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Regulation of polar auxin transport by protein and lipid kinases

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Abstract

The directional transport of auxin, known as polar auxin transport, allows asymmetric distribution of this hormone in different cells and tissues. This system creates local auxin maxima, minima and gradients that are instrumental in both organ initiation and shape determination. As such, polar auxin transport is crucial for all aspects of plant development but also for environmental interaction, notably in shaping plant architecture to its environment. Cell-to-cell auxin transport is mediated by a network of auxin carriers that are regulated at the transcriptional and post-translational levels. Here we review our current knowledge on some aspects of the 'non-genomic' regulation of auxin transport, putting an emphasis on how phosphorylation by protein and lipid kinases controls the polarity, intracellular trafficking, stability and activity of auxin carriers. We describe the role of several AGC kinases, including PINOID, D6PK and the blue light photoreceptor phot1, in phosphorylating auxin carriers from the PIN and ABCB families. We also highlight the function of some Receptor-Like Kinases (RLK) and two-component histidine kinase receptors in polar auxin transport, noticing that there are likely RLKs involved in coordinating auxin distribution yet to be discovered. In addition, we describe the emerging role of phospholipid phosphorylation in polarity establishment and intracellular trafficking of PIN proteins. We outline these various phosphorylation mechanisms in the context of primary and lateral root development, leaf cell shape acquisition as well as root gravitropism and shoot phototropism.

Keywords

kinase; auxin; intracellular trafficking; polarity; gravitropism; phototropism; root; endocytosis; Arabidopsis; receptor-like kinase; cytokinin; phosphoinositide

Introduction

Auxin (Indole 3-Acetic Acid, IAA) is a small molecule plant hormone derived from the amino acid tryptophan that controls virtually all aspects of the plant life. In particular, auxin is of paramount importance for both development and response to the environment and as such is a determining factor in the acquisition of the final shape of plants (Finet and Jaillais,

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30 words statement: This review highlights our current knowledge on how phosphorylation by protein and phosphatidylinositol kinases controls the polarity, intracellular trafficking, stability and activity of auxin carriers.

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2012; Weijers and Wagner, 2016). A particularity of auxin is that it is actively transported across cells and tissues by specialized, plasma membrane localized, influx and efflux carriers. The combined activity of these transporters allows the generation of auxin gradients, as well as auxin maximums and minimums that are critical for organ patterning and differential growth during tropic responses (i.e. growth of the plants toward or against an environmental stimulus) (Finet and Jaillais, 2012). The combination of each auxin concentration in a given cell induces specific transcriptional programs that can have a wide range of outputs, including cell differentiation into various cell types and activation or inhibition of elongation growth. Auxin regulates transcription downstream of its perception by the TIR1 auxin receptor (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005; Weijers and Wagner, 2016). Briefly, TIR1 (and related family members called AUXIN SIGNALING F-BOX, AFBs) are F-BOX E3 ubiquitin ligases that interact with and trigger the degradation of AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional co-repressors in the presence of auxin (Weijers and Wagner, 2016). Aux/IAA degradation releases Aux/IAA-mediated inhibition of AUXIN RESPONSE FACTORs (ARFs), which are transcription factors that regulate the transcriptional output regulated by auxin (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005; Weijers and Wagner, 2016). Furthermore, this transcriptional profile is plugged into an intricate network of hormone, developmental and environmental responses, which together decide the output cellular responses (Finet and Jaillais, 2012; Jaillais and Chory, 2010). These downstream events in auxin signaling have been reviewed elsewhere and will not be further discussed in this review (Habets and Offringa, 2014; Hagen, 2015; Korasick *et al.*, 2015; Larrieu and Vernoux, 2015).

Upstream of gene regulation, a decisive and characteristic feature of auxin is the establishment of auxin gradients and/or maxima, which are established through cell-to-cell polar auxin transport. The chemiosmotic model of auxin transport that was formulated by Rubery and Sheldrake as well as by Mary Helen and Timothy Goldsmith, proposed that the protonation state of auxin, as a weak acid, is dictated by the pH of its environment (Goldsmith and Goldsmith, 1981; Goldsmith *et al.*, 1981; Rubery and Sheldrake, 1973, 1974). The cell wall is acidic (pH $\approx$ 5.5), and therefore a significant proportion of auxin ($\approx$ 20%) is in its protonated state (IAAH), which can freely diffuse across biological membranes. Inside the cell, the pH of the cytosol is neutral (pH $\approx$ 7) and most auxin is in its anionic form IAA^-, which cannot diffuse across membrane and thereby, is trapped inside the cell (Box 1). As a consequence, the chemiosmotic hypothesizes postulated that plasma membrane auxin efflux carriers must exist to ensure the transport of auxin outside the cell. Indeed, it was latter demonstrated that there are three main families of transmembrane proteins that transport auxin across the plasma membrane: i) AUX1/LIKE AUX1 (AUX1/LAX), which are auxin influx permeases, ii) ATP-binding cassette subfamily B (ABCB) transporters, which are auxin efflux transporters and iii) PIN-FORMED proteins (PIN), which are auxin efflux carriers (Box 1) (Finet and Jaillais, 2012). All these subfamilies have been shown to independently transport auxin in planta and in heterologous systems (Barbez *et al.*, 2013; Geisler *et al.*, 2005; Grones and Friml, 2015; Petrasek *et al.*, 2006; Yang and Murphy, 2009; Yang *et al.*, 2006; Zourelidou *et al.*, 2014). However, these studies also suggest that PINs and ABCBs interact and function both independently and interdependently to control polar auxin transport in planta (Bandyopadhyay *et al.*, 2007; Blakeslee *et al.*,

2007; Titapiwatanakun *et al.*, 2009). In particular, PINs/ABCBs form protein complexes that are stabilized by ABCB proteins (Blakeslee *et al.*, 2007; Titapiwatanakun *et al.*, 2009). The PIN family is itself divided into several subfamilies based on the size of their intracellular loop: type-I PIN proteins (PIN1, PIN2, PIN3, PIN4 and PIN7) present a long hydrophilic loop (PIN^{HL}) and are mainly localized at the plasma membrane (box 1), PIN6 presents a partially reduced hydrophilic loop, while type-II PINs (PIN5 and PIN8), present a short hydrophilic loop and are localized in the Endoplasmic Reticulum (Cazzonelli et al., 2013; Ganguly et al., 2014; Krecek et al., 2009; Zazímalová et al., 2010)(Bennett, 2015; Finet and Jaillais, 2012; Habets and Offringa, 2014). In addition, the NRT1.1 nitrate sensor/transporter also facilitates auxin uptake at low $NO3^-$ concentration (Krouk *et al.*, 2010).

All these auxin carriers have been shown to be important for auxin fluxes in planta (Band *et al.*, 2014). However, the auxin carrier family that has received the most attention is the PINs, because it confirmed another visionary prediction from the chemiosmotic theory: that a polar localization of an “auxin secreting system” could explain the directionality of auxin flow, providing that this polarity is coordinated at the tissue and organ level (Rubery and Sheldrake, 1974). In many cell types, type-I PIN proteins are indeed polarly localized and this polar localization was shown to directly correlate with the auxin flow direction (Adamowski and Friml, 2015; Band *et al.*, 2014; Benkova *et al.*, 2003; Blilou *et al.*, 2005; Friml *et al.*, 2002a; Friml *et al.*, 2003; Galweiler *et al.*, 1998; Habets and Offringa, 2014; Muller *et al.*, 1998; Petrasek *et al.*, 2006; Wisniewska *et al.*, 2006). Although, to date, it is not possible to directly visualize the direction of auxin flow in plants, the polar localization of PIN proteins has been widely used as a proxy to deduce these fluxes (Wisniewska *et al.*, 2006). The assumption that auxin transport route can be deduced from PIN polar localization is solidly backed-up by both experimental data and computer simulations (Band *et al.*, 2014; Blilou *et al.*, 2005; Grieneisen *et al.*, 2007). PIN localization also correlates with expected auxin accumulation and depletion sites visualized by the synthetic output (transcriptional) reporters *DR5, DR5rev and DR5v2,* as well as auxin input reporters DII and R2DII (Benkova *et al.*, 2003; Brunoud *et al.*, 2012; Larrieu and Vernoux, 2015; Liao *et al.*, 2015; Sabatini *et al.*, 1999; Ulmasov *et al.*, 1997; Vernoux *et al.*, 2011). However, computational modeling also revealed the importance of both auxin efflux (by PINs and ABCBs) as well as influx (by AUX1/LAXs) carriers for the correct accumulation of auxin in the primary root tip (Band *et al.*, 2014). Furthermore, PIN loss-of-function mutants (and higher order mutants, due to high functional redundancy between PIN family members and compensatory mechanisms) failed to form normal auxin maximum (Adamowski and Friml, 2015; Benkova *et al.*, 2003; Blilou *et al.*, 2005; Friml *et al.*, 2002a; Friml *et al.*, 2003; Vieten *et al.*, 2005). Localizing the various PIN proteins therefore allowed drawing maps of auxin flow in various developmental contexts. As an example, Figure 1 shows putative auxin transport routes in the primary root, as inferred from PIN1, PIN2, PIN3, PIN4 and PIN7 localization. In the primary root, the most noticeable PIN localization are as follow: i) PIN1 (together with PIN3 and PIN7) in the stele, PIN2 in the cortex and PIN4 in three to four cells above the quiescent center (QC) localize at the basal end of the cell thereby directing auxin toward the tip of the root (acropetal auxin flow) (Band *et al.*, 2014; Blilou *et al.*, 2005; Friml *et al.*, 2002a; Muller *et al.*, 1998), ii) PIN4 in the QC and surrounding initials and PIN3/PIN7 in the columella are not polarly localized, redirecting auxin away from this region

(basipetal auxin flow) (Band *et al.*, 2014; Friml *et al.*, 2002a; Friml *et al.*, 2002b), iii) PIN2 in the epidermis is localized in the apical end of the cells, moving auxin back up the root and (Abas *et al.*, 2006; Band *et al.*, 2014; Blilou *et al.*, 2005; Muller *et al.*, 1998), iv) PIN1 in the endodermis and pericycle is not only localized at the basal pole of the cell, but also on the inner lateral plasma membrane domain (facing the stele), which allows recirculating auxin from the epidermis into the stele (Band *et al.*, 2014; Blilou *et al.*, 2005). This fountain-like pattern of auxin flow in the root creates auxin maximum in the QC, surrounding initials and to a lesser extent in the columella and protoxylem. Importantly, the differences in PIN localization are not only regulated by tissue specificity but are also dependent of the PIN proteins themselves. For example, when ectopically expressed in the epidermis, PIN1 is localized at the basal and apical end of the cell, while PIN2 is only apical (Marhavy *et al.*, 2014; Wisniewska *et al.*, 2006).

While the map of auxin flows drawn in figure 1 appears relatively static, PIN protein localizations are in fact highly dynamic and they can be remodeled by both developmental and environmental cues. For example, a change of PIN protein localization reorients auxin toward the tip of young emerging primordia (e.g. lateral root), which is necessary for the emergence of the new organ (Benkova *et al.*, 2003). In addition, PIN protein localizations, abundance, but also their activities, are altered during tropic responses (Rakusova *et al.*, 2015). This high adaptability of the auxin transport machinery to various developmental and environmental responses has been shown over the years to not only rely on genomic changes, but also on post-translational control of the auxin carrier network. Among post-translational modifications, phosphorylation has been implicated at multiple stages in the control of auxin carriers' localization and activity. Here, we will review how protein and lipid kinases dynamically set up and control polar auxin transport.

Phosphorylation and the establishment/maintenance of PIN polarity

Regulation of PIN polar targeting by AGCVIII-mediated PIN phosphorylation

In 1991, a recessive mutation in Arabidopsis was described to produce the so-called pin inflorescences, i.e: inflorescences that elongate but that do not produce flower primordia (Okada *et al.*, 1991). The inflorescences of this mutant, named *pin1*, had reduced downward polar auxin transport. Moreover, wild-type plants grown in media containing high concentrations of auxin transport inhibitors, such as 1-N-Naphthylphthalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA), showed the same inflorescence phenotype, suggesting a role for PIN1 in polar auxin transport (Okada *et al.*, 1991). PIN1 turned out to encode for a multipass membrane protein localized at the basal side of xylem cells in the stem (Galweiler *et al.*, 1998) and later shown to directly transport auxin from the cytosol out of the cell (Petrasek *et al.*, 2006).

In 1995, the *pinoid* (*pid*) mutant was described to have a very similar phenotype as *pin1*, with pin-like inflorescences harboring no or few flowers, which contained an aberrant number of flower organs, altered venation pattern and fused or multiple cotyledons (Benjamins *et al.*, 2001; Bennett *et al.*, 1995; Christensen *et al.*, 2000). This phenotype suggests that PID might regulate polar auxin transport together with PIN1. Genetic analyses showed an enhanced phenotype of the double *pin1pid* mutant as compared to each single

mutant, with a portion of double mutant seedlings having no cotyledon and fully fused first leaves (Furutani *et al.*, 2004; Jaillais *et al.*, 2007). This genetic analysis suggests that PIN1 and PID might act in two separate pathways. However, it was later revealed that this enhanced phenotype of the double mutant very likely reflects the ability of PID to regulate other PINs that act redundantly with PIN1 during embryogenesis (Friml *et al.*, 2004). Alternatively, this phenomenon might be partially explained by the reported interaction between PID and ABCB1 (Henrichs *et al.*, 2012).

The predicted PID protein is a serine-threonine protein kinase. In particular, PID protein falls into the AGC3 group of the plant specific AGCVIII protein kinase family (Christensen *et al.*, 2000; Galvan-Ampudia and Offringa, 2007). PID is an active kinase in vitro (Christensen *et al.*, 2000). Furthermore, overexpression of a kinase dead version of PID fails to induce any phenotype in transgenic Arabidopsis. By contrast, overexpression of wild type PID shows severe developmental defects, including small and dark green curled leaves, as well as reduced apical dominance and impaired elongation of the inflorescence (Christensen *et al.*, 2000). Therefore, PID kinase activity is required for its function in planta. Moreover, *35S::PID* plants are sterile or semifertile and, strong PID overexpression produced embryo lethality. At the seedling stage, both roots and hypocotyls are shorter and strongly agravitropic. In addition, emergence of lateral roots is delayed and only occurred upon collapse of the primary root meristem (Benjamins *et al.*, 2001; Christensen *et al.*, 2000). The *35S::PID* seedling phenotypes are partially rescued by treatments with the polar auxin transport inhibitor NPA. In addition, the expression of the *DR5::GUS* auxin reporter in the root tip of *35S::PID* plants is reduced, arguing that auxin content in the root tip might be lower due to enhancement of upwards auxin transport (i.e. towards the shoot apex). All these data suggested a role of PID kinase in the regulation of polar auxin transport (PAT), which turned out to be via direct phosphorylation of both PINs and ABCBs auxin efflux carriers (Henrichs *et al.*, 2012; Michniewicz *et al.*, 2007) (Fig. 2). Because of the intertwined relationships between PINs and ABCBs (Bandyopadhyay *et al.*, 2007), it is still unclear to which extent the phenotypes reported above for *PID* loss- and gain-of-function can be directly attributed to phosphorylation on PINs and/or ABCBs.

