture.

Experiments in which directional light from the side was used to induce phototropism were already conducted by Darwin (1880). He showed that only the tip of the coleoptiles could sense the light direction; however, the actual bending took

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place just below the tip. Darwin proposed that some kind of (chemical) signal must be transmitted downwards from the tip to the elongating side of the coleoptile. Went (1926) confirmed this hypothesis experimentally using agar blocks; by letting this chemical diffuse into the agar and subsequently placing these agar blocks, he was able to induce coleoptile curvature. When these blocks were placed on decapitated coleoptiles, it induced an elongation response, but when the blocks were placed off-centre, the coleoptiles responded as if responding to directional illumination. For this chemical messenger, Went chose the name auxin, which translates "to increase" in Greek. These early studies by Went thus showed that the differential distribution of auxin underlies the unequal growth at the two sides of a bending organ. Since then a large number of studies have shown that auxin and polar auxin transport are involved in many processes such as embryogenesis, stem cell differentiation, cell division and cell elongation (reviewed by Teale et al. 2006).

Plants usually grow in dynamic environments with neighbouring plants and consequently severe competition for light. In dense stands relatively smaller plants will be shaded by their (slightly) taller neighbours, which will lead to asymmetric competition and results in a lower fitness of the suppressed individuals (Schmitt et al. 1995; Weinig 2000). Most plants can detect early signals of (a)biotic stress and adjust their physiology to limit the impact. Crowding is an example of stress as it results in competition for limited resources. Generally, plants will respond to crowding by elongating their shoot and move their leaves upward to escape from this light limitation stress. This so-called shade avoidance syndrome results in a relative higher light interception since the leaves will be placed into more favourable light conditions (reviewed by Franklin 2008). Elegant data from the Ballaré laboratory established that plants can even demonstrate the shade avoidance response to neighbouring plants before actual shading and competition occur (Ballaré et al. 1987, 1990), thus limiting the future chances of being shaded.

To respond appropriately to these environmental dynamics, plants need reliable signals to detect changes in their environment. In a dense canopy, several facets of the light reaching this smaller plant will change. Not only the direction of the light source may alter, as mentioned above, but also the light intensity and the composition of the light reaching the leaves will change. Perception of such neighbour detection signals, mostly light signals perceived by photoreceptors, can induce rapid changes in gene expression and physiological processes, which regulate phenotypic plasticity (reviewed by Keuskamp et al. 2010a). Morelli and Ruberti (2000) were the first to hypothesise that stem elongation during shade avoidance would be driven by a redistribution of auxin through polar auxin transport.

Although it has been long known that auxin plays a role in plant growth responses to light signals, the last five years have delivered major discoveries regarding the mechanisms through which auxin controls plant responses to light signals that help plants compete for light, using the model species *Arabidopsis thaliana*.

2 Phototropism

Sensing the environment through signals such as altered light quality, quantity and/or direction is of great importance for plants to survive competition for light with surrounding neighbours. These light signals are being perceived through the plants' photoreceptors. Higher plants have four different families of photoreceptors in order to detect UV-B, blue, red (R) and/or far-red (FR) light cues. The cryptochrome and phototropin families are sensitive to blue light fluence rates, whereas phytochromes are mostly sensitive to R and FR light (Ahmad and Cashmore 1993; Quail et al. 1995; Briggs et al. 2001). Recently the fourth photoreceptor, the UV-B photoreceptor UV-RESISTANCE LOCUS 8 (UVR8), has been characterised (Rizzini et al. 2011).

It has been shown that the blue light receptor PHOTOTROPIN (PHOT) is the main regulator of the phototropic response of *A. thaliana* by sensing the direction of the (blue) light. Phototropins belong to the AGC family of kinases and are sensitive to blue (390–500 nm) and ultraviolet-A (320–390 nm) light (Christie 2007). The NONPHOTOTROPIC HYPOCOTYLS 3 (NPH3) protein interacts with phototropin and its activity depends on the blue light-induced phosphorylation of phototropin. Interestingly, NPH3 is also known to be involved in auxin distribution during phototropism (Pedmale and Liscum 2007).

Friml et al. (2002) showed, using the expression pattern of DR5::GUS, that during phototropic bending of the hypocotyl of *A. thaliana* seedlings, auxin activity is up-regulated in the elongating cells. In a study by Esmon et al. (2006), it was shown that during the phototropic response of *Brassica oleracea* seedlings, a close relative of *A. thaliana*, 20 % more indole-3-acetic acid (IAA; auxin) accumulated in the elongating flank. These authors also showed that members of the expansin protein family displayed a differential expression pattern upon phototropic stimulation, having more expression of expansins on the elongating side. Expansins are involved in cell wall loosening, which is an essential process to promote turgor-driven cell expansion in a pH-dependent manner (Cosgrove 2005), a process referred to as acid-induced extension (AIE). Cell wall loosening is at the heart of cell expansion-driven processes such as phototropism of hypocotyls. Up-regulation of expansin activity has also been shown to occur during non-differential internode elongation in response to shade cues in the non-model species *Stellaria longipes* (Sasidharan et al. 2008). Interestingly, auxin can induce acid-induced extension of cell walls (Takahashi et al. 2012), providing a direct link between auxin gradients and elongation responses. Thus, auxin is clearly involved in the phototropic response, but how is this auxin gradient established?

Recent studies showed that curvature of the hypocotyl upon phototropic stimulation was (partly) dependent on the auxin efflux carriers PIN-FORMED3 (PIN3) and PIN1 (Friml et al. 2002; Blakeslee et al. 2004). PIN proteins are a family of eight members in *A. thaliana*, which are known to perform a rate-limiting function in cellular auxin efflux, thereby controlling the direction of the auxin flow (Blilou et al. 2005; Petrasek et al. 2006; Wisniewska et al. 2006). PIN proteins are therefore important players in polar auxin transport (PAT) and primary candidates for regulating auxin concentration gradients. An important feature of auxin hence is that the exact

level of auxin in a specific cell or tissue can be tightly controlled through auxin transport control, thus providing the opportunity for differential regulation of cell expansion rates in different organs, tissues and even cells.

In a more recent study, it was shown that exposure to light coming from one lateral direction polarised the cellular localisation of PIN3 to the lateral side of endodermal hypocotyl cells on the non-illuminated side. This resulted in an auxin flux away from the illuminated side towards the non-illuminated side. As a result, the non-illuminated side accumulated more auxin, which stimulates cell expansion leading to higher growth rates compared to the illuminated side and bending of the organ towards the light source (Ding et al. 2011).

Not only PINs are involved in the phototropic response but also AUXIN-RESISTANT1 (AUX1), a facilitator of the auxin flux into the cell that has been shown to regulate phototropic curvature of the hypocotyl (Stone et al. 2008). Seedlings of *aux1* loss-of-function mutants showed a decreased phototropic response of the hypocotyl upon unidirectional light. Stone et al. (2008) also showed that the expression of *AUX1* was altered as a result of the unidirectional light treatment. AUX1 is known to play an important role in the auxin flux towards the root, but unfortunately the cellular localisation of AUX1 during phototropism is not known, nor is it known exactly how AUX1 influences polar auxin transport during the phototropic response of the hypocotyl.

A direct molecular link between phototropin and polar auxin transport has been made by Christie et al. (2011), who showed that PHOT1 can phosphorylate ATP-BINDING CASSETTE B19 (ABCB19/MDR1/PGP19). Members of the ABCB/MDR/PGP family are also involved in auxin efflux and mediate the polar auxin flow from the shoot apex to the roots. When dark-grown seedlings become exposed to unilateral blue light, PHOT1 is activated and on its turn inactivates ABCB19, which results in auxin accumulation around the shoot apex followed by lateral redistribution and subsequent transport to the elongation zone by PIN3. These studies show that plants are able to translate directional light into a change in polar auxin transport resulting in differential expansion of specific cells. As a result of these photoreceptor-auxin transporter interactions, seedlings will direct growth towards the light source.

Not only the direction of the light is an important source of information about a plant's competitive environment in dense stands but also the composition of the light harbours information. Plants are able to respond to these changes in light quality, which are sensed by the photoreceptors, and induce the so-called shade avoidance response, in which auxin (transport) plays an important role as well.

3 Shade Avoidance

Plants can respond to crowding with phenotypically plastic responses, such as the shade avoidance responses. Shade avoidance responses include various phenotypic traits, including elongation of petioles and hypocotyls, apical dominance, early

Fig. 1 Shade avoidance responses in *Arabidopsis thaliana*. The plant on the *left* represents a control plant and the plant on the *right* represents a plant which received a low R:FR treatment (R:FR = 0.25). Note that low R:FR-exposed plant displays elevated leaf angles and elongated petioles

flowering and upward leaf movement (hyponasty) (Franklin 2008; Keuskamp et al. 2010a) (Fig. 1). These growth and developmental responses help plants to outgrow shade imposed by neighbours. The ability to perceive neighbouring plants and appropriately respond to their presence subsequently determines the chances to survive competition for light in dense stands.

Most plants display shade avoidance responses upon detection of specific light cues that indicate crowding. In a canopy there is not only a reduction in the light availability but also changes in light composition. Plant leaves strongly absorb red (R: λ = 600–700 nm) and blue (B: λ = 400–500 nm) light for photosynthesis whilst transmitting and reflecting most of the far-red (FR) light (λ = 700–800 nm) (Smith 2000). The resulting relative enrichment of FR light, leading a reduced R:FR ratio, is therefore an accurate and early indicator of plant-specific neighbour proximity even in stages of vegetation development where leaf overlap and actual shading have not occurred yet (Ballaré et al. 1990). Reduced photon fluence rates of B light are, on the other hand, a reliable indicator of light intensity and thus actual shading (Ballaré et al. 1991). *A. thaliana* has the capacity to perceive both these light signals, which can subsequently induce pronounced shade avoidance responses (Franklin 2008; Keuskamp et al. 2010a). Over the past decade, major progress has been achieved in understanding how auxin controls shade avoidance responses in *A. thaliana*.

As stated above, phytochromes are sensitive to both R and FR light and this feature is exploited by plants to process the R:FR ratio as a measure for neighbour proximity. Several studies have shown that genes involved in auxin biosynthesis, transport and signal transduction are differentially regulated upon low R:FR light conditions (e.g., Devlin et al. 2003). Also marker genes for auxin activity showed increased expression levels as a result of the low R:FR light treatment (Pierik et al. 2009; Keuskamp et al. 2010b). Other studies showed that indeed enhanced auxin biosynthesis, transport and intact signalling are essential to low R:FR-induced elongation (e.g., Tao et al. 2008; Pierik et al. 2009; Keuskamp et al. 2010b; Kozuka et al. 2010). These studies show that different aspects of auxin play a crucial role in the shade avoidance response.

Low R:FR enhances auxin biosynthesis in a TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1)-dependent manner (Tao et al. 2008), which

facilitates auxin biosynthesis through a dedicated tryptophan-dependent pathway (Tao et al. 2008; Stepanova et al. 2008). Mutants that are null for the function of TAA1 showed no shade avoidance response to low R:FR conditions (Tao et al. 2008; Moreno et al. 2009; Keuskamp et al. 2010b). These results show that TAA1-mediated up-regulation of auxin biosynthesis is an important step in the low R:FR-induced shade avoidance response of *A. thaliana*. YUCCA is a family of proteins which function downstream of TAA1 in the tryptophan-dependent biosynthesis pathway of auxin (Stepanova et al. 2011; Won et al. 2011). YUCCA involvement in the low R:FR-induced shade avoidance response is comparable to TAA1, since YUCCA mutants show the same inhibition of the low R:FR-induced hypocotyl elongation and also lack the low R:FR-induced up-regulation of auxin levels (Won et al. 2011). However, unlike *TAA1*, several *YUCCA* genes are up-regulated in seedlings as a result of the low R:FR treatment (Li et al. 2012).

An important phytochrome-mediated regulatory pathway is through interaction with PHYTOCHROME INTERACTING FACTOR (PIF) proteins. PIFs are part of the BASIC HELIX-LOOP-HELIX (bHLH) family of transcription factors that bind to DNA to regulate gene transcription as part of the phytochrome signal transduction (Duek and Fankhauser 2005). Several studies have shown that PIFs are involved in the hypocotyl elongation response to low R:FR treatment (Hornitschek et al. 2012; Leivar et al. 2012). Hornitschek et al. (2012) show that PIF4 and PIF5 are involved in the expression of auxin-regulated genes and hypothesise that PIFs could directly induce the expression of *YUCCA* genes, since, for example, PIF4 and PIF5 are able to bind to YUCCA promoters. The link between PIFs and auxin biosynthesis was also made by Franklin et al. (2011) who showed that PIF4 regulates expression of *TAA1* at high temperature. Recently it was shown that PIF7 accumulates in its dephosphorylated form upon low R:FR light conditions and increases the expression of auxin biosynthetic *YUCCA* genes (Li et al. 2012). Li et al. (2012) also show that these higher auxin levels initiated by the PIF7-regulated pathway are required for the low R:FR-induced hypocotyl elongation as part of the low R:FR-induced shade avoidance response of *A. thaliana* seedlings. However, elevated auxin biosynthesis is not sufficient to induce shade avoidance since auxin has to be transported to the target tissues for growth stimulation.

