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Novel factors modulating AGC kinase signaling-controlled polar auxin transport

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**Novel factors modulating AGC kinase signaling-
controlled polar auxin transport**

Yao Xiao

肖瑶

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Front cover: Inflorescence of the *pdk1 pdk2* mutant

Back cover: A maze designed by Yao Xiao

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Novel factors modulating AGC kinase signaling- controlled polar auxin transport

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Chapter 1

General introduction

Yao Xiao and Remko Offringa

1 Auxin, a crucial regulator of plant development

Auxin (derived from the Greek word αὐξάνειν for to grow or to increase) was the first plant hormone to be identified in plants. It was predicted by Charles and Francis Darwin in their book *The Power of Movement in Plants* in 1881 (Darwin and Darwin, 1881). Its existence and growth promoting ability were demonstrated with coleoptile bending experiments by the Dutch botanist Frits Warmolt Went in 1926 (Went, 1926). Subsequently, auxin has been isolated, its structure determined as indole-3-acetic acid (IAA), and its biosynthesis-, transport- and signaling pathways have been well-studied during the past century (Leyser, 2010; Weijers and Wagner, 2016).

Auxin is involved in various cell activities during almost every plant developmental stage. It comprehensively directs developmental processes, such as embryo patterning, shoot and root meristem formation, vascular patterning, flower development, and gamete specification. (Cheng, Y. and Zhao, 2007; Möller and Weijers, 2009; Pagnussat et al., 2009; Vernoux et al., 2010; Overvoorde et al., 2010; Biedron and Banasiak, 2018). Auxin occupies a central place in the crosstalk network of responses to environmental factors (gravity, light, temperature, mechanical stress, osmotic stress etc.) and endogenous signals (cytokinin, ethylene, abscisic acid, gibberellic acid, jasmonic acid, salicylic acid, brassinosteroids, strigolactones, nitric oxide, peptides, phospholipids, calcium etc.) (reviewed in Saini et al., 2013; Mroue et al., 2018). The concentration of auxin provides crucial information for local tissue development, and it is strictly regulated through metabolism and polar transport to create patterns of maxima and minima, which direct cell elongation, -division and -differentiation in these tissues (Perrot-Rechenmann, 2010; Di Mambro et al., 2017).

2 Auxin biosynthesis and inactivation

The natural auxin IAA can be synthesized from the amino acid tryptophan (Trp) or through a Trp-independent pathway (Figure 1). The Trp-independent pathway was first proposed in maize and subsequently proven through the *Arabidopsis thaliana* (*Arabidopsis*) mutant *indole synthase* (*ins*) (WRIGHT et al., 1991; Wang et al., 2015). INS has the same catalytic activity as the plastid-localized TRYPTOPHAN SYNTHASE ALPHA CHAIN (TSA), but it has a cytosolic subcellular localization. Detailed analysis of the *ins* mutant phenotype has shown that the Trp-independent pathway plays a vital role in hypocotyl elongation at high temperature and apical-basal patterning during early embryogenesis in *Arabidopsis* (Wang et al., 2015). However, how exactly IAA is synthesized from indole in the Trp-independent way is still unknown.

By taking advantage of the genetic tools in *Arabidopsis* and using different physiological approaches and feeding experiments, also several Trp-dependent auxin biosynthesis routes have been revealed (Figure 1). Multiple key intermediates between Trp and IAA, such as indole-3-acetaldoxime (IAOx), Indole-3-acetamide (IAM), indole-3-acetonitrile (IAN), tryptamine (TAM), indole-3-acetaldehyde (IAAld) and indole-3-pyruvate (IPA) have been proposed, but the enzymes involved in the biosynthesis of these intermediates have mostly remained unidentified (summarized in Kasahara, 2016). AMIDASE-LIKE PROTEIN 1 (AMI1), NITRILASEs (NITs) and ALDEHYDE OXIDASEs (AAOs) can convert respectively IAM, IAN and IAAld to IAA, but the corresponding *Arabidopsis* knockout mutants did not show strong