PID phosphorylates ABCB1, likely on Ser634. Heterologous expression of PID and ABCB1 in tobacco suggests that PID enhances ABCB1 auxin transport activity (Henrichs *et al.*, 2012) (Fig. 2). However, co-expression of PID and ABCB1 with the immunophilin-like TWISTED DARF1 (TWD1) inhibits ABCB1-mediated auxin efflux (Henrichs *et al.*, 2012) (Fig. 2). Therefore, PID has a dual regulatory action on ABCB1 auxin transport, depending on the presence of TWD1. Interestingly, PID not only acts on ABCBs transporters, but also acts directly on PINs. In particular, Friml and colleagues (2004) reported that PID regulates auxin fluxes by determining the subcellular localization of PIN proteins (Fig. 2). PID activity is necessary to specify the delivery of these auxin transporters to the apical side of the cells. The authors showed that overexpression of PID induced apicalization of PINs (localization at the top of the cell or shootward) in root cells where they are normally localized in the basal membrane (at the bottom of the cell or rootward) (Friml *et al.*, 2004). Basal localization of PIN proteins in subepidermal cell files ensures the auxin flux towards the root tip (Figure 1), which is necessary to maintain meristem activity. In *35S::PID* plants, apical localization of PIN proteins enhances upwards auxin flux and, as a consequence,

reduces the auxin maximum at the root tip and produces the collapse of the root meristem (Friml *et al.*, 2004). By contrast, PIN2 localization in the apical membrane of root epidermal cells was not changed by PID overexpression, but was found in endomembrane compartment in the *pid-9* loss-of-function (Friml *et al.*, 2004; Sukumar *et al.*, 2009). In addition, PID is mainly expressed in the epidermis of root meristem where PIN2 is apical and PID expression is absent in subepidermal cell files where PINs are basally localized (Friml *et al.*, 2004; Kleine-Vehn *et al.*, 2009; Michniewicz *et al.*, 2007). PIN1 apicalization was also observed in embryo cells of *35S::PID* plants and more clearly in transactivated *RPS5A>>PID* plants (*RPS5a* promoter being strongly expressed in dividing cells early during embryogenesis) (Friml *et al.*, 2004). An apical-to-basal auxin flow established by basal PIN localization determines auxin maxima in the embryo hypophysis, which maintains primary root meristem formation during embryogenesis (Friml *et al.*, 2003). Apicalization of PIN1 (and other PINs) in *RPS5A>>PID* embryo cells produces strong embryo patterning defects due to accumulation of auxin at the embryo proper to the detriment of the hypophysis (Friml *et al.*, 2004). As a consequence, neither the root nor the cotyledons are properly specified in these plants. On the other hand, an apical-to-basal shift of PIN1 was observed in the epidermal cells of the *pid* inflorescences apex (Friml *et al.*, 2004). PID expression at the flanks of the inflorescence apex nicely correlates with the PIN1 apical localization at these sites (Christensen *et al.*, 2000). PIN1 mislocalization in *pid* plants disrupts the levels of auxin required for floral organ initiation leading to pin-like inflorescences similar to those of *pin1* mutants (Friml *et al.*, 2004).

Similar phenotypes to those of *35S::PID* seedlings and *RPS5A>>PID* embryos were found in a set of loss-of-function mutants for multiple isoforms of the A subunits of protein phosphatase 2 (PP2AA1 (also known as ROOT CURLING ON NPA1, RCN1 (Garbers *et al.*, 1996; Rashotte *et al.*, 2001)), PP2AA2 and PP2AA3) (Michniewicz *et al.*, 2007). PP2AAs are part of the PROTEIN PHOSPHATASE6 (PP6) holoenzyme and form a heterotrimeric complex with the catalytic subunit FYPP1/3 (for PHYTOCHROME-ASSOCIATED SERINE/THREONINE PROTEIN PHOSPHATASE1 and 3) and SAL (for SAL DOMAIN-LIKE which is the regulatory B subunit) (Fig. 2B) (Dai *et al.*, 2012). The PP6 holoenzyme is a PP2A-like complex that incorporate the regulatory subunit PP2AA and SAL and will be referred with the generic name PP2A hereafter. Similar to PID overexpression, apicalization of PIN proteins was also observed in multiple mutants for *pp2aa* subunits (*pp2a1;3* double mutants), *fypp* subunits (*fypp1;3* double mutants) and the *sal* subunit (artificial microRNA targeting all four SAL genes) (Dai *et al.*, 2012; Michniewicz *et al.*, 2007). These observations indicated that PID and PP2A are part of the same pathway and that PID activity on PIN subcellular localization is counteracted by protein phosphatase PP2A (Michniewicz *et al.*, 2007) (Fig. 2B). Mechanistically, PIN proteins where shown to be direct substrates of PID and PP2A (Ballesteros *et al.*, 2013), suggesting that (de)phosphorylation is the signaling event that determines PIN polarity: apical when phosphorylated and basal when dephosphorylated (Dai *et al.*, 2012; Michniewicz *et al.*, 2007). In addition, the PP2A holoenzyme is regulated by ROTUNDA3 (RON3), which copurify with various PP2A components and controls PIN polar targeting (Karampelias *et al.*, 2016).

PID phosphorylation of PIN proteins mainly occurs at the plasma membrane (Simon *et al.*, 2016), where both type of proteins colocalize (Kleine-Vehn *et al.*, 2009; Michniewicz *et al.*,

2007) (Fig. 2A). Similar to *35S::PID* and *pp2a* plants, PIN1 basal localization is also lost in *gnom* loss-of-function mutants or treatment with the fungal toxin brefeldine A (BFA) (Kleine-Vehn *et al.*, 2009). PIN1 constitutively cycles from the plasma membrane to endosomal compartments and BFA blocks the recycling of PIN1 towards the plasma membrane, inducing its accumulation in intracellular compartments and, thus, the depletion from its polar membrane localization (Geldner *et al.*, 2001). Basal PIN polar recycling is maintained by the activity of the GNOM protein, a GDP/GTP exchange factor for small G proteins of the ARF class (ARF-GEF) (Geldner *et al.*, 2003; Steinmann *et al.*, 1999). GNOM is targeted by BFA, which induces its ectopic localization in endosomes and as a result inhibits endocytic recycling (Geldner *et al.*, 2003; Naramoto *et al.*, 2014). Indeed, transgenic plants expressing BFA-resistant GNOM versions did not present changes in PIN1 polar localization upon BFA treatment (Geldner *et al.*, 2003). Consistently with PIN apicalization in the root, *gnom* partial loss-of-function mutants ($gnom^{R5}$) displayed reduced auxin maxima at the root tip and meristem collapse similar to *35S::PID* plants (Geldner *et al.*, 2004; Kleine-Vehn *et al.*, 2009). Moreover, $35S::PID;gnom^{R5}$ plants showed strong developmental defects such as loss of embryonic apical-basal patterning which leads to aberrant root and shoot formation (Kleine-Vehn *et al.*, 2009). The phenotypic similarities of the single *35S::PID* and $gnom^{R5}$ mutants, together with the enhancement of the developmental phenotypes in the double mutant plants and the apicalization of PIN1 observed in both mutants, suggested that PID and GNOM are antagonist modulators of PIN1's subcellular localization. On the other hand, inhibition of GNOM by BFA treatment in *35S::PID* plants or *pp2a* mutants (in which PIN1 is mislocalized to the apical side of root stele cells) did not induce accumulation of PIN1 in endosomes, suggesting that the apical localization of PIN proteins is GNOM-independent (Kleine-Vehn *et al.*, 2009). It is likely that PIN phosphorylation by PID reduces the affinity of PIN proteins to undergo constitutive recycling at the basal membrane and drives its location to the apical membrane via the GNOM-independent pathway (Kleine-Vehn *et al.*, 2009) (Fig. 2A). This model was confirmed by the observation that PIN2 transcytosis in root cortical cells induced by mild BFA treatments was enhanced in *pp2a* mutant and delayed in *pid* background (Kleine-Vehn *et al.*, 2009). It was further supported by the fact that PIN1 carrying mutations that block its phosphorylation (phosphomutants) accumulated in intracellular compartments upon BFA treatment, while mutations that mimic PIN1 phosphorylation (phosphomimic) did not (see below).

PINOID phosphorylates PIN1 in its hydrophilic loop (HL) in the highly conserved PIN specific motif TPRXS(N/S) (Huang *et al.*, 2010; Michniewicz *et al.*, 2007; Zourelidou *et al.*, 2014). Ser-231 (S1), Ser-252 (S2) and Ser-290 (S3) within the three TPRXS(N/S) motifs of $PIN1^{HL}$ were described as in vitro PID phosphorylation sites (Fig. 2B). The biological significance of PIN1 phosphorylation in planta was tested by generation of *PIN1::PIN1-GFP* transgenic plants that contain none, one, two or three of the phosphorylated Ser residues substituted by Glu (to mimic phosphorylation) or by Ala (a nonphosphorylatable residue) (Huang *et al.*, 2010). *The* $PIN1::PIN1^{S1A}$*-GFP* and $PIN1::PIN1^{S1,3A}$*-GFP* lines showed developmental defects at the seedling and reproductive stages, which resembled those of *pid* loss-of-function mutants: formation of three cotyledons and aberrant number of floral organs. Expression of the triple $PIN1^{S1,2,3A}$-GFP mutant caused severe defects leading to

high frequency of embryo lethality or growth arrest upon germination (Huang *et al.*, 2010). Moreover, expression of the PIN1^{S1,3A}-GFP and PIN1^{S1,2,3E}-GFP failed to complement the *pin1* mutant phenotypes (aberrant number of cotyledons and inflorescence without few or no flowers) and, analysis of the subcellular localization of these PIN1 phosphomutants, revealed that the lack of complementation was at least in part due to mislocalization of PIN1. The WT version of PIN1 is localized at the apical membrane of the topmost cells of the inflorescence apex and the phosphomimic PIN1^{S1,2,3E}-GFP protein showed the same apical localization, consistent with the notion that phosphorylated PIN1 induces its apical localization. By contrast, PIN1^{S1,3A}-GFP and PIN1^{S1,2,3A}-GFP proteins were targeted to the basal side, and were not shifted to the apical side of the cell when coexpressed with *35S::PID* (Huang *et al.*, 2010). In embryos, PIN1-GFP protein is mostly localized at the basal side of the membrane in provascular cells. This PIN1 localization is necessary to maintain auxin maxima and to define apico-basal axis during embryo development. On the other hand, PIN1 is localized at the apical side in epidermal cells of the embryo, and drives the accumulation of auxin at the tips of developing cotyledons. PIN1^{S1,2,3A}-GFP protein was mainly accumulated in intracellular compartments in all cell types of the embryo or it was non-polarly localized when present at the membrane. This mislocalization contributes to the alteration of auxin response maxima establishment and consequently, to aberrant embryo development (Huang *et al.*, 2010).

PID clusters together with PID2, WAG1 and WAG2 into the AGC3 group of plant AGCVIII kinases (Galvan-Ampudia and Offringa, 2007; Rademacher and Offringa, 2012; Santner and Watson, 2006). WAG1 and WAG2 were initially described as negative regulators of root waving, as *wag1*, *wag2* and *wag1wag2* mutants showed enhanced waving phenotype (Santner and Watson, 2006). These proteins were later shown to act redundantly with PID in the regulation of cotyledon development, root meristem size and root gravitropic response by modulating the subcellular localization of PIN proteins (Dhonukshe *et al.*, 2010). The triple mutant plants *pidwag1wag2* lack cotyledons. This phenotype might be explained by the observation of an aberrant basal (and sometimes lateral) localization of PIN1 in the embryo epidermal cells of these mutants (Dhonukshe *et al.*, 2010). Basal PIN1 drives auxin to the bottom half of the embryo and thereby inhibits the formation of auxin maxima required for cotyledon initiation. This phenotype is absent or only partially observed in the *pidwag1* and *pidwag2* double mutants, respectively. *pidwag1wag2* roots are agravitropic and shorter than wild-type roots, due to an increase on the auxin content at the root tip and the inability to create asymmetric auxin distribution upon gravistimulation as visualized using the *DR5::GFP* reporter. These defects are associated with the mislocalization of PIN2 at the basal side of epidermal cells where it is normally apical. Notably, and contrary to the case of PIN1 in the inflorescence apex, the basal PIN2 localization in root epidermal cells was not observed in *pid* single mutants, clearly demonstrating the redundant role of these three kinases in root development (Dhonukshe *et al.*, 2010). Moreover, PID, WAG1 and WAG2 phosphorylate PIN2^{HL} at the same Ser residues within the conserved TPRXS(N/S), ie: S1 (Ser-237), S2 (Ser-258) and S3 (Ser-310) and, PIN2::PIN2^{S1,2,3A}-VENUS failed to complement the *pin2* mutant (Dhonukshe *et al.*, 2010). The basal localization of the non-phosphorylable PIN2 protein was not changed by overexpression of PID. Thus, phosphorylation of PIN2 by PID, WAG1 and WAG2 is necessary for its apical localization

in root epidermis and for the correct establishment of auxin fluxes to modulate root meristem growth and response to gravitopic stimulus.

Although the main phosphorylation targets of PID/WAGs are the Ser residues S1, S2, S3 embedded in the TPRXS(N/S) motifs of PIN^{HL}, two additional Ser residues (named S4 and S5) within the hydrophilic loop have been recently shown to be phosphorylated by these kinases (Zourelidou *et al.*, 2014) (Fig. 2B). These two phosphorylation sites are not as well conserved as residues S1 to S3. For example S4 (S271 in PIN1) is mutated to Asparagine (a non-phosphorylable residue) in PIN2 and S5 (position 215 in PIN1) is mutated to an aspartic acid (which might act as a natural phosphomimic or constitutive phosphorylation at this site) in PIN1 (Fig. 2B). S4 and S5 might therefore account for some PIN-specific regulation of their activity. These phosphorylation sites are not necessary to modulate the subcellular localization of PINs but to activate the efflux transport activity of these proteins (Fig. 2B). D6 PROTEIN KINASE (D6PK) phosphorylate PINs at the very same residues as PID/WAGs but with higher affinity for S4 and S5 residues (Zourelidou *et al.*, 2014; Zourelidou *et al.*, 2009). D6PK and its homologs D6PK-LIKE1 (D6PKL1), D6PK-LIKE2 (D6PKL2) and D6PK-LIKE3 (D6PKL3) belong to the AGC1 subgroup of protein kinases (Galvan-Ampudia and Offringa, 2007). D6PK localizes at the basal plasma membrane of the cells where it colocalizes with the PIN auxin efflux transporters. D6PK colocalizes with PIN1 in the main inflorescence stem, with PIN3 in the hypocotyl stem, PIN4 at the lateral root cap cells and PIN1 and PIN2 in the stele and cortex root cells respectively (Barbosa *et al.*, 2014; Willige *et al.*, 2013; Zourelidou *et al.*, 2009). In addition, PIN1-type proteins are direct in vitro phosphorylation targets of D6PK and, PIN1 and PIN3 have been shown to be in vivo substrates as well. Plasma membrane localization of D6PK is essential to accomplish PIN phosphorylation (Barbosa *et al.*, 2014; Willige *et al.*, 2013; Zourelidou *et al.*, 2014; Zourelidou *et al.*, 2009). By contrast to PID and WAG1/WAG2, D6PK phosphorylation is only involved in the regulation of PIN activity (Zourelidou *et al.*, 2014).