Several studies have shown that the inhibition of polar auxin transport was sufficient to inhibit the elongation response of *A. thaliana* upon low R:FR light conditions (Steindler et al. 1999; Kanyuka et al. 2003; Pierik et al. 2009; Keuskamp et al. 2010b; Kozuka et al. 2010; Liu et al. 2011) and the expression of the auxin efflux carriers *PIN3* and *PIN7* are up-regulated under this light condition (Devlin et al. 2003; Keuskamp et al. 2010b). Well before these studies hinting at PIN-mediated control, Morelli and Ruberti (2000) had already hypothesised that polar auxin transport might be redirected in response to canopy shading, either by redistribution of specific auxin efflux carriers or by activation of regulatory proteins that control the activity of those efflux carriers, or both. Keuskamp et al. (2010b) demonstrated that phytochrome inactivation upon low R:FR exposure induces a change in the cellular location of PIN3 (Fig. 2). Under control light conditions, PIN3-GFP is located on the basal side of the endodermal cells of the hypocotyl.

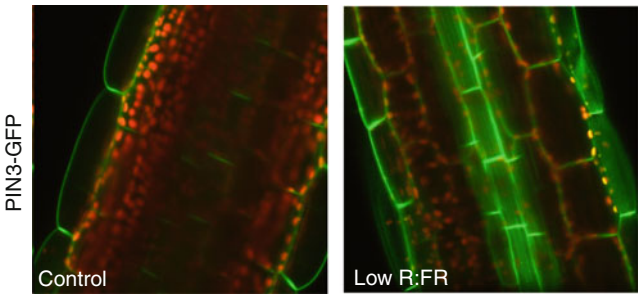

Fig. 2 Low R:FR treatment of seedlings induces a cellular PIN3-GFP protein orientation in the hypocotyl that is more lateral than the basal orientation in control plants

Low R:FR treatment results not only in an increased expression of PIN3 but also in a preferentially high PIN3-GFP abundance on the lateral side of the endodermal cells of the hypocotyl. This change in cellular localisation leads to increased auxin levels in the elongating hypocotyls of the low R:FR-treated *A. thaliana* seedlings. Seedlings of the *pin3-3* mutant lacked this elongation response of the hypocotyl to low R:FR and did not accumulate auxin in the hypocotyl in response to low R:FR (Keuskamp et al. 2010b).

Polar auxin transport is also required for the petiole elongation response to low R:FR. Genetic and chemical inhibition of polar auxin transport reduced low R:FR-induced petiole elongation (Pierik et al. 2009; Keuskamp et al. 2010b; Kozuka et al. 2010). In addition to unravelling the mechanism controlling shade avoidance, also the adaptive value of auxin control of shade avoidance was tested for *A. thaliana* (Keuskamp et al. 2010b). The polar auxin transport mutant *pin3-3*, which grows similar to wild type when grown alone, was used in competition experiments. This was done by first comparing wild type and the *pin3-3* mutant grown in monoculture dense stands (>2,000 Arabidopsis plants m^{-2}). These experiments showed that both the petiole elongation and hyponastic response were induced in response to proximate neighbours in wild-type and *pin3-3* plants, but this was delayed in *pin3-3* as compared to wild type. Next, mixed stands of Col-0 and *pin3-3* (1:1) were grown in a checkerboard design (Fig. 3a). Whereas shoot dry weight and reproductive output were similar between *pin3-3* and Col-0 in monoculture stands, these were dramatically suppressed in pin3 grown in mixture with Col-0 wild-type neighbours (Fig. 3b). The polar auxin transport mutant *pin3-3* is thus clearly outcompeted by wild-type neighbours, which shows that the polar auxin transport-mediated control of shade avoidance has great fitness implications.

As mentioned above, plants can also show shade avoidance responses to blue light depletion. Several studies have shown that the cryptochrome photoreceptors control this shade avoidance response (Pierik et al. 2009; Keller et al. 2011; Keuskamp et al. 2011). Keuskamp et al. (2011) showed that the reduced blue light levels induced hypocotyl elongation as a result of combined action of auxin and brassinosteroids. In the rosette phase, the inhibition of polar auxin transport by 1-*N*-naphthylphthalamic acid (NPA) led to an inhibited elongation response of the

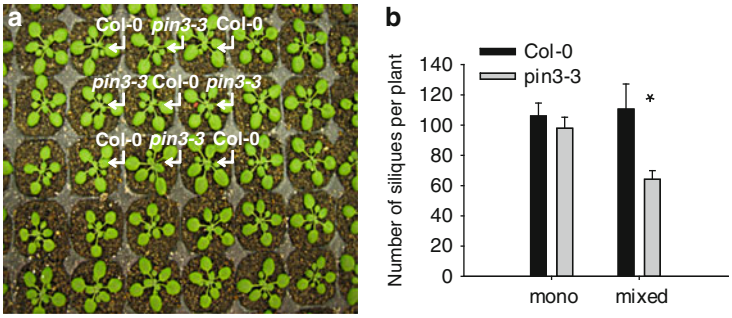

Fig. 3 Competition experiments between wild-type (Col-0) and mutant (*pin3-3*) Arabidopsis plants at a density of >2,000 plants m^{-2}. The mixed competition experiments were performed in a checkerboard design of Col-0 and *pin3-3* (**a**). Competitive ability is reduced in *pin3-3* when competing against Col-0 as indicated by a reduced number of siliques (measured 67 days after potting) relative to Col-0 (**b**). Data are means ($n = 5$) ± SE; *asterisk* indicates a significant difference ($P < 0.05$)

petiole upon blue light attenuation (Keller et al. 2011). Interestingly, inhibiting polar auxin transport with NPA did not affect low blue-induced hyponasty, indicating that the low blue-induced petiole elongation and hyponastic response are regulated through (partly) separate mechanisms. These data show that although the elongation responses are very much alike, a more complex network of hormone interaction appears to be required for the blue light-regulated shade avoidance response, when compared to the low R:FR-induced shade avoidance response.

4 Conclusions and Future Perspectives

This study shows that auxin (transport) plays a crucial role in the light responses of *A. thaliana*. We show that during phototropism and the shade avoidance response, there is a change in polar auxin transport and auxin accumulates in the elongating organs (Fig. 4a, b). Although phototropism is initiated by phototropin and the shade avoidance response is regulated by phytochromes or cryptochromes, PIN3 and its cellular location are major players in both light responses. The cellular location of PINs determines the direction of the auxin flux and can thereby establish differences in auxin accumulation which leads to elongation. Directional light leads to lowered PIN3 abundance on the lateral part of endodermal cells on the illuminated side of the hypocotyl in comparison to the dark side. This leads to differential auxin accumulation within the hypocotyl and thus differential growth towards the light source. Upon low R:FR conditions, PIN3 abundance on the lateral side of endodermal hypocotyl cells is increased, compared to normal light conditions. This leads to higher auxin levels throughout the hypocotyl and thus elongation as part of the shade avoidance response.

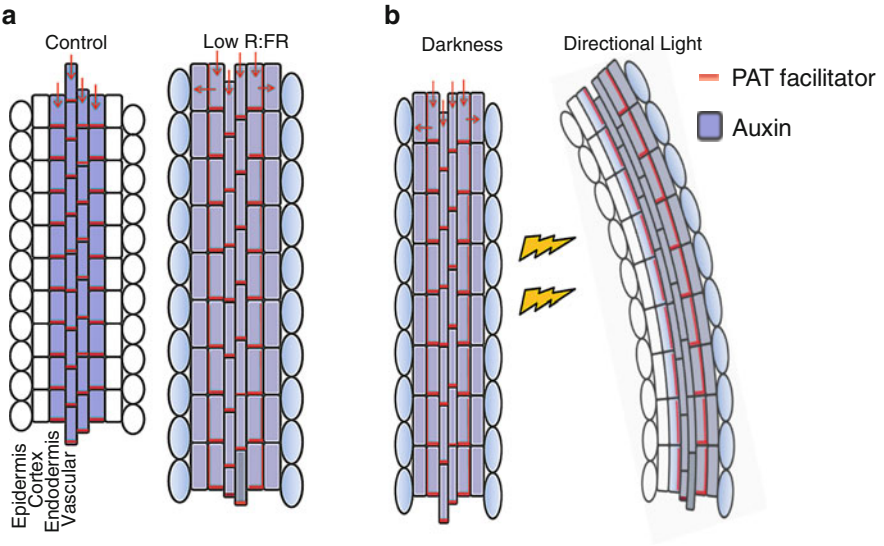

Fig. 4 Polar auxin transport is controlled by light signals. Upon low R:FR treatment, cellular relocation of PIN3 induces changes in auxin accumulation and thereby hypocotyl elongation (**a**). Directional light also changes the cellular location of (polar) auxin transport facilitators, which leads to differential auxin accumulation in the hypocotyl. This results in differential growth of the hypocotyl towards the light source (**b**)

During the green revolution (in the late 1960s), an increased agricultural production was achieved around the world. It involved the development of high-yielding varieties of crops, synthetic fertilisers and pesticides. During the green revolution, farmers started to use shorter (agriculturally advantageous) varieties, which led to higher yields. The investment that plants normally put in their stem to gain height was now used for their seeds. This reduction in plant height in many cases turned out to be the result of gibberellin-associated mutations that made them dwarfed (Peng et al. 1999). Interestingly, some dwarfed varieties of agronomic species were a result of polar auxin transport-related mutations (Multani et al. 2003). When plants are somehow prevented to respond to their neighbours thereby preventing the arms race in the struggle for light, this will lead to increased yields when these plants grow in a monoculture as many agricultural fields are. Obviously, under natural, ecologically meaningful conditions, such mutations would not thrive since well-responding genotypes would quickly outcompete weak shade avoiders (e.g., Keuskamp et al. 2010b). Thus, from an agronomic view point, shade avoidance responses should consider carbon and energy investments that go at the expense of yield, which makes research on the physiological regulation of the shade avoidance response exceptionally relevant.

Competition for light is certainly not the only battle that plants need to fight in dense stands. A plant will encounter biotic stresses created by pathogens and/or herbivores, especially under the favourable microclimate of dense stands. Several

studies have shown there is a trade-off between shade avoidance and the resistance against attackers, and it appears that shade avoidance is generally prioritised over defence (Izaguirre et al. 2006; Griebel and Zeier 2008; Moreno et al. 2009). Moreno et al. (2009) studied the involvement of auxin in this trade-off between the shade avoidance response and herbivore defence. They used the *sav3-2* mutant which is null for TAA1, thus lacking increased auxin biosynthesis upon low R:FR and lacking shade avoidance. Interestingly, the mutant still had lower defence levels against herbivory during the low R:FR treatment, compared to normal light conditions. These data show that the trade-off does not occur through auxin biosynthesis nor through resource allocation between defence and the shade avoidance response.

The search for new mutants or breeds with higher yields should be a search for genotypes with reduced shade avoidance responses but with normal or enhanced defence levels. Research is needed to know where in the phytochrome signal transduction pathway there is crosstalk with the defence levels, so plants can deal with both stresses in an agriculturally optimal way.

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Auxin and Temperature Stress: Molecular and Cellular Perspectives

Kyohei Shibasaki and Abidur Rahman

Abstract Temperature stress is one of the major abiotic stresses that limit plant growth and development and crop productivity worldwide. Plant growth and development is also influenced by endogenous factors such as hormones, and under environmentally stressed conditions. Plants adapt themselves through multiple processes, including a change in hormonal response. Recent evidence indicates that under optimal condition, the plant hormone auxin plays a key role in determining plant development processes through modulating other hormonal responses. However, little is known about the role of auxin under temperature stress. The emerging picture from recent experiments indicates that like under optimal condition, auxin also plays a crucial role in regulating plant growth under temperature stress. In this chapter, we tried to integrate our current understanding on the role of auxin in regulating plant developmental processes under temperature-stressed condition and the future direction of research that may help us in engineering plants/crops for sustainable agriculture.

1 Introduction

As a sessile organism, plant encounters various environmental changes during its life cycle. One of the major environmental changes that affect plant development is temperature. Shift in temperature either to the high or low end drastically affects plant growth and crop productivity. For instance, in 2009, a chilling temperature alone resulted in crop damages equivalent to 158 billion yen in Japan. Similarly,

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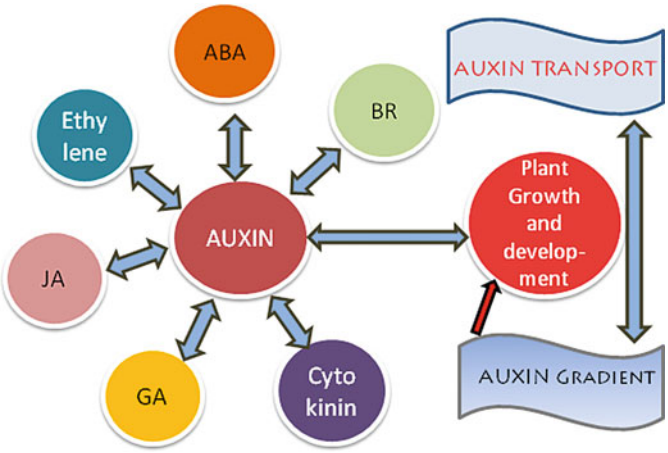

Fig. 1 Auxin at center of hormonal cross talk in regulating the plant growth and developmental processes under optimal condition. Reproduced from Rahman (2012) with permission from Physiologia Plantarum

early and late frost results in damaging vegetable and fruit production equivalent to 5–6 billion yen every year (Rahman 2012). High-temperature limitation of crop yield is also a well-recognized problem in India, Africa, and many other countries. High-temperature stress is a frequent occurring event during rice and wheat growing seasons. It has been shown that heading and flowering stages are most sensitive to the high-temperature stress (Matsui and Omasa 2002). In case of wheat, maize and barley, the combined annual loss rendered by high temperature is $5 billion (Lobell and Field 2007). These data strongly support the notion that temperature stress is one of the major abiotic stresses that limit the plant growth and crop productivity worldwide.