phenotypes that demonstrated the importance of their physiological roles (Seo et al., 2002; Pollmann et al., 2003; Lehmann et al., 2017; Kasahara, 2016; Zhao, 2018). A limited number of genetically proven enzymes are P450 monooxygenases CYP79B2 and CYP79B3 that catalyze the Trp to IAOx conversion, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and TAA1-RELATEDs (TARs) that catalyze the conversion of Trp to IPA, and the flavin-containing monooxygenases YUCCAs (YUCs) that mediate the IPA to IAA conversion (Zhao et al., 2002; Cheng et al., 2006; Stepanova et al., 2008). TAA1 is also named TRANSPORT INHIBITOR RESPONSE 2 (TIR2)/SHADE AVOIDANCE 3 (SAV3)/WEAK ETHYLENE INSENSITIVE 8 (WEI8) in Arabidopsis (Tao et al., 2008; Yamada et al., 2009). The TAA/YUC pathway is the only complete Trp-dependent auxin biosynthesis route which has been verified genetically and biochemically. Moreover, this pathway has been identified in rice, maize, petunias, liverworts and some other plant species as well, suggesting that it is a very conserved auxin biosynthesis pathway in plants (reviewed in Zhao, 2018).

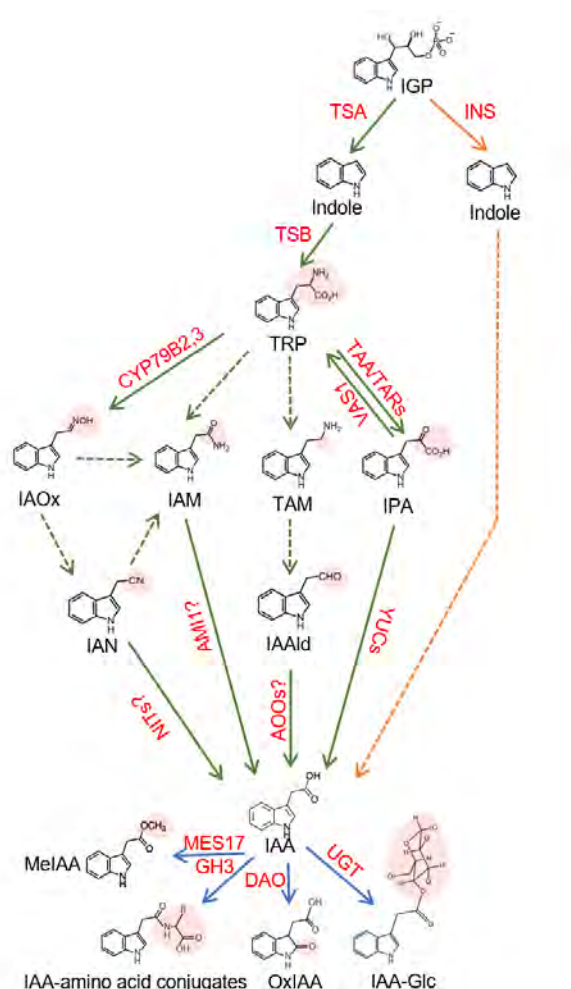


Figure 1. Schematic routes of IAA biosynthesis and inactivation identified in Arabidopsis. The Trp-dependent pathways are shown in green, the Trp-independent pathway is shown in orange, the inactivation pathways are shown in blue, the enzymes are in red. Pink features are used to highlight the modified portion of each chemical structure. Solid or dashed lines represent a direct or indirect link, respectively. Enzymes without genetic evidence are noted with a question mark. TSB, TRP SYNTHASE β -SUBUNIT; VAS1, REVERSAL OF SAV3 PHENOTYPE 1; MES17, METHYL ESTERASE 17; GH3, GRETCHEN HAGEN 3; DAO, DIOXYGENASE OF AUXIN OXIDATION; UGT, UDP GLUCOSE TRANSFERASES. Full names of the remaining enzymes and intermediates or IAA conjugates are explained in the text. The reverse reaction of some inactivation pathways is not shown in this model.

We will not present too much detail about how these auxin biosynthesis routes evolved and how the genes involved are regulated in this thesis. But one thing we want to emphasize is the importance of local auxin biosynthesis, which has been raised before. Auxin has been considered to be synthesized in source tissues and transported to sink tissues to generate gradients for a long time. The contribution of local auxin biosynthesis has been vastly ignored. However, accumulating genetic evidence shows that knock-out of some auxin biosynthesis genes results in tissue-specific phenotypes that cannot be rescued by remote application of auxin (Cheng et al., 2006, 2007a; Chen et al., 2016, 2014; Vandenbussche et al., 2010). For example, overproduction of auxin in the shoot cannot rescue the root phenotype of the auxin biosynthesis deficient *yucQ* mutant (Chen et al., 2014).