Finally, other Ser/Thr residues have been identified as in vivo phospho-residues within PIN^{HL}, but the kinase(s) responsible for this phosphorylation remains unknown. However, phosphorylation in Ser337/Thr340 of PIN1^{HL} is necessary for PIN1 proper subcellular localization in embryo and inflorescence (Zhang *et al.*, 2010b) (Fig. 2B). Phosphorylation of PIN1 at position S337, which is located within a Ser/Thr-Pro motif, a motif that is under the control of the peptidyl-prolyl *cis/trans* isomerase Pin1At (Xi *et al.*, 2016) (Fig. 2B). This protein recognizes phosphorylated Ser/Thr residues in Ser/Thr-Pro motifs and catalyzes the *cis/trans* conformational change of the adjacent Proline residue. Pin1At binds to PIN1 and acts on several Ser/Thr-Pro motifs in the cytosolic loop. To date it is unknown what are the molecular impacts of these conformational changes on the PIN1 protein, but they are important for the PID/PP2A antagonistic effect on PIN1 polarity (Xi *et al.*, 2016). The phosphosites that are recognized by Pin1At are different from the site S1 to S5 that are phosphorylated by PID/WAGs and D6PK and the kinase(s) that is(are) responsible for their phosphorylation is(are) currently unknown (Xi *et al.*, 2016). This suggests that there are a series a kinases/phosphatases that (de)phosphorylate the cytosolic loop of PIN1 successively at different site to control its activity, polarity and trafficking. Interestingly, the Ser337 in PIN1 is conserved in other PINs but is not followed by a proline, suggesting that the Pin1At-mediated conformational change could be involved in PIN1-specific regulations.

The activity of PID/WAGs is itself regulated by phosphorylation, notably by the 3'-Phosphoinositide-dependent protein kinase 1 (PDK1). PDK1 is highly conserved among eukaryotes and is considered as a master regulator of AGC kinases in mammalian cells, due to its ability to activate other AGC kinases (Rademacher and Offringa, 2012). PDK1 possess a pleckstrin homology (PH) domain that is responsible of the interaction of PDK1 to regulatory phospholipids and of the localization of PDK1 at the plasma membrane. Interestingly, most of the AGC kinase substrates of PDK1 do not contain the PH domain and are localized either to the cytoplasm or to the plasma membrane by unknown mechanisms. PDK1 binding to its substrate proteins takes place through interaction between the PIF (PDK1-Interacting Fragment) sequence present at the C-terminus of the substrates and the N-terminal PIF-binding pocket of PDK1 (Rademacher and Offringa, 2012). PDK1 phosphorylates the catalytic domain of the target kinases within their activation loop, which stimulates their autophosphorylation and overall kinase activity.

In Arabidopsis, two homologs of the mammalian PDK1 have been found (PDK1–1 and PDK1–2) and define an independent subgroup of AGC kinases (Galvan-Ampudia and Offringa, 2007). Several members of the plant specific AGCVIII kinases have been shown to interact with and to be phosphorylated by PDK1 in vitro (Zegzouti *et al.*, 2006a; Zegzouti *et al.*, 2006b). Among them, the auxin polar transport regulators PID, WAG1 and WAG2. PDK1 phosphorylates PID on at least one conserved residue (S290) of the activation loop, which enhances PID kinase activity towards myelin basic protein (MBP), an artificial in vitro phosphorylation substrate of PID. The PIF domain of PID is essential for its interaction with PDK1 in vitro (Zegzouti *et al.*, 2006a). On the other hand, WAG1 and WAG2, have been shown to interact with PDK1 in vitro, even if they do not contain a PIF domain within their sequence. However, PDK1 interaction with WAG1 and WAG2 do not produce an increase in the autophosphorylation and activity of these proteins in vitro (Zegzouti *et al.*, 2006b). The requirement of PDK1 regulation of PID, WAG1 and WAG2 activity through its binding and phosphorylation in planta remains to be fully characterized. PID-GUS and PID-VENUS translational fusion lacking the PIF domain, are able to partially complement *pid*-loss of function mutant, arguing against the need of PDK1 modulation of PID activity to regulate auxin-dependent development (Benjamins *et al.*, 2001; Michniewicz *et al.*, 2007; Rademacher and Offringa, 2012). Alternatively to the control of kinase activity, PDK1 might modulate other aspects of the functionality of these proteins, such as their subcellular localization or interaction with downstream substrates.

Phospholipid phosphorylation by PIP Kinases and the control of PIN polarity

Phosphoinositides (also known as phosphatidylinositol phosphates or PIPs) are lipid molecules that are phosphorylated on their polar inositol head, at the inner leaflet of biological membranes (facing the cytosol) (Platre and Jaillais, 2016). They are minor phospholipids, accounting for less than one percent of total membrane lipids, yet they are critical for many membrane-associated events: i) they recruit proteins to membrane compartments through stereospecific interaction domains (*e.g.* Pleckstrin Homology domain (PH), Phox homology domain (PX), Fab1/YOTB/Vac1/EEA1 domain (FYVE)), ii) they are instrumental in cytoskeleton dynamics, iii) they can control enzyme and channel activity, iv) they are precursors of second messengers (*e.g.* Inositol-3-Phosphate, Diacylglycerol) and, v)

they regulate virtually every aspect of membrane trafficking, including endocytosis and exocytosis (Holthuis and Levine, 2005; Jean and Kiger, 2012; Lemmon, 2008). Phosphoinositides form docking platforms at the cytosolic leaflet of membranes and are localized in specific pools in various organelles. Each compartment is composed of a unique combination of phosphoinositides providing a code for membrane identity (Kutateladze, 2010). Phosphoinositides can be phosphorylated on three different positions of the inositol ring, the 3rd, 4th and 5th position, either as single phosphorylation or in multiple combinations (Balla, 2013). Theoretically, there are seven possible PIP species, including three monophosphorylated species (PI3P, PI4P and PI5P), three bi-phosphorylated species ($PI(3,4)P_2$, $PI(3,5)P_2$ and $PI(4,5)P_2$) and one tri-phosphorylated specie ($PI(3,4,5)P_3$). Plant genomes lack type-I and type-II PI3-Kinases that phosphorylate PI4P and $PI(4,5)P_2$ on the 3rd position of the inositol ring. As a result, plants do not produce any $PI(3,4)P_2$ and $PI(3,4,5)P_3$ and have only five different PIP species (Delage *et al.*, 2013; Munnik and Nielsen, 2011). The three main phosphoinositides for which the subcellular localization is known in plants are PI3P, PI4P and $PI(4,5)P_2$ (Simon *et al.*, 2014; Simon *et al.*, 2016; van Leeuwen *et al.*, 2007; Vermeer *et al.*, 2009; Vermeer *et al.*, 2006) (Fig. 3). PI3P is localized in late endosomes, PI4P is mainly presents at the plasma membrane but there exist also a minor pool in early endosomes and $PI(4,5)P_2$ is at the plasma membrane (Fig. 3) (Simon *et al.*, 2014; Simon *et al.*, 2016; van Leeuwen *et al.*, 2007; Vermeer *et al.*, 2009; Vermeer *et al.*, 2006). Both PI4P and $PI(4,5)P_2$ show a slight polar localization in root epidermal cells, being preferentially localized at the apical and basal end of the cell rather than on their lateral sides (Fig. 3) (Ischebeck *et al.*, 2013; Tejos *et al.*, 2014). PI4P is produced by PI4Ks (phosphatidylinositol 4-kinase) that phosphorylate phosphatidylinositol (PI) on the 4th position of the inositol ring (Balla, 2013). In turn, PIP5K (phosphatidylinositol 4-phosphate 5-kinase) phosphorylates PI4P on the 5th position of the inositol ring to produce $PI(4,5)P_2$ (Balla, 2013). Two PIP5Ks (PIP5K1 and PIP5K2) out of 11 present in the Arabidopsis genome are localized in a polarized manner at the plasma membrane of root epidermis, with a preferential localization at the apical/basal pole of the cells (Ischebeck *et al.*, 2013; Tejos *et al.*, 2014) (Fig. 3). It is likely that the polar localization of PIP5K might account for the slight polarized localization of $PI(4,5)P_2$. 12 *PI4K* genes are found in the Arabidopsis genome, but the three type-III PI4Ks (PI4Kα1, PI4Kβ1 and PI4Kβ2) account for almost the entire PI4K activity in planta (Delage *et al.*, 2012). In roots, PI4Kβs are localized to the trans-Golgi Network (TGN), while PI4Kα1 localizes at the plasma membrane (Kang *et al.*, 2011; Okazaki *et al.*, 2015; Preuss *et al.*, 2006).

The polarly localized PIP5K1 and PIP5K2 kinases where shown to regulate polar auxin transport and in particular PIN protein polarity and trafficking (Ischebeck *et al.*, 2013; Mei *et al.*, 2012; Tejos *et al.*, 2014). The single *pip5k2* mutant produces less lateral root, is partially agravitropic, and has altered auxin maximum pattern as visualized by *DR5::GUS* and impaired polar auxin transport (Mei *et al.*, 2012). The *pip5k1pip5k2* double mutant has a stronger phenotype, suggesting functional redundancy between the two kinases (Ischebeck *et al.*, 2013; Tejos *et al.*, 2014). The phenotype of this double mutant is also reminiscent of polar auxin transport defects, including severely impaired primary root growth and altered gravitropic response, abnormal embryogenesis and disconnected vascular tissues in cotyledons (Ischebeck *et al.*, 2013; Tejos *et al.*, 2014). In this double mutant, the polarity of

PIN1 is aberrant in the embryo, the primary root and in leafs, being either apolar or lacking a coordinated polarity from cell to cell (Tejos *et al.*, 2014). This phenotype is reminiscent of the loss of PIN1 polarity in the *gnom* mutant, which is impaired in endocytic recycling (Geldner *et al.*, 2004; Steinmann *et al.*, 1999) and suggest that PIP5Ks might be involved in PIN trafficking. Indeed, the *pip5k2* mutant has decelerated vesicular trafficking and altered response to BFA and the *pip5k1pip5k2* double mutant shows decelerated endocytic recycling of both PIN1-GFP and PIN2-GFP (Ischebeck *et al.*, 2013; Mei *et al.*, 2012). $PI(4,5)P_2$ is known to be involved in clathrin mediated endocytosis in metazoan, as it recruits many endocytic adaptors to the plasma membrane (Schmid and Mettlen, 2013). The *pip5k1pip5k2* double mutant shows altered dynamics and spatial distribution of Clathrin Light Chain 2 (CLC2) at the plasma membrane (Ischebeck *et al.*, 2013). This results suggest that $PI(4,5)P_2$ might directly regulate the localization or function of some molecular actors of clathrin-mediated endocytosis, although this hypothesis has yet to be experimentally tested. It is possible that impaired endocytic trafficking in *pip5ks* mutants induces PIN polarity defects, which in turn might explain the polar auxin transport phenotype of the mutants. However, it is still not clear how the polar localization of $PI(4,5)P_2$ contributes to this phenotype. Indeed, clathrin accumulates preferentially on the lateral side of the cells (Kleine-Vehn *et al.*, 2011), where it presumably stimulates PIN endocytosis, which in turn is polarly recycled at the apical or basal poles of the cell. Therefore, $PI(4,5)P_2$ accumulation does not directly correlate with the recruitment of clathrin (Kleine-Vehn *et al.*, 2011). In addition, CLC2 dynamics in *pip5k1pip5k2* double mutant was analyzed on the outer lateral face of epidermal cells (the side of the epidermis that is in contact with the microscope cover slide), while PIP5Ks are not or less localized in this polar domain. Analyzing PIN endocytosis and clathrin dynamics in apical/basal polar domains is technically challenging, as these poles are not in the plane of the microscope and represent a current limitation in the field.

It is possible that $PI(4,5)P_2$ might also contribute to polarized exocytosis of PIN proteins, perhaps through the recruitment of the exocyst complex. Indeed, the EXO70 exocyst subunit is a known $PI(4,5)P_2$ effector in animal and yeast and has been shown to regulate PIN trafficking in Arabidopsis (Drdova *et al.*, 2013; Martin, 2015). Furthermore, PI4P and $PI(4,5)P_2$ might control the localization of additional proteins involved in PIN protein trafficking, activity and/or polarity. For example, the AGC protein kinases PINOID, and D6PK interact in vitro with these lipids (Simon *et al.*, 2016; Stanislas *et al.*, 2015; Zegzouti *et al.*, 2006a). In particular, PINOID is recruited to the plasma membrane by the electrostatic field generated at this membrane by PI4P (Simon *et al.*, 2016) (Fig. 3). In addition, D6PK localization during root hair initiation is regulated by PIP5K3, another PIP5K expressed in trichoblasts (Stanislas *et al.*, 2015). Finally, the apical-basal polarity of PI4P and $PI(4,5)P_2$ seems to be a variable trait that can be seen only in weak expression lines and was not consistently reported in the literature (Simon *et al.*, 2014; Simon *et al.*, 2016; Tejos *et al.*, 2014; van Leeuwen *et al.*, 2007; Vermeer *et al.*, 2009). The polarity indices (ratio of apical and basal fluorescent signal over lateral signal) of PI4P and $PI(4,5)P_2$ reporters are only around 1.2 to 1.4, while it is around 3 for PIN2-GFP and PIP5Ks (Simon *et al.*, 2016; Tejos *et al.*, 2014). These ratios were shown to be statistically significant as compared to an overexpressed fluorescent aquaporin fusion protein (GFP-aqPIP2) by Tejos et al., (2014), but

not by Simon et al., (2016). It is still unclear whether this apparent phosphoinositide polarity is indeed important for the observed phenotype of *pipk* mutants and PIN polarity establishment or whether it simply reflects the particular topology of root epidermal cells (Simon *et al.*, 2016).

Interestingly, the expression of both *PIP5K1* and *PIP5K2* is induced by auxin, suggesting that auxin itself stimulates the phosphorylation of PI4P into $PI(4,5)P_2$, and as such might induce PIN proteins endocytic trafficking and possibly feedback on their polarity (Mei *et al.*, 2012; Tejos *et al.*, 2014). Consistently with this hypothesis, the inducible overexpression of *PIP5K1* or *PIP5K2* induces PIN1 lateralization (Ischebeck *et al.*, 2013). Such lateralization of PIN1 is reminiscent of the effect of auxin treatment on PIN1 polarity (Sauer *et al.*, 2006). In the root, PIN1 lateralization following auxin treatment is dependent on *ARF7* and *ARF19*, which are also required for *PIP5K1* induction (Sauer *et al.*, 2006; Tejos *et al.*, 2014). As such, PIP5Ks and $PI(4,5)P_2$ could be part of the machinery by which auxin controls the orientation of its own flux, a theory known as the canalization hypothesis. This model proposes a feedback effect of auxin on the directionality of intercellular auxin flow as a mean to polarize tissue and organ. This theory is at the base of many polar auxin transport computational models, although a clear understanding of the molecular mechanisms behind this auxin effect is lacking (Bennett *et al.*, 2014). Furthermore, *PIP5K1* or *PIP5K2* gain-of-function induces the endocytosis and degradation of PIN2-GFP (Ischebeck *et al.*, 2013), which is similarly triggered by either long-term auxin accumulation or depletion (Baster *et al.*, 2013).