Plant growth is also influenced by endogenous factors such as hormones. In fact, every aspect of plant growth from germination to senescence is under hormonal regulation. In regulating plant developmental processes, hormones act in concert, resulting in a complex web of interactions. Interestingly, over the past decades with the aid of Arabidopsis genetics, it became evident that among all plant hormones, auxin plays a central role in determining plant developmental processes through modulating other hormonal responses (Fig. 1). Further, it has been established that an auxin gradient, which is regulated by auxin transport, plays a major role in regulating hormonal cross talks and plant development (Fig. 1; Rahman 2012). Under environmentally stressed conditions, plants adapt themselves to the adverse condition through multiple processes, including changes in hormonal responses. To date, a great deal of information is available about transcriptional regulators that play key roles in plant temperature stress responses (Hua 2009; Rahman 2012). However, the roles of hormones in regulating temperature stress responses are far from understood. Among the plant hormones, although auxin plays a central role in regulating plant developmental processes and responses to other hormones at an

optimal condition, our understanding of the role of auxin in temperature stress responses is limited. However, some recent work shed a light on this aspect and the emerging picture suggests that like under optimal condition, auxin also plays an important role in regulating plant development under temperature stress conditions. This chapter integrates auxin, temperature stress, and plant growth and development.

2 Auxin

2.1 Auxin Synthesis

Auxin biosynthesis is a complex process involving multiple pathways acting in concert. The biosynthesis of the major form of auxin, indole-3-acetic acid (IAA) largely relies on tryptophan (Trp)-dependent pathway (Zhao 2010). Biochemical and physiological experiments indicated the existence of a Trp-independent pathway that may contribute to in vivo IAA biosynthesis. However, no genetic basis has been established for this pathway (Cohen et al. 2003; Strader and Bartel 2008; Zhao 2010). Four major pathways contribute in Trp-dependent IAA biosynthesis: (1) indole-3-acetaldoxime (IAOx) pathway, (2) indole-3-acetamide (IAM) pathway, (3) indole-3-pyruvic acid (IPA) pathway and (4) YUCCA (YUC) pathway (Zhao 2010; Mashiguchi et al. 2011). IAOx pathway was elucidated on the basis of three auxin overproduction mutants, *superroot 1* (*sur1*), *superroot 2* (*sur2*), and *CYP79B2* overexpression lines (Hull et al. 2000; Zhao et al. 2002). However, this pathway is probably not the major IAA biosynthesis pathway as CYP79B2 and its homologue CYP79B3 is absent in monocots (Sugawara et al. 2009). The IAM pathway possibly widely exists in plants. IAM is found in Arabidopsis and an *AMIDASE1* (AMI1) gene has been cloned in Arabidopsis, which can convert IAM to IAA (Pollmann et al. 2003). However, the physiological significance of this pathway is still elusive. The significance of IPA pathway has been recently demonstrated through genetic studies. Three independent studies identified an Arabidopsis aminotransferase, TAA1 (tryptophan aminotransferase of Arabidopsis 1) that can convert Trp to IAA in vivo (Stepanova et al. 2008; Tao et al. 2008; Yamada et al. 2009). The YUC pathway has been proposed as the major IAA biosynthesis pathway as YUC genes are identified ubiquitously across the plant kingdom (Zhao 2010). Further, the genetic evidence also indicates that *YUC* genes play a central role in IAA-regulated developmental processes (Zhao et al. 2001, Cheng et al. 2006; 2007; Yamamoto et al. 2007). *YUC* gene family encodes flavin monooxygenases and showed to catalyze the conversion of tryptamine (TAM) to N-hydroxy-tryptamine (HTAM) in vitro (Zhao et al. 2001; Kim et al. 2007). Based on this IAOx and indole-3-acetonitrile (IAN) were previously reported as possible intermediates of YUC pathway (Zhao et al. 2001). In a metabolite study, Sugawara et al. (2009) showed

that IAOx and IAN are not common intermediates of IAA biosynthesis in plants, raising a question about the validity of the in vitro study. The same group recently convincingly showed that TAA and YUC families function in the same pathway and YUC catalyzes the conversion of IPA to IAA, a rate-limiting step for IAA-regulated plant developmental processes (Mashiguchi et al. 2011).

Traditionally, it is believed that shoot is the sole source of auxin biosynthesis and the auxin supply to other parts of a plant relies on the polar transport of auxin. Recent evidence clearly suggests that root and shoot both can synthesize auxin (Cheng et al. 2006; Stepanova et al. 2008; Pagnussat et al. 2009). This indicates that each organ is possibly self-sufficient in terms of synthesizing auxin, but the question remains open how they maintain the optimal auxin gradient required for plant development and how polar transport of auxin contributes in maintaining the gradient.

2.2 Auxin Transport

Among the plant hormones, auxin is unique as it moves from the site of synthesis through an active transport system (Goldsmith 1977). Auxin is transported both in rootward and shootward directions (Baskin et al. 2010). In the aerial part, auxin moves unidirectionally towards the root and in root, it moves in both directions (Muday and Rahman 2008). The components that regulate the transport pathway have been identified by using Arabidopsis genetics. There are two major protein families that regulate auxin transport, AUX/LAX family, which functions as auxin influx carriers (Bennett et al. 1996), and PINs, which function as auxin efflux carriers (Feraru and Friml 2008). These proteins show tissue-specific expression and regulate transport of auxin in specific tissues (Swarup et al. 2004; Feraru and Friml 2008). In general, influx and efflux transporters regulate intercellular auxin transport and maintain the local auxin gradient which is extremely important in regulating plant developmental processes (Muday and Rahman 2008). Besides these protein families, ABC transporter family has also been shown to mediate auxin transport (Peer et al. 2011). The functional specificity of the PINs has been substantiated by genetic evidence in Arabidopsis. For instance, PIN1 functions in rootward auxin transport and primarily expressed in the vascular tissue (Geldner et al. 2001; Blilou et al. 2005). PIN2 is expressed in outer cell layers with opposite polarity in lateral root cap cells, epidermis, and cortex and regulates the root gravity response (Muday and Rahman 2008; Rahman et al. 2010). The direction of PIN-mediated auxin transport depends polar targeting of the PIN proteins to the right plasma membrane domain of the cells (Friml et al. 2004; Michniewicz et al. 2007). PIN3 is localized symmetrically but relocates asymmetrically to the direction of gravity and partially involved in regulating the gravity response both in root and shoot (Friml et al. 2002b; Harrison and Masson 2008; Rakusova et al. 2011). PIN4 appears to work in the establishment of an auxin sink below the quiescent center of the root meristem (Friml et al. 2002a), and PIN7 is expressed at lateral and basal

membranes of provascular cells in the meristem and elongation zone and plays a role in forming and maintaining the rootward auxin transport (Blilou et al. 2005). Other members of the PIN family (PIN 5, 6, 8) are expressed in the endoplasmic reticulum (Mravec et al. 2009; Friml and Jones 2010).

One of the major factors that regulate the functionality of the auxin efflux carriers is intracellular trafficking of these proteins. PIN proteins continuously cycle between the plasma membrane and endosomes (Geldner et al. 2001). Efficient targeting to the plasma membrane and turnover of PIN proteins determine the proper functionality of these proteins to transport auxin and form an auxin gradient (Geldner et al. 2001; Vieten et al. 2007; Shibasaki et al. 2009; Rahman et al. 2010), which regulates many developmental fates of plants (Friml 2003).

2.3 Auxin Gradient

Multiple experimental approaches (physiological, molecular, and cellular) revealed that intracellular auxin distribution, regulated by auxin homeostasis, results in formation of local auxin gradient that functions as a regulatory factor for plant developmental processes (Sabatini et al. 1999; Bhalerao and Bennett 2003; Leyser 2006; Prusinkiewicz and Rolland-Lagan 2006; Tanaka et al. 2006; Benjamins and Scheres 2008; Ikeda et al. 2009). The patterning or formation of plant organs starts with the accumulation of auxin followed by its redistribution to form a cellular or tissue-specific auxin gradient, which directs major developmental decisions, such as specification of the apical and basal poles and establishment of root and cotyledon (Friml 2003). Auxin gradient also regulates organogenesis of leaves, flowers, and lateral roots, as well as tropisms (Benkova et al. 2003; Muday and Rahman 2008). Auxin gradient formation largely relies on the intracellular targeting of PIN proteins, which is a highly dynamic process with continuous cycling of the PINs between the cell membrane and intracellular compartments (Geldner et al. 2001). Recent evidence suggests that clathrin-dependent endocytosis (Dhonukshe et al. 2007) and ARF-GEF-dependent exocytosis (Geldner et al. 2001) regulate the constitutive cycling of PINs. Additionally, the phosphorylation status, which is regulated by the counterbalancing activities of PINOID kinase and protein phosphatase 2A, also regulates PIN polarity and hence the flow of auxin (Michniewicz et al. 2007; Sukumar et al. 2009; Rahman et al. 2010). Taken together, these results suggest that under an optimal condition, the intracellular trafficking of PIN proteins contributes in maintaining the cellular auxin homeostasis that functions as a prime regulator of plant developmental processes.

3 Temperature Stress

3.1 Cold Temperature

The cold stress can be divided into two major categories, freezing and chilling. Compared with the chilling stress, freezing stress is severe and inhibits the expression of plant's full genetic potentials (Thomashow 1999). However, some of the plant species counter this severe stress through a process called cold acclimation. Cold acclimation is defined as a process by which plants acquire freezing tolerance through prior exposure to low nonfreezing temperatures. Winter-habit plants, such as barley, oat, and rye, require a low-temperature period (vernalization) to acquire the reproductive (flowering) phase (Kim et al. 2009). In Arabidopsis, vernalization induces histone modifications via the plant-homeodomain-PRC2 complex to generate high H3K27me3 levels (Angel et al. 2011). Interestingly, after vernalization, cold acclimation ability gradually decreases (Fowlerl et al. 1996). This phenomenon indicates that the effect of cold stress is reversible (Fowlerl et al. 1996). Many chilling-sensitive crop plants are incapable of cold acclimation. The chilling stress inevitably inhibits plant growth and development (Fukaki et al. 1996; Shibasaki et al. 2009). However, the stress is reversible as plants have the ability to regrow when they are returned to an optimal temperature (Fukaki et al. 1996; Wyatt et al. 2002, Shibasaki et al. 2009).

The cellular and molecular mechanisms that are integrated to cold stress responses are relatively well defined. At cellular level, although debatable, the plasma membrane has been suggested to be the primary site of cold perception as membrane composition changes both qualitatively and quantitatively in response to cold. Cold stress decreases the membrane fluidity due to changes in the fatty acid unsaturation and lipid-protein composition in the cell membrane (Wang et al. 2006). Rigidification of membrane either by mutation or by exogenous application of membrane rigidifier results in expression of cold-inducible genes even at room temperature (Inaba et al. 2003; Orvar et al. 2000; Sangwan et al. 2002). In addition, several membrane-localized proteins such as calcium-permeable channels, histidine kinases, and receptor kinases have been suggested to be putative sensors for cold response (Solanke and Sharma 2008). Calcium has been shown to affect the membrane composition through modulating phospholipid signaling (Vergnolle et al. 2005). Hence, it is thought that membrane rigidification may play an important role in cold perception as well as the cellular response to cold. However, in a recent experiment, it has been shown that membrane rigidification did not alter proper protein trafficking within plant cells and cold stress did not change intracellular localization and trafficking properties of the cold-inducible protein, LTI6b (Shibasaki et al. 2009).

At the molecular level, several cold stress-induced transcription factors have been identified and defined as transcriptional regulators of cold stress-induced genes. Currently, a molecular model predicts that after cold perception, plant uses a phosphorylation cascade counterbalanced by protein phosphatases and MAP

kinases and regulated by cytosolic calcium to transduce the signal to downstream signaling components (Hannah et al. 2005; Rahman 2012). Transcription factors such as ICE1 (inducer of CBF expression 1), MYB15, and zinc finger proteins have been suggested to be the primary regulators of cold-responsive gene expression (Xiong et al. 2002; Zhu et al. 2007). Recent evidence suggests that phosphorylation and SIZ1-mediated SUMO conjugation and deconjugation of ICE1 are the key processes to regulate ICE1 binding to its target genes. Once ICE1 is activated, it binds to MYC *cis*-elements in the *CBF* (C-repeat binding factor) promoter to induce the expression of target genes (Chinnusamy et al. 2003). The induction of the CBF genes at low temperature and the enhanced freezing tolerance of the transgenics overexpressing the CBFs suggest that this pathway plays a central role in regulating cold-stress response (Vogel et al. 2005).

Although hormones play a major role in regulating plant development at the optimal condition, the role of hormones in regulating cold stress is still elusive. The only hormone that has a potential link to abiotic stresses, including cold stress, is abscisic acid (ABA). However, the direct role of ABA in cold stress remains a mystery as it has been shown that the low-temperature-regulated gene expressions occur relatively independently of ABA (Thomashow 1999; Xiong et al. 2002; Shinozaki and Shinozaki 2006).

3.2 High Temperature

In contrast to low temperature, which severely inhibits plant growth and development, high temperature affects plant developmental processes differently. It stimulates the hypocotyl elongation, promotes flowering and inhibits pollen production (Gray et al. 1998; Balasubramanian et al. 2006; Sakata et al. 2010; Kumar et al. 2012). Like cold stress, at cellular level, high-temperature stress also affects cellular functions and membrane-linked processes due to alterations in membrane fluidity and permeability (Sangwan et al. 2002). Enzyme function is also sensitive to high temperature. High-temperature-induced alterations in enzyme activities can lead to shifts in metabolic pathways and can cause enzyme inactivation due to protein denaturation (Vierling 1991; Kampinga et al. 1995). The damaged membrane and nonfunctional proteins facilitate the production of reactive oxygen species (ROS) (Dat et al. 1998a, b; Gong et al. 1998; Larkindale and Knight 2002), which ultimately lead to programmed cell death (PCD) (Vacca et al. 2004). As a defense to high-temperature stress, plants produce heat shock proteins (HSPs) to obtain thermotolerance (Vierling 1991). These proteins are induced in plants during high-temperature acclimation and proposed to act as molecular chaperons to protect cellular proteins against irreversible high-temperature-induced denaturation and to accelerate refolding of damaged proteins (Boston et al. 1996; Hong and Vierling 2000, 2001). Heat-stress transcription factors, which bind to the promoter of HSPs, regulate the expression of these chaperons (Larkindale and

Vierling 2008). Recent evidence suggests that HSP 1011, Hsa32, HSFA2, HSP110, HSFA7a, and HSFA3 play important roles in thermotolerance (Hua 2009).

Unlike cold stress, the hormonal involvement in regulating high-temperature-mediated developmental processes is better understood. Several hormones including auxins, gibberellins, and brassinosteroids have been implicated in regulating plant growth under high-temperature stress (Gray et al. 1998; Stavang et al. 2009).

4 Auxin and Temperature Stress

4.1 Low Temperature and Auxin

Despite the fact that auxin regulates almost all aspects of plant development, little is known about the response of auxin under cold stress conditions. The first demonstration of involvement of auxin in cold stress responses came from the study of Morris (1979), who showed that cold stress inhibits auxin transport in a variety of species. Fukaki et al. (1996) showed that cold treatment inhibits the inflorescence gravity response in Arabidopsis. They further revealed that the cold stress effect is reversible as the gravistimulated inflorescence in cold bends to the gravity vector when it is returned to room temperature. This demonstrates the existence of a gravity-persistent signal and suggests that cold stress affects steps after the gravity perception (Fukaki et al. 1996). Consistently, rootward auxin transport was found to be abolished at 4 °C but was restored to the wild-type level when the plants were returned to the room temperature (Wyatt et al. 2002; Nadella et al. 2006). Wyatt et al. (2002) took a genetic approach to identify components that separate the perception events from the response and screened for mutants in which the gravity-persistent signal was aberrant. Although these *gps* mutants respond abnormally to the gravity stimulus, amyloplast sedimentation is apparently normal, suggesting that the aberrant response is caused by an event (or events) that links gravity perception to auxin transport. Further studies of *gps* mutants revealed that these mutants fail to establish the proper auxin gradient in the inflorescence after gravistimulation and also show altered polar and lateral auxin transport (Nadella et al. 2006). Taken together, these results clearly demonstrate that cold stress affects the auxin response *in planta*. However, what remains obscure is the molecular mechanism that regulates the response of auxin under cold stress. In a recent study, using Arabidopsis root as a model, we tried to answer the question (Shibasaki et al. 2009). The physiological studies revealed that cold stress inhibits both root growth and gravity response in a reversible manner as the plants start to regrow and respond to gravity when they are returned to room temperature, albeit with a lag period. Genetic studies confirmed that the primary target of cold stress is auxin transport as the auxin signaling mutants (*axr1* and *tir1*) respond to cold stress like wild type. Direct transport assay with radiolabeled IAA further confirmed the notion. Under cold, the shootward auxin transport is drastically reduced, which also alters the

intracellular auxin gradient, as visualized by the auxin-responsive marker (Shibasaki et al. 2009). These results suggest that cold stress alters the intracellular auxin homeostasis, which possibly leads to altered growth and development. To further understand the cellular mechanism behind cold stress-induced changes in auxin homeostasis, polar targeting and trafficking of the auxin efflux carriers PIN2 and PIN3 were analyzed. As described earlier, both polar deployment and efficient targeting of the PIN proteins are essential for their functionality. Cold stress does not alter polar localization of PIN2 proteins but inhibits the intracellular cycling of PIN2, indicating that the reduced intracellular cycling affects PIN's functionality, resulting in reduced shootward auxin transport and altered intracellular auxin homeostasis. The selectivity of cold stress on PIN trafficking was substantiated by several markers, representing different pathways such as GFP-ARA7 (Ueda et al. 2004) for endosomal trafficking; NAG-GFP (Essl et al. 1999) for Golgi trafficking; and a GFP-LTI6b (Kurup et al. 2005), for trafficking induced by low temperature and FM4–64, a general endocytic tracker (Bolte et al. 2004). All the results indicated that cold stress inhibits a selective trafficking process and targets the early endosomal cycling pathway, including PIN trafficking (Shibasaki et al. 2009). Collectively, these results provide a mechanistic explanation of cold stress-induced changes in auxin response and shed light on the importance of intracellular trafficking in regulating cold-stress response. This is not surprising as intracellular trafficking pathways have been emerging as central regulators of plasma membrane protein homeostasis, controlling multiple signaling pathways, mediating interactions between multiple hormones, and controlling growth and development in both animals and plants (Grant and Donaldson 2009; Reyes et al. 2011).

4.2 Auxin and High Temperature

In contrast to cold temperature, the role of auxin is better understood in high-temperature stress responses. High temperature affects the growth of the plant in a tissue-specific manner. For example, it promotes the hypocotyl growth (Gray et al. 1998) and flowering (Kumar et al. 2012) but inhibits pollen formation (Sakata et al. 2010). Auxin has been found to be a common factor in regulating these distinct developmental processes under high temperature. Interestingly, current literature indicates that high-temperature-mediated alteration in developmental processes is tightly linked to auxin biosynthesis (Franklin et al. 2011; Sun et al. 2012). Several molecular studies indicated that phytochrome-interacting factor 4 (PIF4) is the primary regulator of the signaling mechanism that integrates auxin and plant development under high temperature (Koini et al. 2009; Franklin et al. 2011; Kumar et al. 2012). High-temperature-induced elongation of Arabidopsis hypocotyl is under regulation of PIF4-mediated auxin biosynthesis (Franklin et al. 2011; Sun et al. 2012). PIF4 directly activates the auxin biosynthetic gene YUCCA 8 (*YUC8*) by binding to the G-box-containing promoter region of *YUC8* and stimulates the auxin biosynthesis under high temperature (Sun et al. 2012). *yuc8* mutation can largely

suppress the long-hypocotyl phenotype of PIF4-overexpression plants and also can reduce the high-temperature-induced hypocotyl elongation, confirming that under high temperature PIF4 regulates auxin biosynthesis through activating the IPA auxin biosynthesis pathway (Franklin et al. 2011; Sun et al. 2012). PIF4 has also been shown to be instrumental in activating the *Flowering Locus T* (*FT*), which promotes the flowering at high temperature in short photoperiods (Kumar et al. 2012). Interestingly, the opposite effect of high temperature on auxin biosynthesis has been observed in developing anthers of barley and Arabidopsis (Sakata et al. 2010). In contrast to hypocotyls, high temperature inhibits the expression of auxin biosynthesis genes in developing anthers, which possibly results in a decrease in endogenous auxin level and results in male sterility. The notion that high-temperature-induced male sterility is due to reduction in tissue-specific auxin was supported by the fact that exogenous application of auxin completely reversed the male sterility (Sakata et al. 2010). These results are consistent with the previous finding that in high-temperature-tolerant rice variety Shanyou63, much slower rate of decrease of pollen activity, pollen germination, and floret fertility was observed compared with the high-temperature-susceptible variety Teyou559 (Tang et al. 2008). Not surprisingly, Shanyou63 showed an elevated level of endogenous auxin compared with Teyou559 (Tang et al. 2008). Taken together, these results support an elegant model where auxin plays a central role in regulating the high-temperature-induced alteration in plant developmental processes and also confirms that high-temperature-induced injury occurs in a tissue-specific manner.

5 Concluding Remarks

The emerging trend from the recent research clearly indicates that like optimal condition, auxin plays an important role in regulating plant developmental processes under temperature stress conditions. Both auxin synthesis and transport are found to be potential targets of temperature stresses. Our current understanding on the effect of temperature on cellular auxin response and plant developmental processes is summarized in Fig. 2. Although these findings bring new insights on how the plant hormone auxin is integrated in regulating plant developmental processes under temperature stresses, there are still many unanswered questions. Current data set represents involvement of two distinct processes that alter auxin response under temperature stress. Low temperature primarily targets auxin transport through modulating a subset of intracellular trafficking pathway that includes the trafficking of auxin efflux carriers, resulting in alteration of cellular auxin homeostasis as well as auxin gradient. On the other hand, high temperature primarily targets auxin biosynthesis through PIF4 transcriptional factor and affects the plant developmental processes. What still remains to be elucidated are whether auxin biosynthesis is affected under low temperature, what is the effect of cold

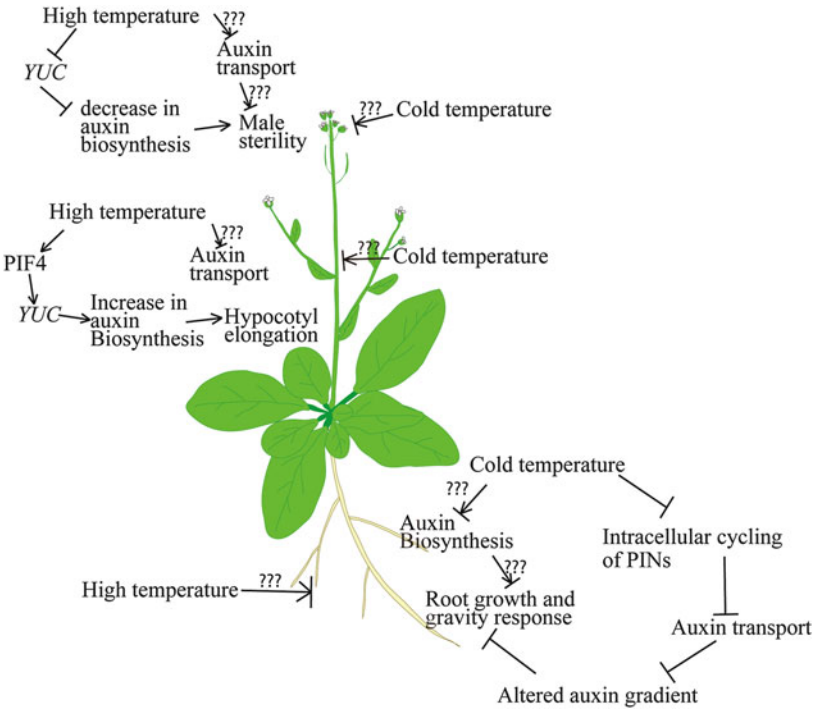

Fig. 2 Schematic summary of our current understanding on the effect of temperature on cellular auxin response and plant developmental processes. *Question marks* represent yet unidentified pathways that may influence plant auxin response under temperature stress

stress on transcriptional regulators of auxin response, and which transcriptional regulator plays a primary role in regulating the auxin responses under cold? Similarly, under high-temperature stress auxin biosynthesis is stimulated or reduced resulting in a change in cellular auxin homeostasis. However, the mechanism by which plants respond to this change is still unclear. Does high temperature also affect auxin transport process? Like cold, does protein trafficking play any roles in high-temperature-induced alteration of auxin homeostasis? Which protein trafficking pathway plays a central role in regulating the temperature stress? How do the changes of auxin response affect the other hormonal responses under temperature stress? Which components link the hormonal response to downstream signaling factors? A concerted effort is required to answer all these questions. Addressing these issues in future research will facilitate our understanding of temperature stress pathways and help engineer crops tolerant to temperature stresses.

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A Fertile Field: The Mutual Influence and Parallel Histories of Auxin and Flavonoids

Elinor Thompson

Abstract Auxin is notable for its influence on almost every aspect of plant growth and development. Its effects are so familiar but they continue to pose questions, features that are mirrored by the flavonoids. Although these secondary metabolites' biosynthesis is one of the best studied of all pathways, their functions are so broad as to be perplexing. The ubiquity of flavonoids in fruit and flowers and the appearance of anthocyanins in the plant stress response often make these pigments' presence obvious. Their effects, however, extend beyond these visible roles to communication with other organisms and to auxin transport inhibition. Decades of study have shown that they are capable of altering auxin flow in the root, and inflorescence phenotypes of flavonoid mutants suggest a perturbation of development that could also be related to altered auxin distribution in aerial tissues. A compound that is able to regulate such an important hormone as auxin might be expected to have an enormous impact in the plant, and now quite a body of evidence has accumulated to support this hypothesis—but there remain, nevertheless, questions over its relevance in "real-life" plant growth.