Altogether, PI phosphorylation into PI4P and then $PI(4,5)P_2$ has a direct impact on PIN protein trafficking, polarity and auxin flow and this phosphorylation machinery is under transcriptional control of auxin.

Histidine Kinase Cytokinin Receptors and the regulation of PIN polarity and stability

Auxin has long been shown to interact antagonistically with the phytohormone cytokinin (El-Showk *et al.*, 2013). Cytokinin signaling is executed by a histidine-to-aspartate phosphorelay pathway (Hwang *et al.*, 2012) (Fig. 4). This signaling cascade, also called two-components signaling system, is found in bacteria. In its simplest form, it involves a membrane histidine kinase, which acts as sensor or receptor, and response regulator proteins. Histidine kinase auto-phosphorylates on histidine residues, while response regulators are phosphorylated on conserved aspartate residues. Cytokinin signaling involves one intermediate between the histidine kinase and the response regulators in the form of a histidine phosphotranfer protein. In Arabidopsis, there are four cytokinin receptors (CYTOKININ-INDEPENDENT1, CKI1; and three ARABIDOPSIS HISTIDINE KINASE, AHK2 to AHK4), six histidine phosphotransfer proteins (ARABIDOPSIS HISTIDINE PHOSPHOTRANSFERT PROTEIN, AHP1 to AHP6) and two sub-families of response regulators (ARABIDOPSIS RESPONSE REGULATORs, ARRs), five type-A ARRs (ARR3, 9, 15-17) and eleven type-B ARRs (ARR1, 2, 10-14, 18-21) (Hwang *et al.*, 2012).

Binding of cytokinin in AHK's extracellular ligand-binding domain triggers autophosphorylation on a conserved histidine residue in the N-terminal sensor kinase domain. The phosphoryl group is then transferred from this histidine to a conserved aspartate residue in the C-terminal receiver domain of the same AHK. The phosphoryl is then transferred from the aspartate in the AHK to a conserved histidine in AHPs, which are soluble proteins, unlike AHKs that are transmembrane receptors (Hwang *et al.*, 2012). The phosphoryl on AHP's histidine is then finally transferred to ARRs on conserved aspartate residues, hence the term "phosphorelay" that describes this signaling system. Type-B ARRs localize in the nucleus and are DNA binding transcription factors. They directly regulate the expression of primary cytokinin responsive genes, including type-A ARRs, which are negative feedback regulators. This canonical cytokinin signaling pathway regulates the expression of many genes and in particular it directly controls the expression of *PINs* (Simaskova *et al.*, 2015). As such, the genomic response to cytokinin directly impact auxin fluxes (Fig. 4).

In addition, cytokinin also regulates polar auxin transport via non-genomic means, notably in the context of lateral root formation (Marhavy *et al.*, 2011; Marhavy *et al.*, 2014). Live imaging during lateral root outgrowth shows that cytokinin treatment induces rapid and pronounced PIN1 degradation (Marhavy *et al.*, 2011). This effect is dependent on AHKs, in particular AHK4 as well as some type-B ARRs (ARR1 and ARR12). However, it is independent of protein synthesis and transcription as it still occurs following treatment with cycloheximide or cordycepin, which inhibit translation and transcription, respectively (Marhavy *et al.*, 2011). PIN1 degradation following cytokinin treatment requires an intact endocytic pathway to route PIN1 from the plasma membrane to the lytic vacuole, where it is degraded. Interestingly, this cytokinin-induced degradation is not a general effect on membrane proteins: it seems to be specific for PIN1, PIN3 and PIN7, but has little effect on PIN2 or AUX1 localization in Arabidopsis root (Marhavy *et al.*, 2011).

In addition to this effect on PIN1 stability, cytokinin also controls PIN1 polarity during lateral root emergence. During lateral root initiation, PIN1 polarity switches from a basal localization to a lateral localization, so that PIN1 is facing away from the stele and toward the tip of the new initium (Benkova *et al.*, 2003). This redirects auxin flow and allows the formation of an auxin maximum at the tip of the new primordium, which is required for lateral root emergence. This lateralization of PIN1 is known to require endocytic trafficking (Geldner *et al.*, 2004; Jaillais *et al.*, 2007). Cytokinin treatment at low concentration specifically depletes PIN1-GFP from the basal end of the cell but not from the periclinal side (Marhavy *et al.*, 2014). Again, this effect is dependent on an intact cytokinin phosphorelay pathway and is specific for PIN1. To show that cytokinin specifically depletes PIN1 at the basal end of the cell, Marhavý et al., missexpressed PIN1 in the PIN2 expression domain (epidermis and cortex). In this context, PIN1 is basaly localized in the cortex (similar as PIN2) but is localized at both basal and apical poles of the cell in the epidermis (Marhavy *et al.*, 2014). Strikingly, cytokinin selectively depletes PIN1 at the basal pole but not the apical pole of epidermal cells. This effect was dependent on PIN1 phosphorylation status as cytokinin effect on PIN1 degradation was mitigated in *pp2a* loss-of-function mutants, PID overexpression lines or transgenic lines that constitutively express a PIN1 phosphomimic mutant (Marhavy *et al.*, 2014). Overall, this study suggests that cytokinin not only induces

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PIN1 trafficking to the vacuole, but that this effect is specific for a specialized polar domain, very likely based on PIN1 phosphorylation status. This finding is highly relevant to lateral root formation, since the removal of PIN1 at the basal pole of the cell allows accumulation of PIN1 selectively at the periclinal plasma membrane and thereby redirection of the auxin flow toward the tip of the emerging primordium (Benkova *et al.*, 2003). This suggests that cytokinin acts as a polarizing clue during organogenesis. However, the exact mechanisms by which cytokinin induces PIN1 degradation in the absence of transcription are currently unknown and one of the challenges for the coming years will be to understand these underlying mechanisms.

The TMK Receptor-Like Kinase (RLK) family in the control of 'non-genomic' auxin signaling and establishment of cell shape

Leaf pavement cells are highly polarized cells that present an interdigitated growth with the presence of lobes and necks. As such, they form an epidermal cell layer, which is characterized by its jigsaw-puzzle pavement. Exogenous auxin treatments promote lobe formation in a dose-dependent manner, while auxin biosynthesis mutants present a reduction of lobes that can be rescued by auxin treatments (Xu *et al.*, 2014). In addition, PIN1 overexpression positively regulates the number of lobes in leafs and cotyledon pavement cells (Guo *et al.*, 2015; Li *et al.*, 2011). Moreover, the *pin1* loss-of-function mutant also presents an altered PCs phenotype; the cells being long and narrow (Xu *et al.*, 2014). By contrast to auxin biosynthesis mutants, exogenous auxin treatment does not rescue the *pin1* cell shape phenotype, suggesting that PIN1-mediated polar auxin transport is essential for the formation of lobes and necks in PCs. However, plants grown on NPA have wild-type pavement cell morphology (Ringli *et al.*, 2008; Xu *et al.*, 2014). This result, together with the fact that auxin deficient mutants have relatively mild PC shape phenotype, suggests that polar auxin transport is a modulator, rather than a major regulatory pathway, of leaf cell morphogenesis.

PIN1 is slightly polarized at the tip of the lobes of pavement cells in young cotyledons (Guo *et al.*, 2015; Xu *et al.*, 2014). Evidence that this localization might be important for pavement cell morphogenesis came from a forward genetic screen for mutants with impaired PC shape. This screen isolated a loss-of-function mutant in FYPP1, the catalytic subunit of the PP2A holoenzyme (Fig. 2B), suggesting that the *fppy1* mutants might have altered PIN1 phosphorylation and localization (Li *et al.*, 2011). Indeed, PIN1 is delocalized to both necks and lobes of pavement cells in the *fypp1* mutant (Li *et al.*, 2011). In addition, overexpression of PINOID, which is known for its antagonistic action with PP2As on PIN1 phosphorylation, also induces a reduction of the number of lobes in PCs (Li *et al.*, 2011). However, by contrast to the *fypp1* loss-of-function mutant, PID gain-of-function delocalizes PIN1 from the lobe into the neck of pavements cells. Similar to the *pin1* mutant, auxin treatment does not rescue pavement cell shape phenotype in *fypp1* mutant and *35S::PID* lines. Together, these results suggest that the PP2As and PID antagonistic effect on PIN1 phosphorylation status controls PIN1 targeting at the tip of the pavement cell lobes and that this polar localization is essential for the proper establishment of pavement cell morphogenesis (Li *et al.*, 2011). TYPE-ONE PROTEIN PHOSPHATASE 4 (TOPP4), a

catalytic subunit of PROTEIN PHOSPHATASE1 (PP1), is also required for pavement cell morphogenesis, PIN1 polar targeting in lobes and PIN1 dephosphorylation (Guo *et al.*, 2015). Similar to FYPP1, TOPP4 directly interacts with PIN1 and antagonistically contributes to PIN1 dephosphorylation (Guo *et al.*, 2015). Altogether, these results suggest that the phosphorylation switch observed in meristematic and embryo tissues, is active in pavement cells to control PIN1 polar targeting in lobes and cell morphogenesis.

Auxin activates ROPs [Rho-like guanosine triphosphatases (GTPase)], in particular, ROP2/ROP4 and ROP6 (Craddock *et al.*, 2012; Yang and Lavagi, 2012). Interestingly, ROP2 and ROP6 have different subcellular localization, with ROP2 accumulating slightly more in the lobes than the neck regions and ROP6 slightly more in the neck than the lobes regions (Fu *et al.*, 2005). Gain-of-function mutants of ROP2 and ROP6 have squared PCs that almost entirely lack lobes and necks (Fu *et al.*, 2002; Lin *et al.*, 2013; Poraty-Gavra *et al.*, 2013). In addition, corresponding loss-of-function mutants also have a reduction in their number of lobes and necks, although these phenotypes are relatively weak (Fu *et al.*, 2005; Fu *et al.*, 2009; Lin *et al.*, 2013). *Rop2* and *rop6* mutants present similar phenotypes, but they affect the cytoskeleton in different ways (Fu *et al.*, 2005). On one hand, ROP2 and its effector ROP INTERACTIVE CRIB MOTIF-CONTAINING PROTEINS4 (RIC4) promote the assembly of filamentous actin in lobes, likely promoting targeted exocytosis and/or endocytosis events required for cellular outgrowth. On the other hand, ROP6 and its effector RIC1 promote the bundling of microtubules in the necks, which likely restrict growth notably through the thickening of cell wall by microtubule driven cellulose synthase activity (Fu *et al.*, 2005; Fu *et al.*, 2009; Lin *et al.*, 2013; Nagawa *et al.*, 2012; Sampathkumar *et al.*, 2014). The downstream ROP6 effector RIC1 controls microtubule dynamics by directly binding to and controlling the activity of KATANIN (KAT), a microtubule-severing enzyme (Lin *et al.*, 2013).

Both ROP2 and ROP6 are rapidly activated by auxin (i.e. auxin induces the production of GTP-loaded ROP2 and ROP6- the active form of these GTPases- that interact with their downstream effectors) (Wu *et al.*, 2011; Xu *et al.*, 2010). Auxin-mediated activation of ROP2 in the lobe region activates its downstream effector RIC4, which stabilizes actin in the lobe region and decreases PIN1 internalization (Nagawa *et al.*, 2012). Reduction of PIN1 endocytosis in turn might increases PIN1 localization at the tip of the pavement cell's lobes a localization that seems to be required for normal pavement cell shape establishment (Nagawa *et al.*, 2012). Polarized PIN1 exports auxin preferentially at the lobe region, which activates ROP2 in this region, providing a positive feedback mechanism and self-organizing system for the polar PIN1 distribution in PCs. However, the effects of ROP/RIC signaling on PIN localization are relatively minor, suggesting that other signaling pathways might redundantly control PIN1 localization in PCs (Nagawa et al., 2012).

The receptor like kinases (RLK) from the TRANSMEMBRANE RECEPTOR KINASEs (TMKs) family (TMK1 to TMK4) have been identified upstream of ROP2 and ROP6 activation by auxin (Xu *et al.*, 2014; Xu *et al.*, 2010). This mutant presents squared pavement cells and this phenotype is not rescued by auxin. TMKs, which are receptor-like kinases are required for the auxin-mediated activation of ROP GTPases (Xu *et al.*, 2014). Multiple mutants in TMK genes also present squared PCs and this phenotype is not rescued after

auxin treatment (Xu *et al.*, 2014). In addition, the ROP2 effector RIC4, which is localized at the plasma membrane in the tip of the lobe region and promotes the accumulation of actin microfilaments in wild-type plants, is mislocalized in the cytoplasm in the quadruple *tmk1234* mutants (Xu *et al.*, 2014; Xu *et al.*, 2010). The delocalization of RIC4 in this mutant suggests that ROP2 is inactive in the absence of these proteins. In addition, RIC1 association with cortical microtubules was impaired in *rop6-1* single mutant as well as in the *tmk1234* quadruple mutant (Xu *et al.*, 2014; Xu *et al.*, 2010). Altogether, these results suggest that TMKs might be important for auxin perception upstream of ROP activation. Activated TMKs likely control downstream substrates by phosphorylation. These downstream effectors are currently unknown, but it could be ROP GTPases themselves. However, it is more likely that TMKs might activate ROP activators, such as ROP-GUANINE EXCHANGE FACTOR (ROP-GEF) (Miyawaki and Yang, 2014). Indeed, ROP-GEF have been shown to act downstream of other RLKs in plants, such as for example the FERONIA receptor (Duan *et al.*, 2010; Miyawaki and Yang, 2014). Consequently, how exactly are ROPs activated by auxin and what is the role of TMK receptor kinases in this activation will require further investigations.

RLKs in the regulation of root gravitropism

Auxin and PIN dynamics during gravitropism

Root reorientation upon gravistimulation requires dynamic changes of auxin distribution at the tip of the root, which involve auxin transporters (Fig. 5). Using the synthetic auxin promoter *DR5::GFP* and *DR5rev::GFP*, it was detected that auxin accumulates in the lower part of the root (Ottenschlager *et al.*, 2003; Paciorek *et al.*, 2005). This auxin accumulation likely triggers a rapid growth arrest that is required for root bending. Using live imaging of the DII-VENUS auxin input sensor, the timing of this asymmetric auxin distribution was studied together with the dynamics of root bending (Band *et al.*, 2012). In this study, Band *et al.,* found that auxin accumulates at the lower side of the root within 30 minutes of a 90° gravistimulation. However, this auxin asymmetry is disappearing when the root tip is reaching a 40° angle to the horizontal. This phenomenon presumably corresponds to a tipping-point mechanism that allows to re-establish symmetric vertical growth (Band *et al.*, 2012). Using *DR5rev::3xVENUS-N7* (auxin output reporter), Baster et al., (2013) found an accumulation of the *DR5* signal in the lower part of the root starting from 2 hours of gravistimulation up to 8h and then a decrease of the signal at 12h and 24h (Fig. 5). In parallel, they monitored a decrease of the auxin response at the upper side of the root notably at 4h and 8h post gravistimulation, which then came back up at 12h and 24h (Baster *et al.*, 2013). Importantly, at 24h after gravistimulation, auxin maxima on both side of the root have a similar intensity and the root is growing straight down. This suggest that not only auxin accumulates at the lower side of the root but also is depleted at the upper side of the root, thereby increasing the auxin asymmetry and resulting elongation growth between the two sides (Fig. 5).