1 Introduction

Direct regulatory links between auxin and flavonoids is a neat conclusion to their many shared characteristics. These are simple molecules which have an impressively wide spectrum of action, impacting on numerous aspects of plant (and microbial) growth and development. Pleasingly, they also share long histories in the chemical and biological sciences.

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Fig. 1 Anthocyanin pigments in *Cotinus*

Flavonoids are a diverse group of secondary metabolites, ubiquitous in the plant kingdom and including the characteristic red, blue and purple anthocyanin pigments of plant tissues. Besides pigmentation of plant leaves (Fig. 1) and floral organs, they are involved in numerous pathways and processes. Flavonoids function in protection from ultraviolet (UV) light, biotic interactions, defence, root nodulation, pollen viability and function, the regulation of reactive oxygen levels and root formation from callus in tissue culture (Djordjevic et al. 1997; Imin et al. 2007; Li et al. 1993; Napoli et al. 1999; Taylor and Grotewold 2005; Treutter 2006; Wasson et al. 2006). We are interested in them for nutritional and therapeutic aspects and, as discussed here, they also affect plant architecture (Buer and Djordjevic 2009).

Flavonoids and their derived pigments have also been the colourful facilitators of much of modern genetics—they have helped us to elucidate transposon biology, control of structural and regulatory gene expression; gene silencing, gene duplication and the evolution of novel compounds and functions and how rates of evolution vary between genes (for review, see Grotewold 2006). The founding studies of the flavonoid pathway are the basis of the classical genetic analysis of flower colour inheritance patterns proposed by Mendel in 1865: in fact, flavonoid biosynthesis is one of the longest-studied examples of coordinated gene and enzyme regulation in response to environmental factors.

Around the same time as Mendel's work, the concept also arose of a mobile phytohormone in plants: Charles Darwin was among those at the end of the nineteenth century to examine the growth of plants in response to light. This first described plant hormone, then, was auxin (indole acetic acid, IAA). Roles attributed to auxin have expanded so that the wide-ranging list now includes not just plant phototropism but geotropism, embryogenesis and vascular differentiation, via the hormone's regulation of cell expansion, division and differentiation (for review, see Abel and Theologis 2010). In fact, auxin is now somewhat notorious for its influence on almost every aspect of plant growth and development.

Although we have at our disposal so many chemistry, genetics and biology tools, and we know so much about the fundamental biosynthesis, storage, regulation, evolution and biological activities of flavonoids, many features of these pigments and of auxin remain unknown. Their very ubiquity, and such numerous roles and interactions, makes a complicated task of dissecting their effects, not least on one another.

2 Evolutionary Origins

The plant growth regulator auxin is distinct from flavonoids in being produced not only by seed plants but by other organisms as disparate as bacteria, algae and even fungi, such as Rhizopus species and Saccharomyces cerevisiae (Rao et al. 2010). The microbially synthesised auxin is important because it modulates plant growth in pathogenic and symbiotic plant–microbe interactions alike (Glick et al. 1999). In contrast, less evidence has been found for microbial flavonoids. They have been declared absent even in algae, despite the likely origins of land plants among the macroscopic algae, the Charales (Lewis and McCourt 2004; Markham 1988). Flavonoids have been identified in red algae such as *Cyanidioschyzon* and *Acanthophora,* however, and recently sequenced genes of the green alga *Chlamydomonas reinhardtii* are similar to those encoding plant flavonoid biosynthetic enzymes, such as dihydroflavonol 4-reductase (see plants.Ensembl.org). Thus the hypothesis that evolution of the flavonoid pathway took place after the colonisation of land, with bryophytes (mosses), liverworts and hornworts the first land plants to produce flavonoids, may be incorrect (Rausher 2006). The first chemicals of this class have been hypothesised to be the chalcones, flavonols and flavones (Markham 1988), since these are derived from the first three enzymes of the flavonoid pathway (chalcone synthase—CHS, chalcone flavanone isomerise—CHI and flavanone 3-hydroxylase/flavonol synthase; Fig. 3). CHS and CHI interestingly appear to have been derived from genes encoding enzymes of the primary metabolism, being similar to bacterial proteins involved in fatty acid synthesis (Ngaki et al. 2012; Verwoert et al. 1992).

3 Auxin Synthesis and Function

As detailed fully elsewhere in this volume, biosynthesis of higher plants' native auxin (IAA; Fig. 4) has profound effects on plant growth and development. As well as playing essential roles in developmental processes such as gametogenesis, embryogenesis, seedling growth, vascular patterning and flower development (for review, see Zhao 2010), local auxin biosynthesis may function in more short-term regulatory responses, such as in stress responses by helping to control stomatal opening (Dietrich et al. 2001) and by allocating resources under poor growth conditions

(Palme and Gälweiler 1999). As we will see later with flavonoids, dissection of auxin biosynthesis pathways using the fantastic resource of Arabidopsis mutants is complicated by aspects of the plant's metabolism, here the metabolism of tryptophan. In this species, indole glucosinolates and other defence compounds are synthesised via a metabolite of tryptophan and the indole-3-acetaldoxime pathway which, it has been noted, can be diverted to synthesise auxin (Normanly 2010).

Plant morphogenesis and development are dependent upon the dynamic poising of metabolism, physiology and gene activity. Auxin does not act alone: it functions in concert with other plant hormones. For example, auxin and cytokinin have long been known to act synergistically or antagonistically to regulate growth and development, such as in embryogenesis or the formation and maintenance of the meristem (Su et al. 2011). To regulate and integrate many different signals, auxin itself is controlled at many different levels—all aspects of auxin's presence in plants are regulated, from its biosynthesis to degradation to transport. Auxin transport occurs in a strictly controlled direction from the shoot apex towards the root tips via a specialised transport system (Benjamins et al. 2005).This allows gradients of the hormone to form and influence such an enormous range of developmental and environmental responses. The key to all auxin-mediated processes is the movement of the hormone around the plant, and this is an aspect that can be influenced by flavonoids.

As well as regulating auxin's presence and distribution, it is also possible to modify the capacity of plant cells to respond to the hormone by differentially expressing signalling pathway components. Regulating many developmental processes, one of the important auxin receptors in Arabidopsis is the transport inhibitor response 1 (TIR1) protein, along with the Aux/IAA proteins and auxin response factor (ARF) which are transcriptional repressors or activators that regulate auxin signalling (Lau et al. 2008).The large numbers of different Aux/IAA and ARF proteins allow different combinations to be expressed in specific cell types, so the output of different subsets is numerous developmental and physiological responses to auxin (Friml and Jones 2010).

Modulators of auxin activity, then, have been proposed to include the flavonoids. The possibility of phenolic modulation of polar auxin transport was proposed by Kenneth Thirmann in the 1960s (Thirmann 1965), supported by flavonoids' similarity to 1-*N*-naphthylphthalamic acid (NPA). Despite only slight structural similarity between flavonoids and auxin, flavonoids do resemble this synthetic auxin transport inhibitor (Fig. 3). Thus flavonoids and NPA can, for example, bind to the auxin protein facilitator and other regulatory proteins controlling auxin transport around the plant (Santos-Buelga et al. 2010).

The effects of NPA have been used in the study of flavonoid function for nearly half a century, and the regulation of auxin transport by flavonoids is consequently supported by a substantial body of data. This is not to say that there is no remaining scepticism over this role in 'real life', in plant growth outside the laboratory.

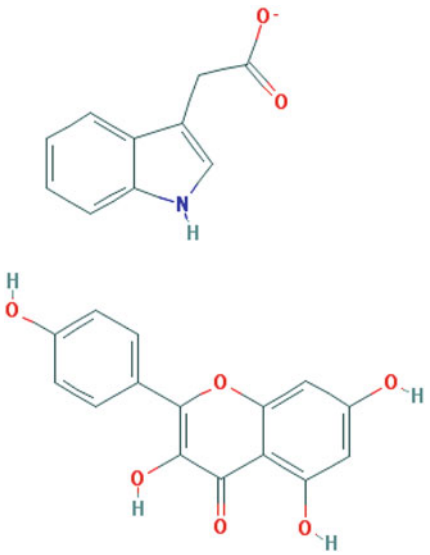

Fig. 2 Auxin (indole acetic acid) and the C6–C3–C6 flavonoid backbone of flavonol (here kaempferol; PubChem Compound)

4 Flavonoids: Synthesis and Distribution

Flavonoids are phenolic secondary metabolites. Among the molecules sharing the same chemical backbone (Figs. 2 and 4) are three major classes, the pink and purple anthocyanins (Fig. 1), the pale yellow flavonols and the proanthocyanidins of seed coats (Fig. 3). They are bioactive across kingdoms, and over 9,000 structural variants are known (Williams and Grayer 2004) including lignin, a specialised structural compound, molecules that can prevent photodamage (Li et al. 1993) and the subset of flavonols in the plant that are able to regulate plant growth and development (Peer et al. 2004).

Synthesis occurs via the phenylpropanoid pathway, from conversion of phenylalanine to cinnamate and formation of *p*-hydroxycinnamate from cinnamate and 4-coumarate. The condensation of a molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA then yields the molecule at the base of the flavonoid biosynthetic pathways, naringenin chalcone (Figs. 3 and 4). Hydroxylation of naringenin (4′,5,7-trihydroxyflavanone) forms the dihydroflavonols, which, along with the subsequent product, flavonols, are often extensively glycosylated—the key to the specification of role and localisation of the metabolites (Jones et al. 2003; Kerhoas et al. 2006; Ringli et al. 2008; Winkel-Shirley 2001). Reduction of dihydroflavonols forms flavan-3,4-diols which lead to the colourful anthocyanins and proanthocyanidins of vegetative tissues, fruits and flowers (see Siegler 1995, for more detail).

Most of the aglycone flavonols become glycosylated in the C-3 and C-7 positions, and the principal sugar substitutions are glucose and rhamnose (Kerhoas et al. 2006).The flavonoid pathway has been used to demonstrate how a biochemically complex trait can evolve in stages (Fig. 3),with each addition conferring the

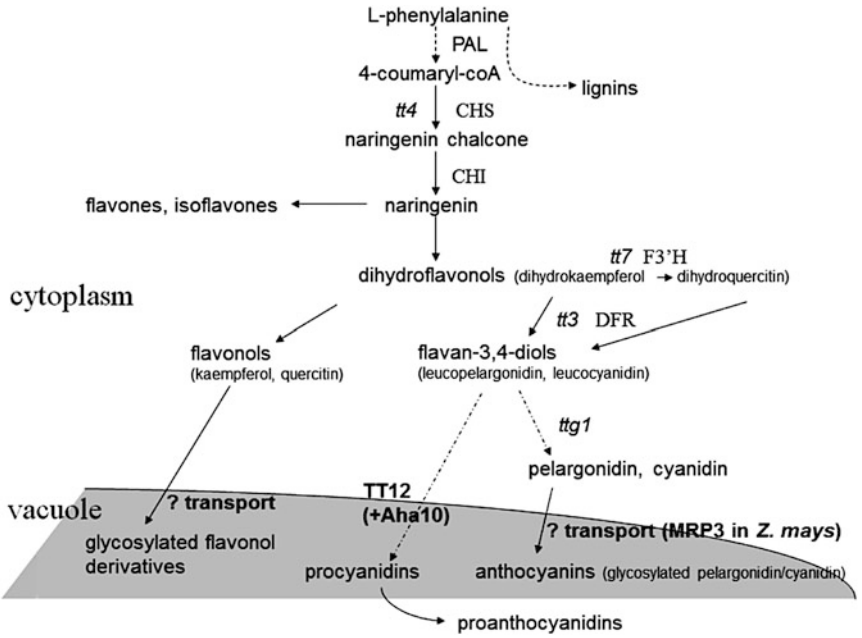

Fig. 3 Summary of flavonoid biosynthesis pathways [reproduced from Thompson et al. (2009)]

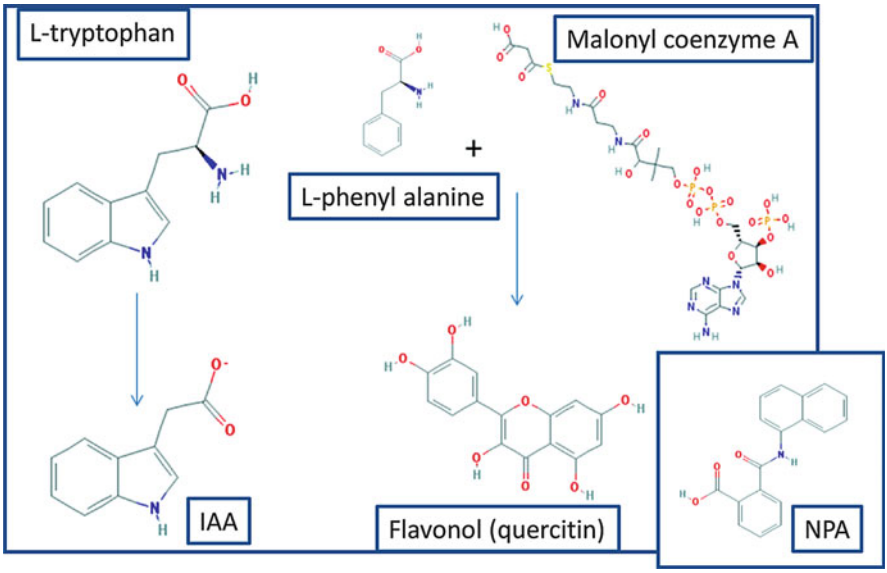

Fig. 4 Summary of auxin (IAA) and flavonoid synthesis from amino acid precursors tryptophan or phenylalanine, respectively, showing limited structural similarity between the flavonol quercetin and the auxin transport inhibitor 1-*N*-naphthylphthalamic acid [adapted from Winkel-Shirley (2002)]

ability to adapt to different conditions (Rausher 2006).The existence of kaempferol-3-(2-*O*-α-l-rhamnopyranosyl)-β-*d*-glucopyranoside-7-*O*-α-l-rhamnopyranoside in Arabidopsis indicates the existence, for example, of a pathway beyond the biosynthesis of the flavonol aglycone kaempferol with at least three glycosyltransferase genes for the modification of the aglycone (Saito and Matsuda 2010).