Both auxin influx and efflux carriers are involved during the gravitropic response. The *aux1* mutant is almost completely agravitropic, which highlight the importance of this auxin influx carrier in the root gravitropic response (Bennett *et al.*, 1996). AUX1 is loading auxin

that accumulates at the root tip into the lateral root cap, where it is then redistributed to the epidermis (Marchant *et al.*, 1999; Swarup *et al.*, 2005). Although *aux1* is agravitropic, there is no evidence that AUX1 directly regulate the gravitropic responses. Rather, AUX1 is the driving force for redirection of auxin from the columella and, thus provides the source of auxin utilized in gravitropism. However, the first auxin transporter to dynamically change its localization following gravistimulation is PIN3 (Friml *et al.*, 2002b). PIN3 is expressed in the root columella cells (Fig. 1), and it is localized in a non-polar fashion at the plasma membrane (Fig. 5A). Upon gravistimulation, PIN3 is rapidly (within minutes) relocalized by trancytosis (from one plasma membrane pole to another via endocytosis and recycling) at the lateral side facing the direction of the new gravity vector (Friml *et al.*, 2002b; Kleine-Vehn *et al.*, 2010) (Fig. 5B). This change of PIN3 localization triggers the redirection of auxin flow that initiate an asymmetric accumulation of auxin and the differential growth towards the new gravity vector. The auxin transporter PIN7 partially colocalizes with PIN3 in columella cells (Blilou *et al.*, 2005) (Fig. 1). PIN7 also relocalizes according to the new gravity vector (Kleine-Vehn *et al.*, 2010). Accordingly, PIN3 and PIN7 might act redundantly in redirecting the auxin fluxes during the gravitropic response (Kleine-Vehn *et al.*, 2010). However, the agravitropic phenotype of *pin3pin7* is relatively weak suggesting that other PINs might be involved as well or that some compensation mechanism exists. Gravity is mainly perceived in the columella by sedimenting statoliths, however the signal transduction leading to PIN3/PIN7 transcytosis and at what point during the gravitropic bending PIN3 localization is reset to apolar remains unknown (Fig. 5). The initial auxin asymmetry triggered by PIN3/PIN7 relocalization will then be amplified by differential trafficking of the PIN2 auxin carrier on the lower and upper side of the root depending on their respective auxin concentration (Fig. 5C). Two hours following gravistimulation and correlating with the change in auxin distribution, PIN2 plasma membrane localization is enhanced at the lower part of the root reinforcing auxin accumulation in this area (Baster *et al.*, 2013; Paciorek *et al.*, 2005) (Fig. 5). However, Peer and collaborators and Band and collaborators detected that auxin redirection occurs within 30 minutes (Band et al., 2012; Peer et al., 2014), therefore it is likely that all of the changes in PIN localization function in combination to amplify polar movements that follow cell to cell gradients. The accumulation of PIN2 at the lower part of the root is triggered by the initial increase in auxin, which inhibits endocytosis (Paciorek *et al.*, 2005) (Fig. 5 and 6). Auxin inhibition of endocytosis is transient (Robert *et al.*, 2010). As such, PIN2 plasma membrane accumulation is also transient and goes back to its original state 4h following gravistimulation (Baster *et al.*, 2013) (Fig. 5D). Concomitantly, the level of PIN2 in the upper part of the root decreases (starting at 2h and culminating at 4h), before going back up to the original level 12h post gravistimulation (Abas *et al.*, 2006; Baster *et al.*, 2013; Jaillais *et al.*, 2006) (Fig. 5D). On the upper part of the root, low auxin triggers PIN2 routing toward the vacuole, in a process that depends on PI3Kinase activity (Jaillais *et al.*, 2006) (Fig. 3). PIN2 routing to the vacuole is regulated at the post-translational level by ubiquitination rather than phosphorylation but also requires genomic auxin signaling from the TIR1/AFBs auxin-signaling pathway (Abas *et al.*, 2006; Baster *et al.*, 2013; Korbei *et al.*, 2013; Robert *et al.*, 2010). The molecular steps between the perception of an auxin minimum by this TIR1/AFBs pathway and ubiquitination of PIN2 are so far unknown (Figure 6).

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‘Non-genomic’ auxin signaling and the inhibition of PIN2 endocytosis on the gravi-stimulated side - is TMK or another RLK upstream of ROP6?

Short-term auxin accumulation (or treatment) inhibits endocytosis, which is important to accumulate PIN2 at the lower side of the root and thereby amplify the asymmetric auxin accumulation between the two root sides (Paciorek *et al.*, 2005; Robert *et al.*, 2010). This pathway is independent of the canonical auxin ‘genomic’ perception that involves TIR1/AFB receptors but relies on a ‘non-genomic’ pathway that does not require de novo transcription (Robert *et al.*, 2010). Similar to the ‘non-genomic’ pathway described above for pavement cell shape establishment, auxin inhibition of endocytosis involves ROP small GTPases (Chen *et al.*, 2012; Lin *et al.*, 2012). Auxin inhibits PIN internalization following BFA treatment, an inhibitor of protein recycling (Paciorek *et al.*, 2005) (Fig. 5 and 6). Interestingly, loss-of-function mutants in *rop6* or its downstream effector *ric1* are insensitive to auxin inhibition of endocytosis and thereby show a general enhanced endocytosis (Chen *et al.*, 2012; Lin *et al.*, 2012). By contrast, *ROP6* and *RIC1* gain-of-function mutants have increased sensitivity to auxin inhibition of endocytosis and show lower endocytic rate. Consistently, *rop6* and *ric1* loss-of-function mutants respond more slowly to gravity, while their respective gain-of-function mutants had the opposite phenotype, their root bending faster than the wild type (Chen *et al.*, 2012; Lin *et al.*, 2012).

In addition, SPIKE1 (SPK1) was also shown to act directly upstream of ROP6 (Lin *et al.*, 2012). SPK1 is a member of the dock homology region 2 (DHR2)-type Dock family of Rho guanine nucleotide exchange factors (ROP-GEFs) and acts as a GEF for ROPs *in vitro* (Basu *et al.*, 2008). Similar to *rop6* mutants, *spk1* mutants present a reduced lobe formation in pavement cells and a slow gravitropic response (Lin *et al.*, 2012). In addition, *spk1* mutants present an increase in PIN2 endocytosis that was not recovered after auxin treatment (Lin *et al.*, 2012). Importantly, pull-down assays, *in vivo* fluorescence resonance energy transfer (FRET) analysis and Co-IP show that SPK1 directly interacts with the GDP bound form of ROP6, and is required for ROP6 activation. While FRET analyses suggest that SPK1 might localize at the plasma membrane (PM) (Lin *et al.*, 2012), an earlier report showed that SPK1 localizes in the endoplasmic reticulum (ER) (Zhang *et al.*, 2010a). These results might point toward a role for ER-PM contact sites in ROP signaling, that should be explored further. Altogether, ROPs act in the ‘non-genomic’ auxin-signaling pathway to regulate PIN endocytosis in both roots and leaf pavement cells. However, they use different signaling components in these two cell types, ROP2-RIC4 in leaves and ROP6-RIC1 in roots (Chen *et al.*, 2012; Lin *et al.*, 2012; Nagawa *et al.*, 2012; Robert *et al.*, 2010; Xu *et al.*, 2014; Xu *et al.*, 2010).

It is currently unknown how auxin is perceived upstream of the SPK1-ROP6 module. However, the SPK1 ROP-GEF might be regulated by a receptor kinase upon auxin treatment. Indeed, FERONIA (FER) is a plasma membrane receptor kinase that regulates cell elongation and hormone crosstalks (Cheung and Wu, 2011; Kanaoka and Torii, 2010). This receptor is expressed in all the vegetative tissues and was discovered as a regulator of the communication between the pollen tube and the synergid cell (Escobar-Restrepo *et al.*, 2007; Huck *et al.*, 2003; Ngo *et al.*, 2014; Rotman *et al.*, 2008). However, *fer* mutant has a plethora of phenotypes including short root hairs that are often ruptured, bigger seeds,

altered response to pathogens and abnormal response to mechanotransduction (Deslauriers and Larsen, 2010; Duan *et al.*, 2010; Kessler *et al.*, 2010; Mao *et al.*, 2015; Shih *et al.*, 2014; Yu *et al.*, 2014; Yu *et al.*, 2012). Using yeast two-hybrid, FER was shown to interact with 3 different ROP-GEF in Arabidopsis, GEF1, GEF4 and GEF10 (Duan *et al.*, 2010; Huang *et al.*, 2013). By analogy to the FER-GEF system, we can therefore hypothesize that a receptor kinase is likely to act upstream of the SPK1-ROP6 module to control 'non-genomic' auxin signaling (Miyawaki and Yang, 2014). One obvious candidate would by the TMK receptor kinases, which act upstream of ROP6 for 'non-genomic' auxin signaling during pavement cell shape establishment (Xu *et al.*, 2014). TMK mutants have root phenotypes, but they need to be further examined, in particular for possible defects in endocytosis regulation (Dai *et al.*, 2013). The question of how exactly these potential receptors are activated by auxin, will also require further studies.

GOLVEN peptides: an example of signaling ligands involved *in the regulation of root gravitropism and PIN2 trafficking*

Secreted peptides often act as signaling ligands that binds to RLKs and/or to a receptor-like proteins at the plasma membrane (Dievart and Clark, 2004). In Arabidopsis, GOLVENs belong to a small signaling peptide family encoded by 11 genes (Fernandez *et al.*, 2013a; Fernandez *et al.*, 2013b). The overexpression of all GLV peptides, with the exception of GLV4 and GLV8 causes root gravitropism defects (Fernandez *et al.*, 2013a; Whitford *et al.*, 2012). Furthermore, exogenous treatments with synthetic GLV peptides and GLV3 overexpression inhibit the formation of the lateral auxin gradient observed in the primary root after gravistimulation (Fernandez *et al.*, 2013a). Exogenous treatment with synthetic GLV3 peptide stabilizes PIN2 both at the plasma membrane and endosomes, while PIN2 is degraded in *glv3* loss-of-function mutant (Whitford *et al.*, 2012) (Fig. 6). As such, GLV3 impacts differential PIN2 accumulation between the lower and upper part of the root following gravistimulation (see Fig. 5), which likely account for the agravitropic phenotype observed upon GLV3 treatment or in *GLV3*-overexpression lines. In addition, PIN2 internalization is triggered by exogenous treatment with other GLV peptides, including GLV3, 5, 6 and 11, but not with GLV1 or GLV4 (Fernandez *et al.*, 2013a; Whitford *et al.*, 2012). The molecular components that perceive GLV peptides at the plasma membrane are likely RLKs but they have not been identified yet.

Phosphorylation and the regulation of polar auxin transport during phototropism

Phototropism, etymologically derived from the Greek words *photo* (=light) and *tropos* (to turn), can be defined as the tendency of growing plant organs to move or curve under the influence of light. Growth towards a light source is called positive phototropism, while growth away from the light source is called negative phototropism (Fig. 7). Plant stems generally exhibit positive phototropism, while roots exhibit negative phototropism. The adaptation of plant organogenesis and architecture to changing light conditions by means of tropic growth ensures the maintenance of plant fitness.

The phototropic movement of plants attracted the interest of philosophers and scientists during the past centuries, giving rise to the basis of the actual knowledge and current models of phototropism (for a historical perspective of phototropic movement see (Whippo and Hangarter, 2006, 2009)). Notably, in the manuscript *The Power of Movement in Plants* (1880), Charles Darwin and his botanist son Francis Darwin established that the site of photoperception at the shoot tip and the site of curvature are physically separable in both monocots and dicots. This concept has been proven true to date for coleoptiles of monocotyledonous plants (Holland *et al.*, 2009; Matsuda *et al.*, 2011; Whippo and Hangarter, 2009). By contrast, in the dicot Arabidopsis, it has been reported that light perception and bending occurs at the same region of the hypocotyl and a role of the cotyledons in the phototropic response has been excluded (Christie *et al.*, 2011; Preuten *et al.*, 2013). Moreover, Darwin additionally established that a substance produced in the tip is transmitted to lower regions of the plant and is responsible of the induction of the phototropic curvature. This transmissible substance was posteriorly identified as the phytohormone auxin. The phototropic curvature follows the Cholodny–Went theory, which states that in response to tropic stimulus, the asymmetric auxin distribution causes differential growth of the two sides of the plant organ and, as a consequence, organ bending. In the case of shoot phototropism, unilateral light produces auxin accumulation at the shaded side of the hypocotyls, where it stimulates cell expansion (Fankhauser and Christie, 2015; Liscum *et al.*, 2014; Sakai and Haga, 2012) (Fig. 7). Ultraviolet A (UV-A) and blue light (BL) (320–500 nm) are the most effective wavelengths to induce the phototropic bending. Analysis of Arabidopsis seedlings with altered phototropic responses lead to the identification of the blue-light (BL) photoreceptors responsible for phototropism, named phototropin 1 (phot1) and phototropin 2 (phot2) (Christie *et al.*, 1998; Christie *et al.*, 1999; Huala *et al.*, 1997; Kagawa *et al.*, 2001; Kinoshita *et al.*, 2001; Liscum and Briggs, 1995; Sakai *et al.*, 2001). Phot1 is involved in the phototropic response at both low and high BL intensities, while phot2 is only necessary for the phototropic response at high BL intensities. *Phot1* (also called *nph1*) mutants show non-phototropic hypocotyl at BL intensities <10 $\mu mol \cdot m^{-2} \cdot s^{-1}$, whereas at higher fluence rates (10 and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) the hypocotyls show a clear positive phototropic response, mediated by phot2 activity (Liscum *et al.*, 2014; Sakai *et al.*, 2001). By contrast, the *phot2* (or *npl1*) single mutant shows normal phototropic responses at all BL intensities, due to phot1 activity. Only the *phot1phot2* (or *nph1npl1*) double mutants show strong non-phototropic phenotype at both low and high BL (Sakai *et al.*, 2001).

Analysis of the protein sequence of phototropins lead to their classification into the AGC4 group of AGC kinases and constitute the only members of this clade (Galvan-Ampudia and Offringa, 2007). Structurally, phototropins contain two functional domains: an N-terminal photosensor domain and a C-terminal serine/threonine kinase domain. The N-terminal photosensor domain contains two LOV (light, oxygen, or voltage) domains, LOV1 and LOV2, which bind flavin mononucleotid (FMN) as a cofactor, and function as the blue light-sensing moiety of the protein (Christie *et al.*, 1998; Christie *et al.*, 1999). In the dark, the LOV2 domain acts as a repressor of the C-terminal kinase domain and BL irradiation induces a conformational change that allows the activation of the phot kinase activity, triggering phot1 autophosphorylation and activation of a signaling cascade that ultimately

leads to the establishment of an asymmetric auxin distribution between the illuminated and shaded sides of the hypocotyl (Fankhauser and Christie, 2015; Harper *et al.*, 2003).