The literature states that single-copy genes encode most of the enzymatic machinery for flavonoid synthesis (Fig. 3), although the situation has turned out to be slightly more complex than this with different isoforms of several enzymes in specialised tissues (see Thompson et al. 2005; Dobritsa et al. 2010). Substitution of precursors is also possible since CHS, for example, can accept caffeoyl-CoA-derived metabolites instead of coumaroyl-CoA (Seigler 1995). Mutation of the biosynthetic pathway enzymes nevertheless frequently results in dramatic, plant-wide changes to flavonoid synthesis, from which the well-known 'transparent testa' (tt) phenotypes in seeds are derived. *tt* seeds are deficient or altered in distribution of the flavonoid-derived brown proanthocyanidin pigments (Buer et al. 2010; Lepiniec et al. 2006) and *tt* lines are limited to structural enzymes. Altered expression of transcription factors (TF) that regulate the flavonoid biosynthetic pathway often also results in a *tt* phenotype, such as *tt1* (zinc finger TF), *ttg1* (*transparent testa glabra1*, a WD40 repeat TF), *tt2* (a R2R3 Myb TF), *tt8* (a basic helix–loop–helix TF) and *tt16* (MADS domain TF; Nesi et al. 2001; Zhao 2010).

Flavonoids can be a major component of plants, for example, forming even around a fifth of the dry mass of *Camellia sinensis* leaves (Punyasiria et al. 2004). Particular compounds may be specialist components of particular tissues, and an interesting feature of flavonoid regulation (and pertinent to auxin regulation) is that pathway TFs often exist as multigene families, with each member of the family having a restricted domain of expression (Chopra et al. 2006). Thus, this is another regulatory mechanism that is seen with both flavonoids and auxin. The multitude of Aux/IAA and ARF transcriptional regulators of auxin transport direct gene activity in specific cell types: knockouts of the TF family controlling flavonoids can eliminate pigment production in specific tissues such as flowers (or even in parts of flowers), without affecting anthocyanin or general flavonoid production in other tissues. Cis-regulatory sequences also allow parallel differential regulation in different tissues (Rausher 2006).

Another example of specialisation via localisation is that several of the pathway's biosynthetic enzymes have been identified at the apical end of cortex cells in the root elongation zone of Arabidopsis (Saslowsky and Winkel-Shirley 2001). Such differential distribution can control flavonoids' effect on auxin transport and of course has a visible effect in the case of anthocyanins, which do not show their brilliant colours until they are accumulated in the acidic vacuoles. Just as transport is the key to auxin function, transport is essential to the synthesis and function of proanthocyanidins, the polymers of the flavan-3-ols (viz. the precursor monomers catechin and/or epicatechin). In the seed coat, the precursors once again accumulate in the vacuole, followed by polymerisation and oxidation to proanthocyanidin within this organelle (Debeaujon et al. 2000, 2001; Kitamura et al. 2004; Lepiniec et al. 2006; Xie et al. 2003). These oxidised compounds enable hardening of the seed coat, induction of moderate dormancy in the seeds and the ability to limit detrimental effects of physical and biological attacks (Debeaujon et al. 2000).

In contrast with the progress made in the last decade on auxin transport and despite the well-studied flavonoid biosynthetic pathways, how plants achieve this distribution to different subcellular compartments remains largely unknown. The identification of flavonoid transporters is an emerging area and current data suggest that different plant species may use different mechanisms or multiple mechanisms. Whereas, for example, the maize *bronze2* locus encodes a glutathione transporter which is a key player in vacuolar uptake of flavonoids, the 'MATE' multidrug and toxin efflux transporters (of the multidrug resistance transporter family) have been proposed to operate in Arabidopsis and tomato (Debeaujon et al. 2001; Mathews et al. 2003; Thompson et al. 2009, 2010). In Arabidopsis, the TT12 gene encodes a MATE protein and which appears to control the vacuolar sequestration of flavonoids in the seed coat endothelium (Debeaujon et al. 2001); FFT is another MATE transporter which alters floral flavonoid composition (Thompson et al. 2009, 2010). Altered pH or altered vacuolar morphology also appears to affect subcellular flavonoid accumulation, as Arabidopsis AHA10 mutants, which harbour a defect in a plasma membrane H^+ ATPase, exhibit both altered vacuole formation and flavonoid content (Baxter et al. 2005).

The distribution of flavonoids and the flavonoid biosynthetic machinery in different plant tissues is an interesting aspect of the control of these metabolites and their roles in different locations. Flavonoids have been shown to accumulate in the cotyledonary node, the hypocotyl–root transition zone, and the root tip of young Arabidopsis seedlings (Peer et al. 2001). The principal flavonoids in Arabidopsis leaves have been reported to be the paler-coloured kaempferol and quercetin glycosides (Pelletier et al. 1999), with kaempferol often reported to be the basis of most vegetative flavonol glycosides, with kaempferol-3-*O*-rhamnoside-7-*O*-rhamnoside as a major component (Routaboul et al. 2006). Studies of Arabidopsis using liquid chromatography–mass spectrometry and/or nuclear magnetic resonance reported anthocyanins along with glycosylated kaempferol flavonols in leaves (Tohge et al. 2005; Yonekura-Sakakibara et al. 2008), whereas seeds contained the glycosylated quercetins, epicatechin and proanthocyanidins (Kerhoas et al. 2006; Routaboul et al. 2006). Interestingly, these studies differentiated between monoglycosylated flavonols and proanthocyanidins that were accumulated principally in the seed coat and diglycosylated flavonols which were found in the seed embryo (Routaboul et al. 2006). Proanthocyanidins are the most common flavonoids along with anthocyanins in many fruits, such as apples and grapes (Mane et al. 2007; Zhang et al. 2003), and seeds and grain crops (Lepiniec et al. 2006; Auger et al. 2010). Arabidopsis remains a useful model plant at this level since its seed contains large amounts of proanthocyanidins similar to those found in related crop seeds or fruits (Routaboul et al. 2006, 2012).

Flowers have again been reported to contain predominantly kaempferol compounds, but quercetin derivatives are known to accumulate in stamens (Burbulis et al. 1996; Routaboul et al. 2006; Shirley et al. 1995). Among the quercetin glycosides identified in inflorescence tissue are quercetin–glucoside–rhamnoside, quercetin–glucoside–rhamnoside–rhamnoside and quercetin–rhamnoside–rhamnoside (Jones et al. 2003).

4.1 Function and Visualisation of Flavonoids

Flavonoids have considerable biological activity in plant, animal and bacterial systems—an unusually broad spectrum and spread which is seen with few other compounds. Cellular targets of these secondary metabolites are not fully documented, although inventive use of the numerous mutants available in model plant species such as *Arabidopsis thaliana* and *Medicago truncatula* (such as the *tt* lines mentioned above) are enabling the intricacies of these compounds' effect on plant physiology to be deduced. Mutation is rarely lethal, since these low molecular weight secondary metabolites, unlike primary metabolites, are not essential for plant survival (Buer et al. 2010).

The fact that flavonoids affect such a wide range of processes, from flower pigmentation to photoprotection to molecular signalling, illustrates that they act in both plant development and physiology. Even simple flavonoids such as chalcones, aurones and flavanones are able to absorb strongly UV light. Indeed, both high light and reactive oxygen species upregulate the entire anthocyanin biosynthesis pathways (Vanderauwera et al. 2005), and the accumulation of anthocyanin pigments in vegetative tissues is a marker for plant stress. Hypothetical mechanisms for this generic stress response include the pigments' inherent antioxidant activity along with their ability to absorb harmful radiation. Like other phenylpropanoids (and indeed auxin), they are synthesised by the shikimate pathway, which has been suggested to act as a sink for reduced carbon. Since flavonoids accumulate in stress conditions when photosynthetic capacity concurrently reduces, this pathway has thus been suggested to act as a route for excess energy dissipation, consuming trioses phosphate, ATP and NADPH (Hernández and Van Breusegem 2011). Of course, their ability to control auxin transport could likewise contribute to stress protection via regulation of plant growth and tropism.

One example well-represented in the literature is the role of flavonoids in fertility, a role necessary beyond flower pigmentation and resultant pollination success. Flavonols, particularly kaempferol and quercetin, are essential for pollen germination and tube growth in petunia (*Petunia hybrida*) and maize (*Zea mays*; Mo et al. 1992; Vogt et al. 1995). In the absence of CHS, both petunia and maize anthers contain white pollen that is devoid of all flavonols and which cannot germinate or produce a functional pollen tube in self-pollination (Napoli et al. 1999). Since flavonoid-deficient Arabidopsis plants are able to germinate, grow and set fertile seed, a dogma has developed that Arabidopsis is a special case in not requiring CHS for fertility (Burbulis et al. 1996). It does seem, however, that Arabidopsis does require correct transport of flavonoids for full fertility and normal pollen development (e.g. see Thompson et al. 2009, 2010), and CHS-deficient mutants may be able to reproduce successfully because of redundant or specialised isoforms in the floral tissues (Dobritsa et al. 2010).

Among their many activities in plants and interlinked with anther function, the regulation by flavonoids of auxin must be among the most important. After this proposal by Thirmann (1965), it has even been argued (Stafford 1991) that the earliest function of flavonoids could have been this more subtle role of regulation of

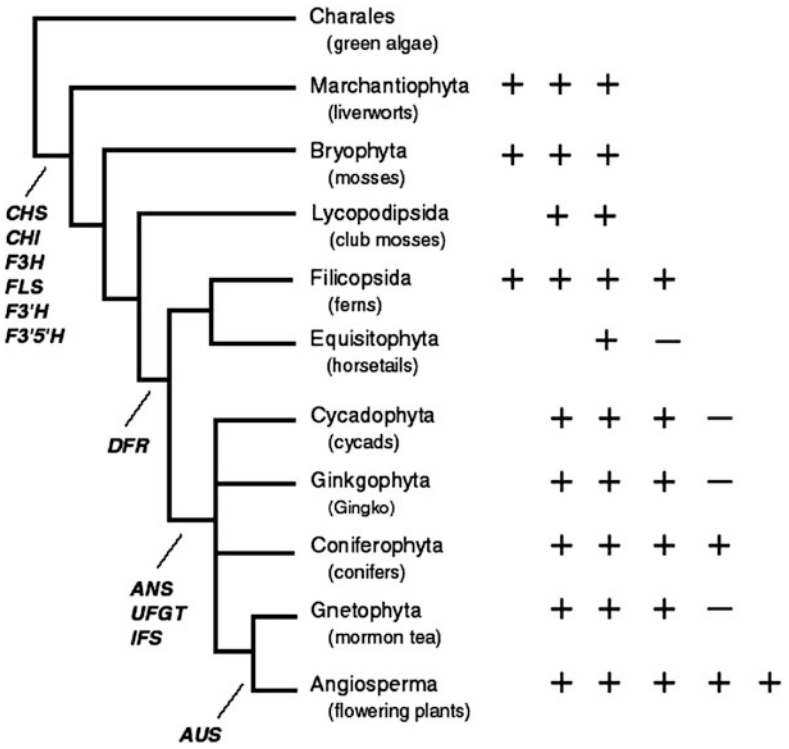

Fig. 5 Evolution of flavonoid biosynthetic genes in the green plants [reproduced from Rausher (2006), *Science of Flavonoids* p180; Springer]

plant hormones. This was postulated because, although flavonoid knockout mutants can be very sensitive to damage from UV light (Li et al. 1993; Lois and Buchanan 1994), the large amounts of flavonoids that would be needed to afford UV protection presumably would not have accumulated during the early evolution of these secondary metabolites (Rausher 2006). In terms of biological effect, data show that flavonols are the most important flavonoids in this regard and their regulatory role is again supported by their existence early in the evolution of the pathway: flavonols are the most ancient and widespread of the flavonoids, synthesised even in mosses and ferns (Fig. 5), and they seem particularly capable of potent physiological activities (Stafford 1991).

4.2 How Are Flavonoids Observed in Plant Tissues?

Although mass spectrometry methods can be used to accurately determine the major flavonoid metabolites present in plant tissues, these pigments are interesting to work with because they can be directly visualised. In situ fluorescence

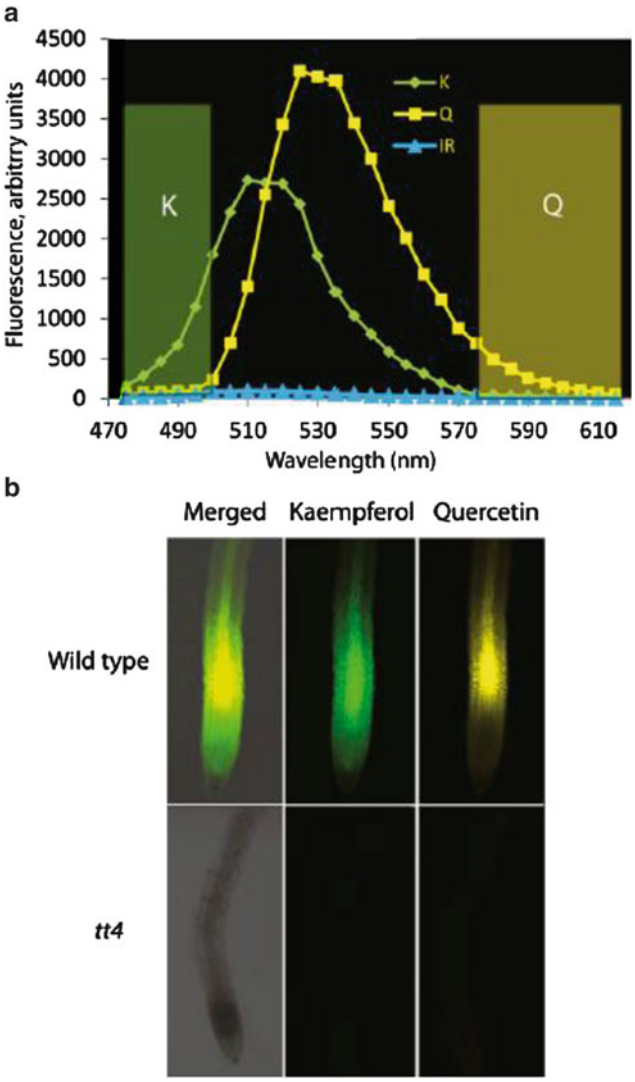

Fig. 6 Use of DPBA in visualisation of flavonoids in wild type and tt4 chalcone synthase mutant Arabidopsis roots [reproduced from Lewis et al. (2011)]

localisation is often used to determine the in vivo levels of different flavonoids in specific tissues since their UV absorption can be enhanced using the compound diphenylboric acid 2-amino ethyl-ester (DPBA). This fluoresces with distinct spectral properties when in complex with kaempferol and quercetin (Peer et al. 2001), quercetin–DPBA having a longer wavelength fluorescence emission than the kaempferol–DPBA complex (Fig. 6). In Fig. 6, therefore, untreated wild-type roots exhibit kaempferol–DPBA staining throughout the meristematic and elongation

zones, whereas quercetin–DPBA is more concentrated in the elongation zone. The *tt4* CHS mutant lacks this fluorescence since flavonoids are apparently absent from the roots (Fig. 6). It should be noted that, in Arabidopsis seedlings, it is not possible to see the most bioactive component, however: DPBA fluorescence results largely from binding to glycosylated flavonols, as aglycone flavonols are of low abundance or nondetectable in these tissues, according to HPLC analyses (Burbulis et al. 1996).

4.3 Flavonoids and Auxin Regulatory Functions

Studies by Jacobs and Rubery (1988) and others using auxin efflux inhibitors such as NPA followed the initial proposals that there could be phenolic modulation of polar auxin transport. Their data provided evidence that flavonoids, particularly apigenin (4′,5,7-trihydroxyflavone) and the flavonol quercetin, could compete with NPA to perturb auxin transport. Additional reports supported the contention, but this topic remained controversial.

While the debate over this has progressed, there has been enormous progress in understanding how the auxin gradients necessary for its function are established. It was hypothesised several decades ago that the polarity of auxin flow results from differences at the single-cell level between apical and basal membranes in their relative permeabilities to the hormone (Rubery and Sheldrake 1974; Raven 1975). Graded concentrations of auxin are today known to be essential for embryonic, root and shoot organogenesis, and these gradients are established and maintained by active, specific transport mechanisms. How this auxin transport occurs has been elucidated much faster than have transport systems for flavonoids. The polarity of auxin transport is controlled by the localisation of auxin transport proteins, where efflux and influx carriers are asymmetrically distributed (reviewed in Kramer and Bennett 2006). This asymmetry then explains how changes in auxin flux can occur and allow tropic responses and patterning.

Apical and basal plasma membrane localisation of AUXIN-RESISTANT1 (AUX1) and PIN-FORMED1 (PIN1) auxin transport components underpin this directionality of intercellular auxin flow in *Arabidopsis thaliana* roots. Auxin influx carriers such as AUX1, belonging to the amino acid permease family of proton-driven transporters, are situated at the apical (upper) side of cells, in contrast with basally (lower side) localised PIN1 auxin transporters in the same cells (Kleine-Vehn et al. 2006). Since the description of the Arabidopsis "pin-formed" mutant in 1991 and the subsequent cloning of AtPIN1, a further seven members of the family have been discovered (Paponov et al. 2005). As would be expected from the ubiquity of auxin, PIN sequences appear to be ubiquitous in vascular plants: genes homologous to the Arabidopsis *PIN* are in fact present in genomes throughout the plant kingdom, including nonvascular plants such as the moss *Physcomitrella* (Friml and Jones 2010).

Changes in auxin concentration alter PIN distribution, and AtPIN1 located in a polar manner in plasma membranes has been shown to be capable of rapid relocalisation after pharmacological disruption (Friml and Palme 2002; Paponov et al. 2005). AtPIN1 can also apparently be replaced by AtPIN4, possibly to compensate for this loss, since incubation with the auxin transport inhibitor NPA results in AtPIN4 becoming localised to the domain of AtPIN1 in the stele of the developing root (Friml and Palme 2002; Paponov et al. 2005).

4.4 Flavonoid Regulation of Auxin Gradients: Growth

As mentioned above, flavonoids are like auxins in showing specialised cellular and tissue distribution (Djordjevic et al. 1997; Mathesius et al. 1998; Peer et al. 2001). Localised accumulation of specific flavonoids in the distal root elongation zone and the root/shoot junction of Arabidopsis (Buer and Djordjevic 2009; Buer et al. 2010; Peer et al. 2001) would allow inhibition of auxin transport in specific tissues. In support of this, the *tt* mutants and other related knockouts, containing altered flavonoid profiles, show elevated auxin transport and altered growth phenotypes. The increased auxin transport and accumulation in the upper root in *tt4* (and other *tt* mutants that make no or few flavonols) than in wild type is consistent with the loss of a negative regulator, and the result is longer primary roots, more secondary roots and a loss of apical dominance in inflorescences (Brown et al. 2001; Buer and Muday 2004; Peer et al. 2003, Winkel-Shirley 2002). Several *tt4* alleles, lacking wild-type flavonoid synthesis, also show a delay in root gravitropism, which can be reversed by chemical complementation with the flavanone naringenin (Buer and Muday 2004; Buer et al. 2006). Presumably flavonoids could promote gravitropism by regulating auxin movement in the root tip and thus modulate differential growth (Buer and Muday 2004). Accordingly, quercetin treatment of the *pin2* mutant—which therefore is defective in the efflux mediating basipetal auxin transport (Chen et al. 1998)—restores the gravitropic response and the formation of gradients of auxin-induced gene expression in gravity-stimulated roots (Santelia et al. 2008). This effect on root growth seems to extend beyond severe lesions in flavonoid biosynthesis to transporters: without correct localisation of the regulatory portion of these metabolites, the *fft* flavonoid-transporter (Fig. 7) mutant seedlings grew and matured more quickly than the wild type, with significantly faster root growth in young seedlings (Thompson et al. 2009).

Vegetative tissues are not exempt from the effects of flavonoids. The *rol1-2* mutant (for repressor of Arabidopsis root hair mutant *lrx1* mutants; Ringli et al. 2008) is defective in rhamnose biosynthesis and thus has an altered flavonoid glycosylation profile and an increase in auxin consistent with loss of flavonol regulation. The *rol1-2* allele demonstrates hyponastic growth (upward movement of the leaf blades), aberrant pavement cell and stomatal morphology in cotyledons and defective trichome formation, but only the hyponastic phenotype was suppressed by blocking flavonoid biosynthesis altogether. Thus only the hyponastic

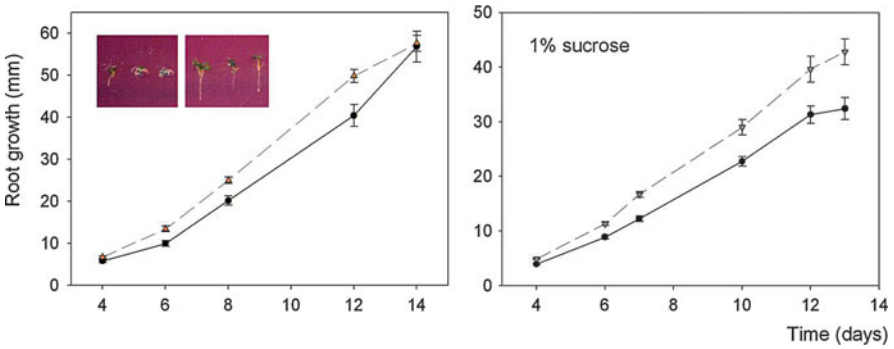

Fig. 7 *fft* flavonoid-transporter mutant root growth phenotype (Thompson et al. 2009)

cotyledons were thought to result from the flavonol-induced increase in auxin concentration (Ringli et al. 2008). In leaf tissues and auxin transport streams beyond the root, therefore, it seems that kaempferol and its derivatives may also have distinct functions.

4.5 *Which Proteins and Flavonoids Are Responsible for Regulation, and in Which Direction?*

Lewis et al. (2011) asked whether kaempferol and/or quercetin are the negative regulators of basipetal or of acropetal auxin transport. Mutant lines *tt4* (CHS) and *tt7* (F3′H; see Fig. 3) exhibited increases in basipetal auxin transport relative to the wild type. Since *tt4* makes neither quercetin nor kaempferol and *tt7* makes kaempferol alone, these data suggested that quercetin, but not kaempferol, is the inhibitor of root basipetal auxin transport (Lewis et al. 2011). Another study, however, did not detect elevated auxin transport from the shoot apex into the root in a *tt7* mutant line, but did detect elevated transport in *tt4*, suggesting that the rootward auxin transport stream requires only kaempferol for its regulation (Peer et al. 2004). Unfortunately, different alleles, tissues and growth conditions in published studies prevent firm identification of the regulator(s) via mutant studies.

Various proteins are known to interact with flavonoids. Studies in animal cell lines have shown that flavonoids alter multiple kinase and phosphatase activities in these cells (reviewed in Williams et al. 2004), and likewise, they seem to be able to interact with proteins such as the PINOID (PID) serine/threonine kinase in plants (Benjamins et al. 2001). PID is a component in auxin signalling, with a role in polar auxin transport, and is upregulated by the hormone. PID mutants also display completely unbranched inflorescences. PID-mediated kinase activity may be modulated by endogenous flavonols (Peer et al. 2004); the PID protein is sensitive to NPA and interacts with Ca^{2+} binding proteins, TOUCH3 (a calmodulin-related protein) and with PID-binding protein, which also has Ca^{2+} binding motifs,

suggesting a connection between auxin and Ca^{2+} signalling (Benjamins et al. 2003). It has been suggested that flavonoids can act in Ca^{2+} signalling pathways too (Montero et al. 2004)—they certainly do in root associations.

4.6 Flavonoid Regulation of Auxin: Root Symbioses

One of the best-studied examples of flavonoid signalling is how flavonoids mediate the interactions between the plants and microorganisms in the environment at both competitive (allelopathy/defence) and cooperative (mycorrhizal association) levels. For example, flavonoid modulation of auxin accumulation has been observed during nodulation, the association between leguminous plants and Gram-negative soil bacteria (rhizobia) that have the ability to establish a N_2-fixing symbioses. In *Medicago truncatula*, auxin efflux carriers of the PIN family are necessary for root nodule formation (Huo et al. 2006), and flavonoid-deficient roots are unable to initiate root nodules, even though normal root hair curling occurred after inoculation with rhizobia. Nodule formation was restored by the addition of naringenin (Wasson et al. 2006).