Among the downstream signaling components of the phot1-dependent phototropic response, NPH3 (for Non-phototropic hypocotyl 3) is required for the formation of the lateral auxin gradient upon directional light treatment (Haga *et al.*, 2005). *nph3* loss-of-function Arabidopsis mutants show defects in hypocotyl and root phototropism under a broad range of light intensities (Inada *et al.*, 2004; Liscum and Briggs, 1995; Sakai *et al.*, 2000). NPH3 is a BTB-containing protein belonging to the plant specific NPH3/RPT2 (or NRL) protein family (Motchoulski and Liscum, 1999; Pedmale *et al.*, 2010; Pedmale and Liscum, 2007). Several members of this family act redundantly in auxin-mediated organogenesis and root gravitropism (which involves asymmetric auxin distribution), by modulating the subcellular localization of the polarly localized auxin-exporters PIN1 and PIN2 (Furutani *et al.*, 2007; Furutani *et al.*, 2011; Wan *et al.*, 2012).

NPH3 and phot1 interact at the level of the plasma membrane where they both localize in dark conditions (Lariguet *et al.*, 2006; Motchoulski and Liscum, 1999). NPH3 is phosphorylated in the dark but not as a direct substrate of phot1. Instead, NPH3 dephosphorylation occurs within minutes upon BL exposure and depends on phot1 activity (Pedmale and Liscum, 2007). NPH3 additionally interacts with Cullin3 (CUL3), forming a Cullin3 Ring E3 ubiquitin ligase that uses NPH3 as a substrate adaptor ($CRL3^{NPH3}$) (Roberts *et al.*, 2011). The $CRL3^{NPH3}$ complex ubiquitinates phot1 upon irradiation with either low or high BL. Low BL induces mono- and multiubiquitination of phot1, and high BL induces mono-/multi- and polyubiquitination (Roberts *et al.*, 2011). The biological significance of phot1 ubiquitination is poorly understood. It has been suggested that mono-/multiubiquitination induces phot1 internalization from the plasma membrane and might promote intracellular phot1 signaling by interaction/phosphorylation of cytoplasmic proteins (Liscum *et al.*, 2014). On the other hand, polyubiquitination has been proposed to target phot1 for degradation via the 26S proteasome as a mechanism of receptor desensitization at high BL intensities (Roberts *et al.*, 2011). However, a recent study using myristoylated and farnesylated (two lipid anchors that target and attach soluble proteins to the plasma membrane) phot1 proteins elegantly showed that the plasma membrane-bound fraction of phot1 represents the active form of the receptor and that internalization is not required for signaling (Preuten *et al.*, 2015). These results would fit in a model in which phot1 modulates asymmetric auxin fluxes within the hypocotyl by phosphorylation of PIN proteins, but efforts to prove this direct interaction have failed (Ding *et al.*, 2011). Nevertheless, phot1 binds in vivo and phosphorylates in vitro the auxin exporter ABCB19, a member of the MDR/PGP family of auxin transporters (Christie *et al.*, 2011). ABCB19 phosphorylation by phot1 produces its inactivation (Christie *et al.*, 2011) (Fig. 7). ABCB19 is a negative regulator of the phototropic response, as demonstrated by the enhanced hypocotyl bending of the *abcb19* mutants in response to unilateral light (Noh *et al.*, 2003). In addition to its intrinsic auxin transport activity, ABCB19 stabilizes PIN1 protein at the basal side of hypocotyl cells and promotes basipetal auxin flux (Noh *et al.*, 2003). Disruption of ABCB19 activity by phot1 phosphorylation (or *abcb19* mutation) reduces the vertical auxin flux (rootward), and produces a local accumulation of auxin at the bending zone of the

hypocotyl. This available auxin might further be redistributed towards the shaded side of the hypocotyl by PIN-mediated lateral auxin transport (Christie *et al.*, 2011) (Fig. 7).

The involvement of PIN proteins in the formation of lateral auxin gradients in response to unilateral light stimulus is still a matter of debate. Nonetheless, some PIN proteins, such as PIN3, have been proposed to function as modulators of lateral auxin transport in phototropism (Ding *et al.*, 2011). PIN3 is mainly expressed in the endodermis of the hypocotyl (Friml *et al.*, 2002b), but also found in the vasculature, cortex and epidermis. In etiolated Arabidopsis seedlings, PIN3 is apolarly localized at both the outer and inner lateral membranes of the endodermis cells (Fig. 7). Upon BL irradiation, PIN3 is depleted from the outer membrane and becomes more abundant at the inner side (facing the vasculature) of these cells. PIN3 polarization occurs only at the illuminated side of the hypocotyl, producing an overall polarization of PIN3 at the shaded side of the organ. Moreover, PIN3 polarization requires GNOM-dependent transcytosis (Ding *et al.*, 2011) (Fig. 7). PIN3 re-localization might drive auxin back to the vasculature, and reduces the auxin content at the illuminated side of the hypocotyl. PIN3 polarization is abolished in *phot1* mutants suggesting a role of phot1 in the modulation of lateral auxin flux through PIN3 phosphorylation. However, PIN3 is not a direct substrate of phot1. By contrast, PIN3 was found to be a direct substrate of PID, and PID activity is necessary for PIN3 lateralization (Ding *et al.*, 2011). In both, *35S::PID* plants and the triple mutant plants *pidwag1wag2*, PIN3 failed to re-localize upon unilateral light stimulus. In addition, *PID* expression is reduced upon light treatment. All these results lead the authors to propose a model in which, in the cells from the shaded side of the hypocotyl, PID activity is high and phosphorylates PIN3. The phosphorylated PIN3 is targeted to both sites of the endodermic cells (inner and outer lateral membranes). On the other hand, in the illuminated side of the hypocotyl, phot1 activation reduces PID activity, and non-phosphorylated PIN3 enters the polar trafficking pathway, with preferential localization at the inner cell side (Ding *et al.*, 2011) (Fig. 7). This model proposes an indirect role of phot1 in the modulation of PIN3 lateralization, by regulating the level of expression and activity of PID through so far uncharacterized molecular mechanisms. A similar regulatory mechanism has been shown to involve PID (as well as PID2, WAG1 and WAG2) in the red-light enhancement of the phototropic curvature (Haga *et al.*, 2014; Haga and Sakai, 2012, 2015). In this case, red-light treatment induces phytochrome-dependent down regulation of *PID* transcription and PID protein abundance, which increases the fraction of non-phosphorylated PINs (for instance, PIN3 and PIN7) delivered to the plasma membrane through the GNOM-dependent pathway (Haga *et al.*, 2014).

Despite these evidences for a prominent role of PIN3 in phototropism, two main results suggest the existence of additional auxin transporters involved in lateral auxin fluxes: i) *pin3* mutant plants only show a slightly reduced phototropic response (and the same is true for the *35S::PID* and *pidwag1wag2* mutant plants) and, ii) lateral auxin transport is not completely impaired in the *pin3* mutant background. However the *pin3pin4pin7* triple mutant have strong phototropic defect, suggesting that they redundantly mediate hypocotyl bending (Ding *et al.*, 2011; Willige *et al.*, 2013). On the other hand, using dark acclimated de-etiolated seedlings, Christie al., (2011) concluded that PIN3 is not responsible for lateral auxin redistribution, as *pin3* mutants have the same pattern of auxin distribution as wild-type plants, as analyzed by the *DR5rev::GFP* reporter. Instead, these authors reported a reduction

of PIN3 protein below the region of the hypocotyl curvature upon BL treatment, thus suggesting that PIN3 could promote auxin accumulation at the bending zone and indirectly contribute to lateral auxin distribution (Christie *et al.*, 2011). Moreover, PIN3 lateralization in etiolated seedlings was detected after 3-4h of unilateral light treatment (Ding *et al.*, 2011), while auxin gradient formation starts within less than one hour of illumination (for a timeline on early phototropic events we refer to the excellent recent review by Fankhauser and Christie (2015)). These observations might exclude PIN3 from the initial asymmetric auxin distribution and argue in favor of a role of PIN4, PIN7 or an unknown PIN-independent lateral auxin mechanism. However, it is also possible that either i) PIN3/PIN4/PIN7 localization happens within 1 hour but can be monitored by confocal microscopy only after 3-4h and/or ii) apparent polar localization is only part of the story and that auxin transport activity itself can also be polarized.

In parallel to the discovery of the strong non-phototropic phenotype of the *pin3pin4pin7* triple mutant, the phenotypic characterization of the quadruple mutant *d6pk0123* lead to the involvement of D6PK proteins in hypocotyl phototropism. In addition to the impaired hypocotyl phototropic response, the *d6pk0123* mutant plants also show impaired gravitropic response in both hypocotyls and roots and, developmental defects such as reduced number of lateral roots (Willige *et al.*, 2013; Zourelidou *et al.*, 2009). All these phenotypes resembled those of auxin transport mutants. The hypocotyls of *d6pk012* plants failed to establish the asymmetric auxin gradient necessary for hypocotyl bending upon unilateral light stimulation, although PIN3 lateralization occurred as in wild-type plants. However, *d6pk0123* plants showed strong inhibition of the basipetal auxin transport, to the same extend as *pin3pin4pin7* mutants. Together these results suggested that the non-phototropic hypocotyl phenotype of these plants was primary associated to defects on the main vertical auxin fluxes and suggested a genetic link between D6PK and PIN proteins (Willige *et al.*, 2013). By contrast to PID/WAG-dependent PIN phosphorylation, D6PK-dependent PIN phosphorylation do not induce changes in the polar localization of PIN proteins (as observed by the proper lateralization of PIN3 in hypocotyls of *d6pk012* mutants upon unilateral light stimulus), but instead it stimulates the auxin efflux activity of these transporters and ensures proper basipetal auxin transport in planta (Barbosa *et al.*, 2014; Willige *et al.*, 2013). Thus, the lack of phosphorylation of PIN3 in *d6pk* mutants results in a reduced auxin transport capacity of this protein, finally impairing the phototropic response of these plants. Hence, stimulation of PIN efflux activity by D6PKs is required for proper phototropism (Willige *et al.*, 2013).

Conclusion

Throughout this review we highlighted the diverse mechanisms by which phosphorylation/dephosphorylation has been involved in ‘non-genomic’ regulation of polar auxin transport. Mainly, phosphorylation controls auxin carriers either by directly acting on their transport activity or their intracellular trafficking. The latter impacts the polarity, degradation and plasma membrane accumulation of PIN proteins. However, it is clear that much still need to be addressed to understand each pathway described in this review. Among the outstanding questions that remain unanswered are: How the differential phosphorylations of PIN proteins act either on their polarity or activity? How auxin and GOLVEN peptides are perceived to

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regulate PIN intracellular trafficking? What are the molecular steps, independent of transcription, between cytokinin perception and PIN polarity regulation? What are the proteins regulated by phosphoinositides that control PIN trafficking and polarity? How other environmental (e.g. halotropism (Galvan-Ampudia *et al.*, 2013)) and developmental (e.g. GA signaling (Lofke *et al.*, 2013; Willige *et al.*, 2011)) regulatory cues coalesce in the regulation of PIN trafficking and activity? Addressing these questions will bring us closer to understanding how polar auxin transport is both a robust system in some contexts but highly dynamic and versatile in others. Integrating these different regulatory modules into one unifying model will also be a challenge for the coming years that will certainly require multidisciplinary approaches ranging from structural biology to quantitative biochemistry and cell imaging as well as mathematical modeling.

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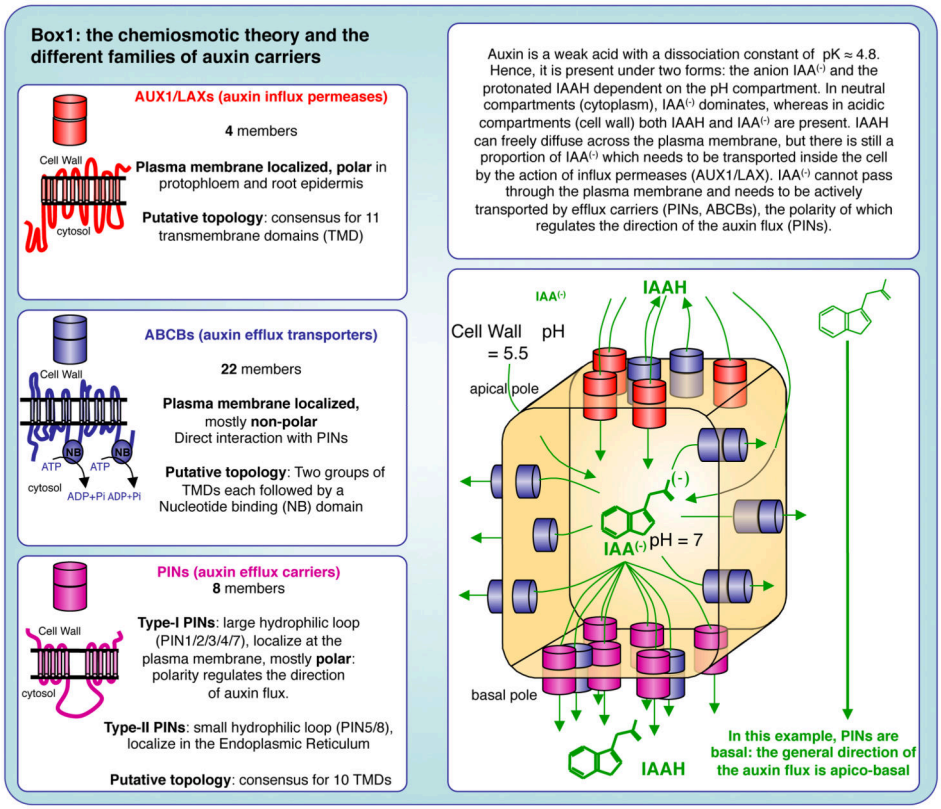

Box1.