Establishment of the nodule symbiosis requires co-ordination of plant and bacterial gene expression, which is regulated through the mutual exchange of molecular signals. The early interaction between the plant signals, flavonoids and the NodD transcriptional regulator activates *nod* gene transcription in rhizobia that are compatible with the plant. The host plant can sense the complementary rhizobial signalling molecules (Nod factors) through transient changes in intracellular free calcium (Moscatiello et al. 2010) and then initiate the nodule primordium (Maj et al. 2010). Notably, flavonoids, NodD1 and NodD2 TFs and the nodulation promoter (nod box) NB15 all modulate expression of a downstream auxin synthesis locus in *Rhizobium* sp. strain NGR234 (Theunis et al. 2004). At a molecular level, flavonoids may be altering the topology of DNA, allowing access of RNA polymerase to nod boxes upstream of nod operons (Maj et al. 2010). This action is specific to particular flavonoids again, as seen with regulation of acropetal or basipetal root auxin transport. Luteolin (3′,4′,5,7-tetrahydroxyflavone) is a *NOD* gene inducer in *Sinorhizobium meliloti* (Peck et al. 2006; Peters et al. 1986), whereas naringenin and 4′,7-dihydroxyflavone induces nod genes in *Rhizobium leguminosarum* biovars (Moscatiello et al. 2010; Zaat et al. 1989).

Flavonoids also play roles in other microbial interactions (Päsold et al. 2010). The pattern of auxin and flavonoid distribution in *Agrobacterium tumefaciens* tumours of *Trifolium repens* suggests that flavonoids may be the endogenous regulator of the basipetal auxin flux and thus contribute to auxin levels in tumours. Three different flavonoids—naringenin, quercetin and kaempferol—and their glycosides accumulate in root galls of Arabidopsis after infection with the obligate biotrophic pathogen *Plasmodiophora brassicae* (which causes economic losses because of brassica clubroot). An increased auxin response occurs during gall development, illustrated by the auxin-responsive DR5 promoter, along with differential transcriptional regulation of

the flavonoid pathway in galls compared with noninfected tissues. In addition, naringenin and quercetin are visible in complex with DPBA as cyan and yellow-orange fluorescence, respectively, whereas the green kaempferol–DPBA fluorescence is observed in few root galls (Päsold et al. 2010).

4.7 Flavonoid Regulation of Auxin: Flowers

There are important roles for auxin in plant floral development, organ formation and pollen development, and the hormone also coordinates maturation of pollen and anther dehiscence (Cecchetti et al. 2008; Cheng et al. 2006; Wilson et al. 2011). Distribution of auxin during floral development has been detected via GUS activity in Arabidopsis plants transformed with an auxin response element, DR5-GUS (Ulmasov et al. 1997), which was mainly active at stages 10–12 of floral development (flower configuration according to Smyth et al. 1990), especially in anthers and, although less so, in sepals during stages 12–15.

Auxin promotes elongation of hypocotyls in high temperatures and Arabidopsis mutants in parts of the auxin biosynthesis pathways responsible (e.g. *yuc2* and *yuc6*) are male sterile (Sakata et al. 2010). In barley, auxin levels were also reduced in anthers in response to prolonged high temperatures (Sakata et al. 2010) reducing fertility in the crop as a result of incorrect anther development and pollen failure. This multifactorial effect, which includes mitochondrial dysfunction and altered anther-wall transcription patterns, occurs in many species (Sakata et al. 2010).

Cecchetti and colleagues (2008) recently discussed how auxin regulates anther dehiscence and pollen maturation in Arabidopsis: local increases in auxin sensitivity in anther somatic tissue, caused by expression of the *ROLB* (*root locus B*) Agrobacterium oncogene, delayed the degradation of the anther circular cell cluster and stomium, supporting a role for auxin in anther dehiscence. Meanwhile, a decrease in endogenous free auxin levels in tobacco resulted in decreased pollen embryogenesis (Yang et al. 1997), suggested by the authors to show how auxin in the tapetum is important for pollen development.

Auxin-transporting PIN proteins are also important in fertility, as well as in plant architecture: use of radiolabelled auxin and observation of the expression of *AtPIN1* both demonstrated polar auxin transport in anther filaments and pollen grains. A block in auxin flow in stamen filaments resulted in shortened filaments and defective pollen grains in both Arabidopsis and tobacco (Feng et al. 2006). A relationship between floral development and flavonoids emerges when considering the double mutant *Atmdr1/Atpgp1*, which is lacking NPA-binding auxin transporters. Auxin transport was altered and the fertility of the flowers was poor because the filaments did not elongate sufficiently to position the anthers above the stigma at anthesis (Noh et al. 2001) in this self-fertile plant. Since these transporters are also flavonoid-binding proteins, it has been suggested that AtMDR1 and AtPGP may modulate the effects of flavonoids on PIN expression and protein localisation (Murphy et al. 2002; Noh et al. 2003).

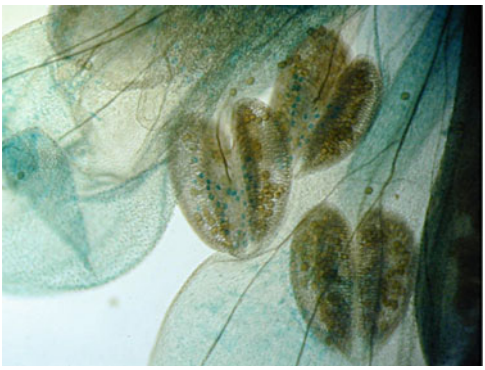

Fig. 8 FFT promoter–GUS transformed Arabidopsis showing GUS activity in particular in anther guard cells around the dehiscence zone (Thompson et al. 2009)

Pollen grains of land plants have evolved strong outer walls (exine) which protect pollen and also interact with female stigma cells. Exine is composed of sporopollenin, a polymer whose composition and synthesis is not fully elucidated, but of which fatty acids and phenolics are likely components. Flavonoids, members of the family of plant phenolics and products of phenylpropanoid metabolism, function in pollen germination and plant fertility in many plant species (e.g. Napoli et al. 1999). As discussed above, the CHS-null mutation in Arabidopsis results in fertile plants despite a reported absence of flavonoids in the mature stamens (Burbulis et al. 1996; Ylstra et al. 1996). The *FFT* MATE transporter is found in nectary and anther, and in the *fft* flavonoid-transporter mutant, however, many anthers fail to dehisce, with no visible pollen outside each (Fig. 8; Thompson et al. 2009). The *tt4* mutant could either be fertile because flavonoids are not required for Arabidopsis male fertility or because *TT4*-independent flavonoid synthesis occurs in anthers, perhaps transiently and at an earlier developmental stage. This was confirmed by the investigation of flavonoid pathway enzyme isoforms in the floral tissues (Dobritsa et al. 2010). Mutations in putative CHS enzyme isoforms, *LAP5* and *LAP6*, reduced the accumulation of flavonoid precursors and flavonoids in developing anthers, which does suggest a role in the synthesis of phenolic constituents of sporopollenin (Dobritsa et al. 2010). Meanwhile, adding kaempferol to flavonoid-deficient petunia pollen causes rapid and synchronous germination and pollen tube outgrowth. The mechanism of the flavonol's action is not yet clear, but a structural role in wall formation has been ruled out (Xue et al. 1996). The sensitivity and specificity of the germination requirement, as well as the rapid response to added flavonoids, again supports their role as signalling molecules, just as seen in the compelling case of the communication between plants and soil bacteria.

5 Auxin and Flavonoid Co-regulation

The regulatory function of flavonoids is subject to a feedback loop, since auxin is able to influence synthesis of the very pigments that regulate its own transport. Flavonoid synthesis is regulated by a whole variety of developmental and

environmental cues, including transcriptional regulation of biosynthetic pathway genes via different R2R3-MYB, basic helix-loop-helix and WD40-type transcription factors as discussed above (e.g. Stracke et al. 2007; Nesi et al. 2001; Winkel-Shirley 2002). Light quality and quantity also influence expression of the biosynthetic pathway genes (Pelletier and Shirley 1996) via distinct photoreceptors (Hemm et al. 2004), and light regulation of auxin transport can also induce synthesis of flavonoids. In fact, two hormones, auxin and ethylene, can act semi-independently to alter the relative abundance of quercetin and kaempferol, with auxin favouring a higher ratio of Q (quercetin) to K (kaempferol) and vice versa (Lewis et al. 2011).

This hormonal crosstalk, its regulation of the synthesis of flavonols and impact on root growth and development were examined in Arabidopsis using wild-type seedlings and a hormone-insensitive mutant lacking the transport inhibitor response1 (TIR1) auxin receptor (which controls degradation of the transcriptional repressor Aux/IAA family, thereby inducing gene expression; Dharmasiri et al. 2005). These authors found that auxin altered both flavonol pathway transcripts and flavonol accumulation: untreated *tir1-1* seedlings exhibited significantly lower CHS and F3′H transcript accumulation in roots than seen in wild type. After auxin treatment of *tir1-1*, both CHS and FLS transcript levels were unchanged and, although auxin did provoke a significant increase in F3′H transcript in the mutant, this was only about half the increase seen in the wild type. This regulation of the branch point enzyme, F3′H, which is required for quercetin synthesis (Fig. 3) maintains a low abundance of F3′H relative to other pathway enzymes and a delayed induction of F3′H expression in response to auxin. The result is a transient peak in kaempferol accumulation and then a later increase in quercetin accumulation, allowing subtle, coordinated timing and sequential activation of genes.

6 The Continued Debate

Questions over the physiological function of flavonoids focus on their occurrence in locations where more obvious nonregulatory roles may be required: in the leaf, are flavonoids present to colour vegetative tissues, deflect light stress and deter herbivores, or are they controlling plant development via auxin transport? Damage from UV light requires mediation and flavonoids in the upper surfaces of leaves are clearly present to filter light that can damage key proteins of the photosynthetic reaction centres. Meanwhile, UV light can also damage nucleic acids: therefore, is the identification of CHS and CHI in the nucleus of Arabidopsis cells (Saslowsky et al. 2005) and of flavonoids in the nuclei of many other plants (e.g. *Brassica*, *Camellia*, *Flaveria*, *Picea* and *Taxus*; Buer and Muday 2004; Feucht et al. 2004; Grandmaison and Ibrahim 1996; Hutzler et al. 1998; Kuras et al. 1999) demonstrating a protective role or the possibility of flavonoids directly regulating transcription of genes required for growth and development—such as auxin transport proteins—via DNA binding? It also reminds us of the question of how

intracellular distribution of the enzymes is regulated. Flavonoid transport experiments performed by external flavonoid application showed that dihydroflavonols are transported *in planta*, whereas flavonol aglycones are not (Buer et al. 2007). This is presumably the key: subsets of flavonoids doubtlessly perform independent and specific functions and are presumably transported and regulated accordingly.

Their mediation of reactive oxygen species means that the possibility that flavonoids as regulators of auxin breakdown by peroxidase has been discussed, as shown in cells undergoing nodule organogenesis in white clover (Mathesius 2001). Several peroxidases can act as nonspecific auxin oxidases (Mathesius 2001; Päsold et al. 2010) and inhibition of this activity by flavonoids could therefore result in decreased auxin degradation. Indeed, kaempferol inhibits mammalian monoamine oxidase/peroxidases, of which the IAA oxidase is a family member (Sloley et al. 2000). Meanwhile, auxin oxidation has been reported to result in increased radical production which could be detoxified by flavonoids (Kim et al. 2005), and auxin levels are typically increased in root galls (Ludwig-Müller et al. 2009).

There is conflicting evidence on the extent to which flavonoids accumulate in roots when shoots are grown in the light and roots are grown in the dark: this is the normal physiological state for plants grown in the environment, but in the laboratory, experiments are usually carried out in semi-transparent Murashige and Skoog agar plates. There is a report of root flavonols visualised by in situ DPBA staining of plants grown with roots in the dark and shoots in the light (Buer and Muday 2004), but another did not find flavonols in roots grown in darkness when samples were examined via HPLC and DPBA staining (Stracke et al. 2010). Accumulation of flavonols in dark-grown roots may indicate long-distance communication of light-dependent signals to the roots or movement of flavonols from the shoot into the root (Buer et al. 2007, 2008, 2010) or consist of flavonoid precursors (e.g. dihydroflavonols) which are able to move from an application site on the cotyledons to the root tip (Buer et al. 2007) or from wild-type shoots across a graft junction into *tt4* roots (Buer et al. 2008). It has been suggested that Arabidopsis roots growing close to the soil surface have light-induced flavonoid synthesis, followed by flavonoid regulation of gravitropism that would guide roots deeper into the soil to benefit the plant.

Flavonoids are non-essential modulators of auxin, since null mutant lines are able to grow if not always reproduce. Treatment with an ethylene precursor [1-aminocyclopropane-1-carboxylic acid (ACC)] was also able to increase auxin transport capacity in the wild type and *tt4* and *tt7*mutants, suggesting a flavonoid-independent mechanism exists for enhanced auxin transport (Lewis et al. 2011). ACC also increased expression of PIN2, and it has been suggested that, rather than acting via PIN proteins, the flavonol modulation of auxin efflux would be via members of the ATP-binding-cassette transporter superfamily (ABCB), another class of auxin efflux transporters (Geisler et al. 2005; Blakeslee et al. 2007; Kuhn et al. 2011).

Questions therefore remain over the likely physiological function of flavonoids, how they are transported around the plant, the auxin regulatory proteins with which they interact and which flavonoids are responsible for which regulatory function.

Although the experimental data are very convincing, there is much to discuss. Whereas auxin is one compound, the community interested in flavonoids has a plethora of bioactive molecules, redundancy and enzyme isoforms to consider. This remains a fertile field for exploration.

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Index

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