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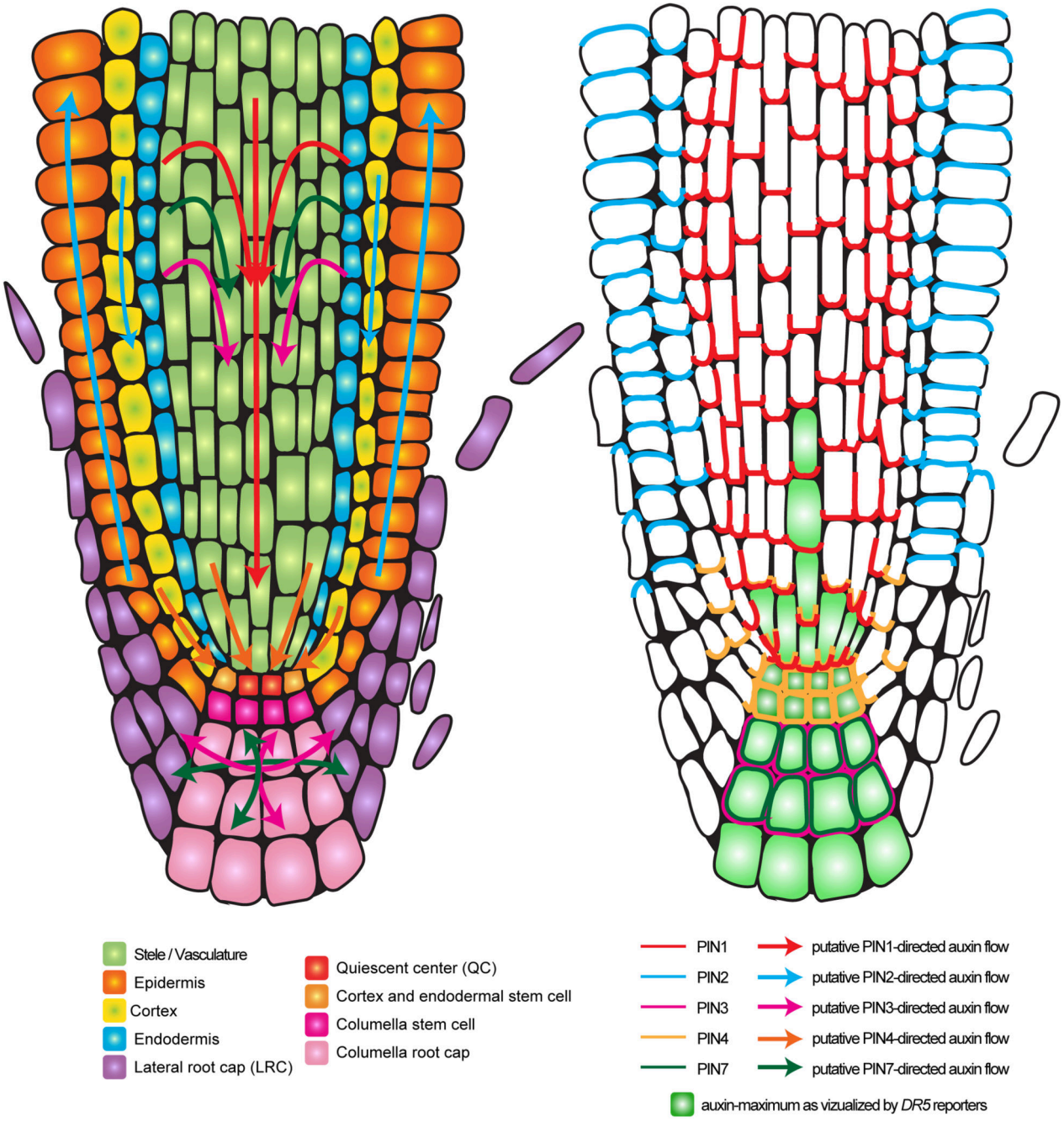

Figure 1. Polar auxin transport and PIN localization in the primary root tip.

Schematic representation of a longitudinal root section showing the different root tissues (left). The arrows represent putative auxin fluxes (left) as deduced from PIN protein localization (right). For clarity, PIN3 and PIN7 proteins have been omitted from the stele since their localization is redundant with that of PIN1.

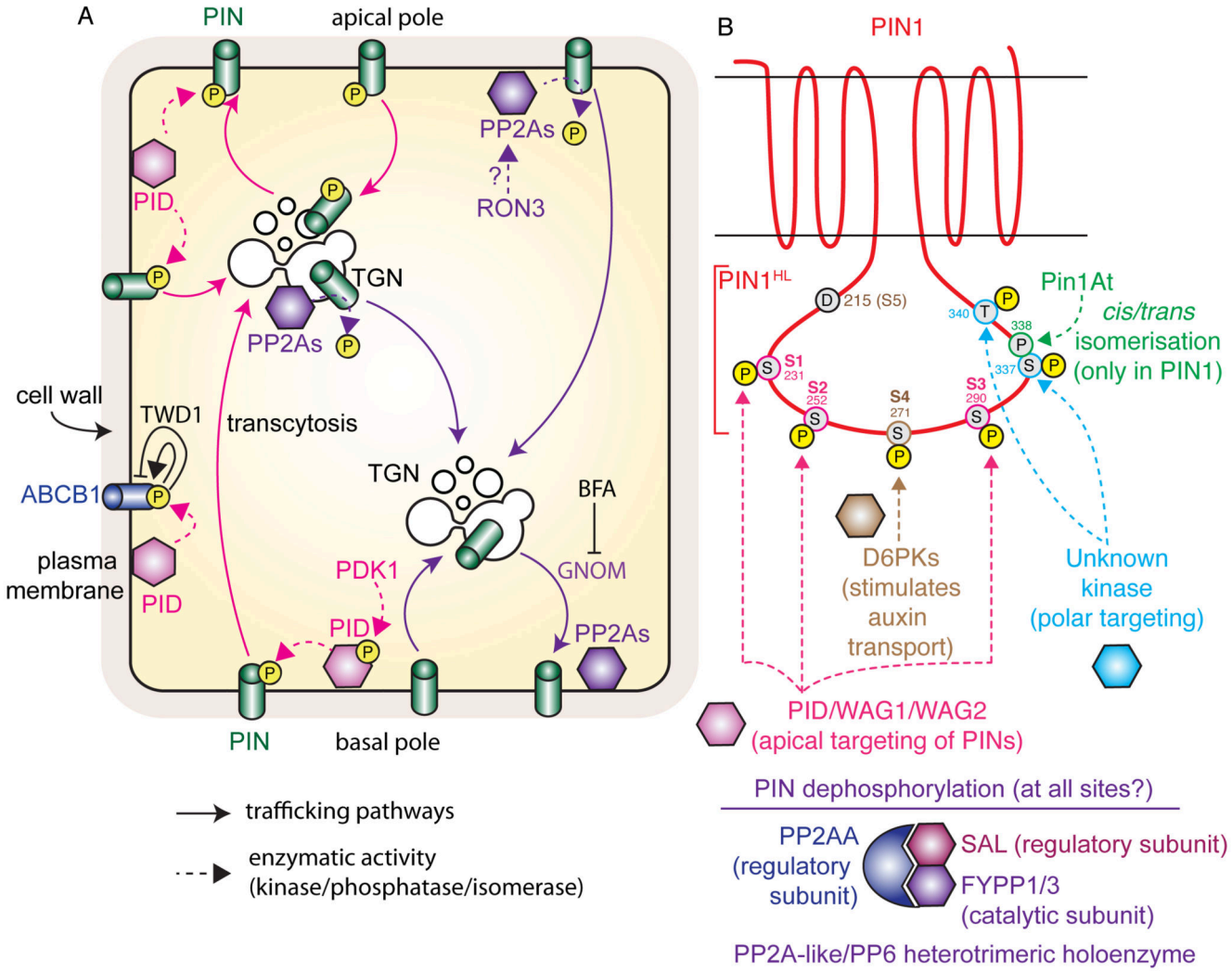

Figure 2. Model for PID and PP2As antagonistic activity on PIN phosphorylation, and the dual action of ABCB1 phosphorylation by PID.

A) Non-phosphorylated PINs undergo continuous endocytic recycling in a GNOM-dependent manner, which mediates their basal localization. The PID kinase phosphorylates PINs at the plasma membrane, which prohibits them from recycling via the GNOM-dependent route. Phosphorylated PINs instead undergo transcytosis to the apical pole of the cell. PINs are dephosphorylated by PP2As phosphatases, presumably at the plasma membrane and endosomes. PID also phosphorylates ABCB1, which promote ABCB1-mediated auxin export in the absence of TWD1, but inhibits it in its presence. B) Schematic representation of PIN1 topology and position of PIN1 phosphorylation site within PIN1 hydrophilic loop ($PIN1^{HL}$). The action of the kinases/phosphatases/isomerase on specific residues is highlighted. Note that only D6PK main phosphorylation sites (S4 and S5) are highlighted, but that S1, S2 and S3 are also phosphorylated by this kinase, albeit less potently. Similarly, PID/WAG1/WAG2 are able to phosphorylate S4 and S5 but preferentially act on S1, S2 and S3. Dashed arrows represent direct phosphorylation, dephosphorylation or isomerisation events; arrows with chevron-shaped arrowhead represent

trafficking pathways and blunt-ended lines represent inhibition. Yellow-filled circles with the letter ‘P’ represent phosphorylation, while grey-filled circles represent individual amino acid (each residue is numbered according to its position within the PIN1 protein).

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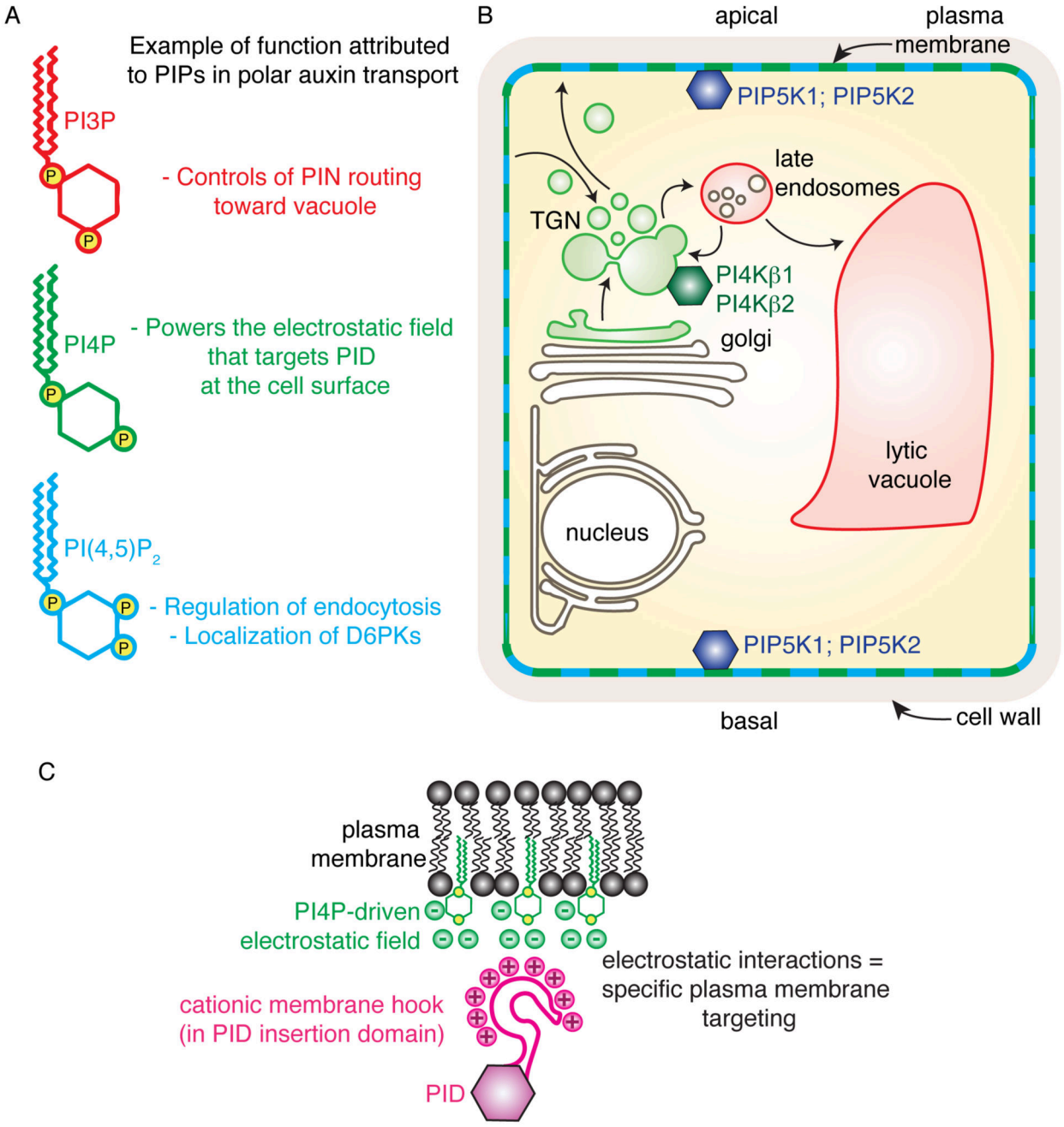

Figure 3. Phosphoinositide localization in Arabidopsis root epidermis and function in regulating polar auxin transport.

A) Schematic representation of PI3P, PI4P and PI(4,5)P_2 and example of their respective function in polar auxin transport. Note that they are likely involved in many more pathways and this represents only the studied examples. B) PIP subcellular localization in Arabidopsis root epidermis. The localization of the respective PIP kinases is indicated when known. Arrows with chevron-shaped arrowhead represent trafficking pathways. C) Model for the plasma membrane localization of PID by PI4P-driven electrostatics.

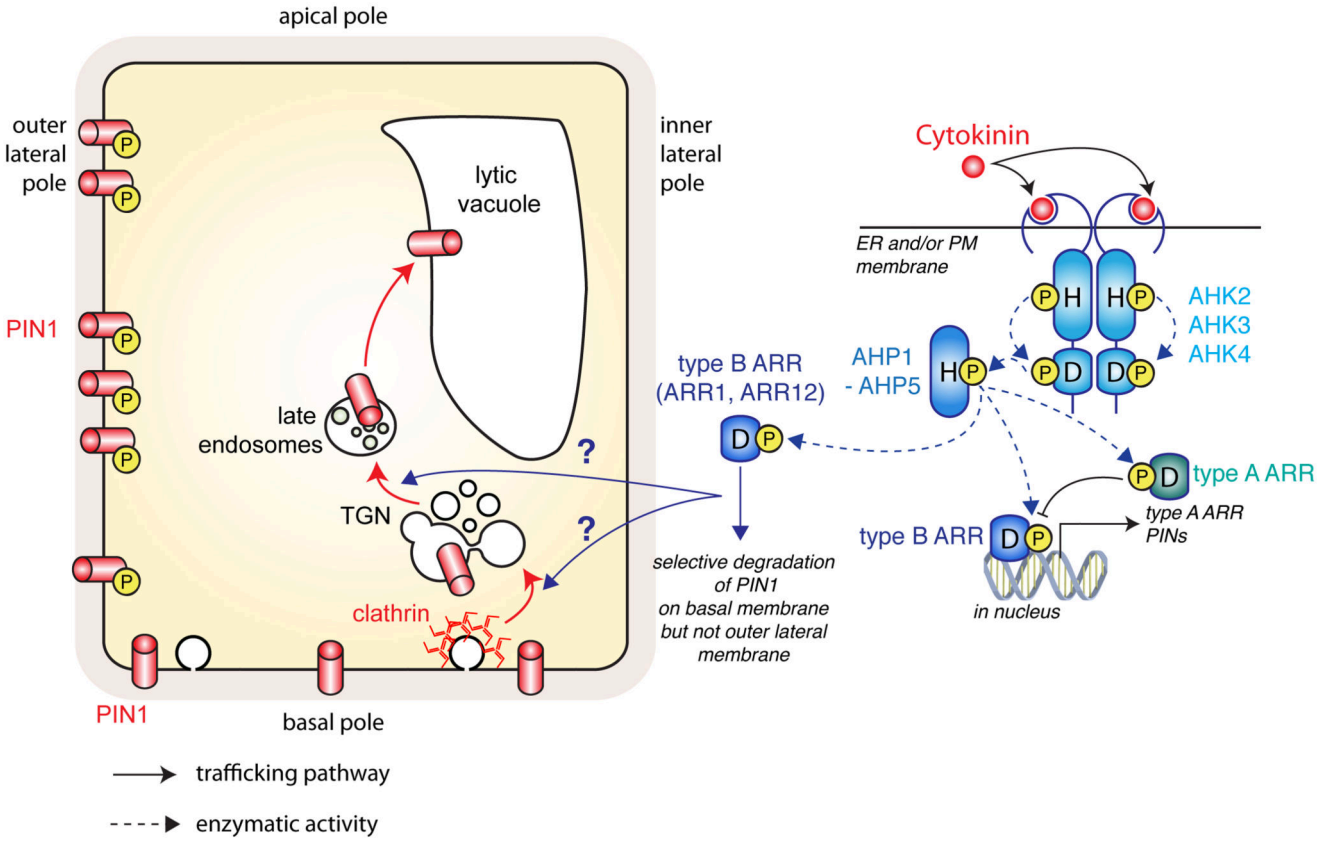

Figure 4. Cytokinin phosphorelay and its role in PIN1 trafficking.

Schematic representation of the ‘classical’ cytokinin phosphorelay pathway from receptor to gene activation (right) and its ‘non-canonical’ role, independent of transcription but relying on AHKs and ARRs, in PIN1 trafficking during lateral root initiation (left). Dashed arrows represent direct phosphorylation events; arrows with chevron-shaped arrowhead represent trafficking pathways and arrows with triangle-shaped arrowhead represent signalling events; blunt-ended lines represent inhibition.

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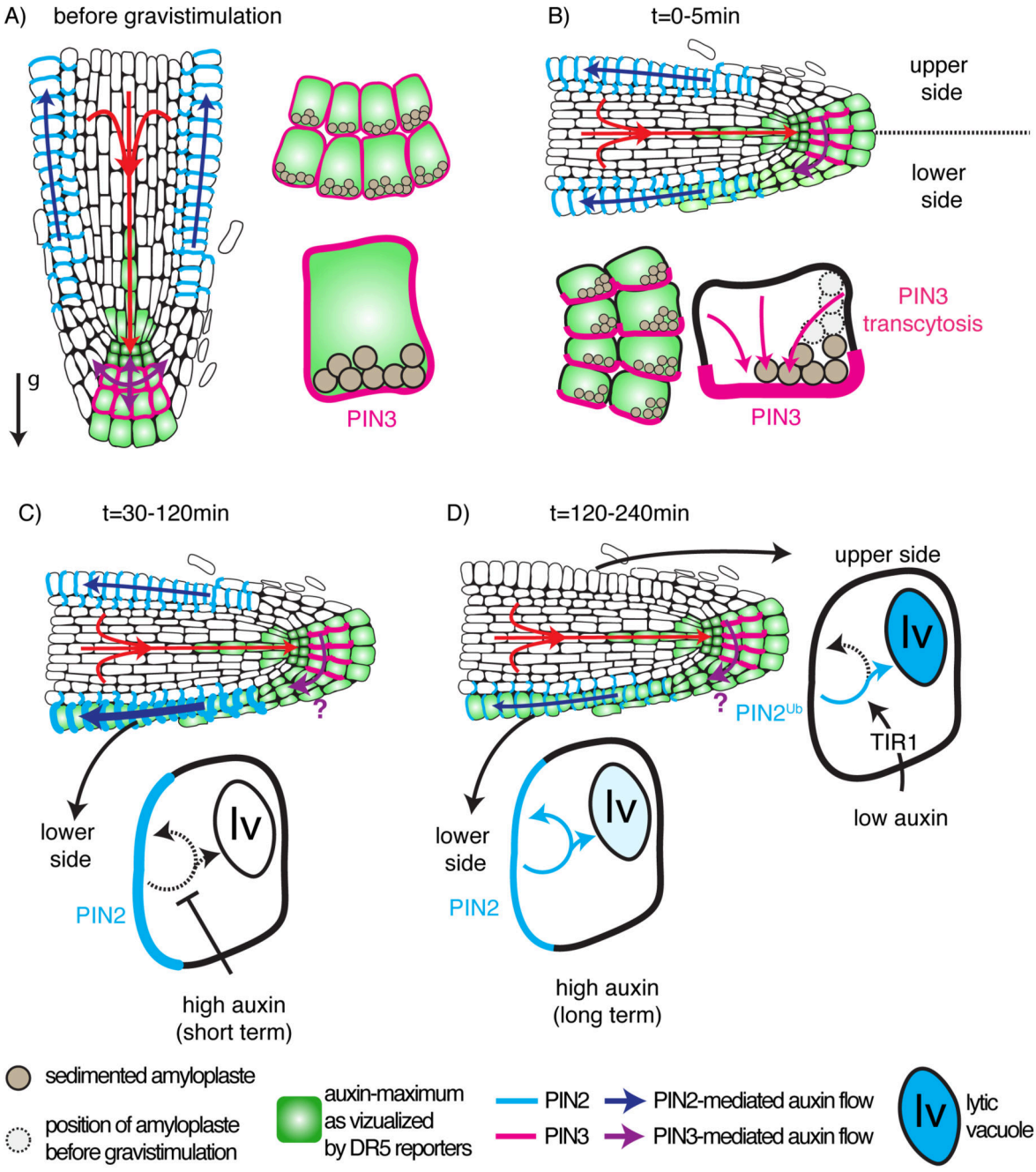

Figure 5. auxin fluxes and PIN trafficking during the gravitropic root response.

A) Auxin fluxes and PIN2/PIN3 localization in the primary root tip before gravistimulation. Note the position of sedimented amyloplasts in the collumela. The arrow with the 'g' letter indicates the gravity vector. B) Auxin fluxes and PIN2/PIN3 localization in the primary root tip from 0 to 30 min after gravistimulation. Note the movement of amyloplasts according to the new gravity vector, which triggers PIN3 transcytosis to the lower part of the cell and initiates the establishment of an asymmetric auxin maximum between the upper and lower part of the root. C) Auxin fluxes and PIN2/PIN3 localization in the primary root tip from 30

to 120 min after gravistimulation. High auxin in the lower part of the root inhibits PIN2 endocytosis, which promotes its localization at the plasma membrane and reinforce asymmetric auxin localization. Accumulation of auxin on the lower part of the root locally inhibits cell elongation, which triggers root bending. D) Auxin fluxes and PIN2/PIN3 localization in the primary root tip from 120 to 240 min after gravistimulation. Inhibition of endocytosis by auxin is transient and PIN2 endocytosis is re-established in the lower part of the root. In the meantime, low auxin on the upper part of the root triggers PIN2 degradation in a TIR1/AFBs-dependent manner. Note that the times indicated are compiled between those reported by Band et al., (2012) and Baster et al., (2013), but may vary according to the experimental setup and are only indicative. It is unknown at what point during the gravitropic response PIN3 localization is reset to apolar, hence the purple question mark in C and D.

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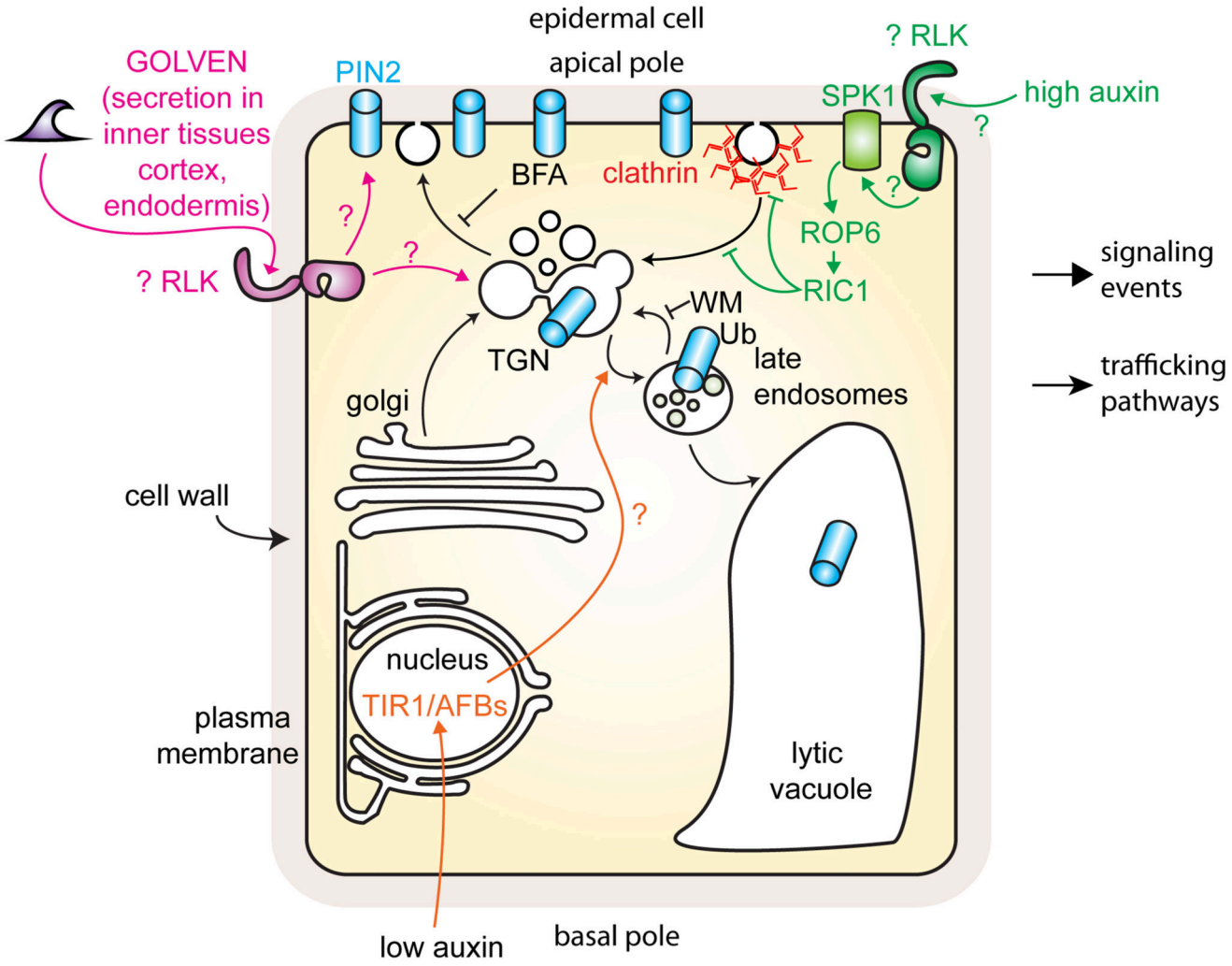

Figure 6. Missing RLKs in the regulation of PIN2 trafficking in epidermal root cells during gravitropism.

Schematic representation of PIN2 trafficking as regulated by the 'non-genomic' auxin pathway and GOLVEN peptides. Note that RLKs are likely involved in both pathways but have yet to be identified. Arrows with chevron-shaped arrowhead represent trafficking pathways and arrows with triangle-shaped arrowhead represent signalling events; blunt-ended lines represent inhibition.

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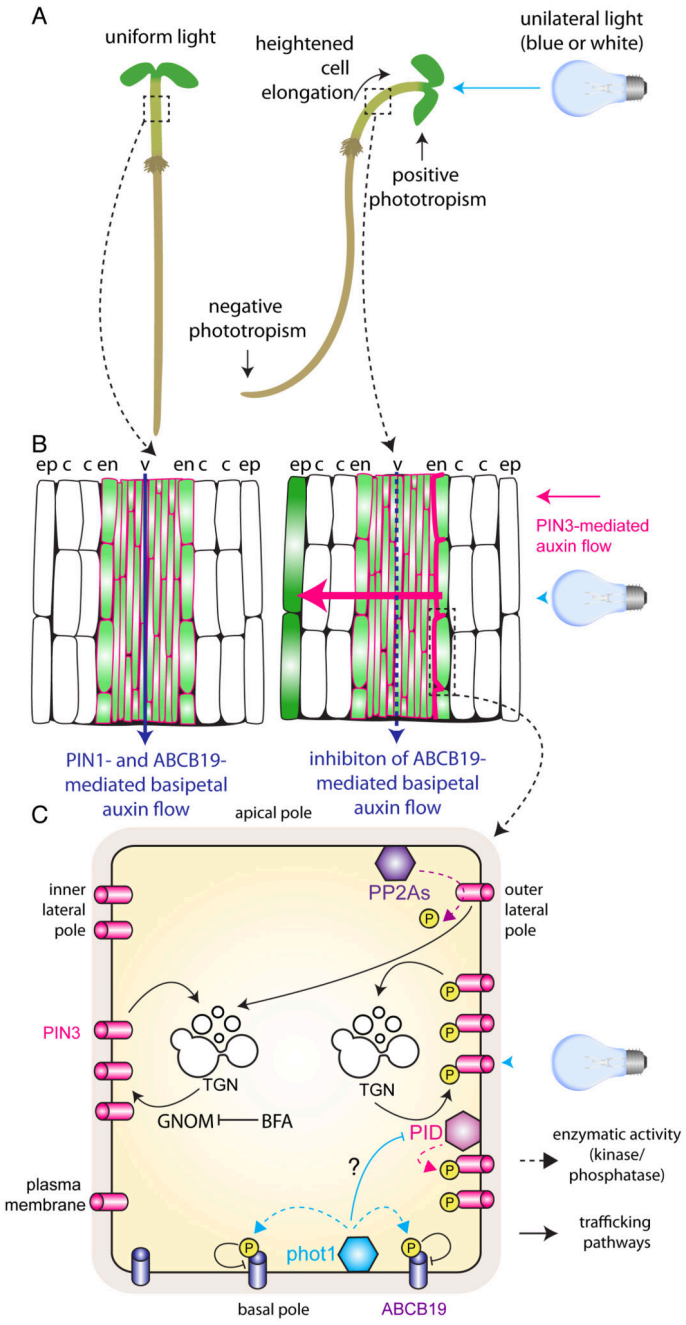

Figure 7. Protein kinases and auxin fluxes during the hypocotyl phototrophic response.

A) Seedling grown in uniform light (left) or under unilateral light (right). B) Schematic representation of a longitudinal section of hypocotyl showing putative auxin maximum (in green) and PIN3 localization (in pink) when seedling are grown in uniform light conditions or in darkness (left) and when they encounter unidirectional light (right). Arrows in dark blue represent PIN1- and ABCB19-mediated rootward auxin flow and the arrow in pink show PIN3-mediated lateralization of auxin flow toward the shaded side of then hypocotyl. C) Schematic representation of PIN3 trafficking in endodermis (illuminated side of the

hypocotyl). Phot1 inhibits PID activity by unknown mechanisms, which limits PIN3 phosphorylation by PID and favours its routing by the GNOM-dependent pathway to the inner lateral pole of the cells. Phot1 also directly phosphorylates ABCB19, which inhibits its efflux activity and limits the downward auxin flow. ABCB19 inhibition and PIN3 lateralization stimulates auxin trafficking toward the shaded side of the hypocotyl, which promotes cell elongation and hypocotyl bending toward the light source. Note that phot1 action on ABCB19 and PIN3 trafficking has been represented in the same cell for convenience, but it is not known whether these events happen in the same place of the hypocotyl. Dashed arrows represent direct phosphorylation events; arrows with chevron-shaped arrowhead represent trafficking pathways and blunt-ended line represents inhibition.

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