Besides a strict regulation of auxin biosynthesis, the inactivation of the hormone by methylation, amino acid conjugation, oxidation or glucosylation is also essential to balance the auxin levels (Abbas et al., 2018; LeClere et al., 2002; Zhang et al., 2016; Jin et al., 2013). Among these reactions, oxidation or conjugation with aspartic acid or glutamic acid are irreversible, while methylation, glucosylation or conjugation with other amino acids are reversible. Moreover, IAA can also be reversibly converted into indole-3-butyric acid (IBA), which is a storage and transport form of auxin that is much used to for the induction of adventitious root formation (Xuan et al., 2015; Olatunji et al., 2017).

3 Canonical and non-canonical auxin signaling pathway

Following the biosynthesis, auxin will be locally retained or polarly transported to target cells to regulate gene expression. Since this thesis mainly focuses on the regulation of auxin transport, an overview of auxin perception and signal transduction will be discussed first, after which we will go in detail on the mechanisms of polar auxin transport (PAT) (Figure 2).

TRANSPORT INHIBITOR RESISTANT1/AUXIN SIGNALING F-BOXs (TIR1/AFBs), AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins, and AUXIN RESPONSE FACTORS (ARFs) are three protein families that form the canonical auxin signaling pathway (Overvoorde et al., 2005; Calderón Villalobos et al., 2012). Under low auxin conditions, Aux/IAA proteins physically interact with the ARF transcription factors to repress their activity on gene transcription regulation (Ulmasov et al., 1997; Tiwari et al., 2001, 2004; Overvoorde et al., 2005). When auxin accumulates in the cell, it more frequently binds to the pocket provided by the N-terminal leucine-rich repeats (LRR) domain of TIR1/AFBs (Tan et al., 2007), where it acts as “molecular glue” to increase the interaction affinity between TIR1/AFBs and domain II of Aux/IAA, which docks in the same groove (Tan et al., 2007). TIR1/AFBs are F-box proteins that are part of the SCF^{TIR1/AFB} ubiquitin ligase complex (Dharmasiri et al., 2005; Parry et al., 2009), and recruitment of Aux/IAA proteins leads to their ubiquitination and subsequent degradation via the 26S proteasome (Gray et al., 2001). This releases the ARFs, allowing them to activate the expression of genes containing auxin response elements (AuxREs) in their upstream promoter sequences (Tiwari et al., 2004).

Several alternative auxin sensing mechanisms have been reported in recent years (Figure 2). Simonini and coworkers found that ARF3/ETTIN interacts with INDEHISCENT (IND) to form a complex to control gynoecium morphogenesis by repressing *PINOID* (*PID*) expression (Simonini et al., 2016). This interaction is directly disrupted by IAA, both *in vivo* and *in vitro* (Simonini et al., 2016). This ARF3/ETTIN-mediated unique IAA-sensing mechanism expands

from IND to many other transcription factors, including REPLUMLESS (RPL), KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1/3 (KNAT1/3), ABERRANT TESTA SHAPE (ATS), BABYBOOM (BBM), TEOSINTE BRANCHED, CYCLOIDEA AND PCF4/18 (TCP4/18) and PLETHORA5 (PLT5) (Simonini et al., 2016). By disrupting these different ARF3/ETT-transcription factor complexes, auxin can affect the expression of a new set of target genes linked to different growth and development processes than the canonical signaling pathway. Another auxin response system in the nucleus is SKP2A-EF2C-DPB complex (Jurado et al., 2010). S-Phase Kinase-Associated Protein 2A (SKP2A) is an F-box protein that is responsible for directing degradation of the cell cycle transcription factors EF2C and DPB (Jurado et al., 2008). Auxin binds to SKP2A directly to promote SKP2A degradation in a TIR1-independent manner (Jurado et al., 2010). In addition, auxin binding enhances the interaction between SKP2A and DPB or EF2C, leading to the degradation of these transcription factors. Through the SKP2A-EF2C-DPB rheostat, auxin adjusts the balance between cell division and endoreduplication.

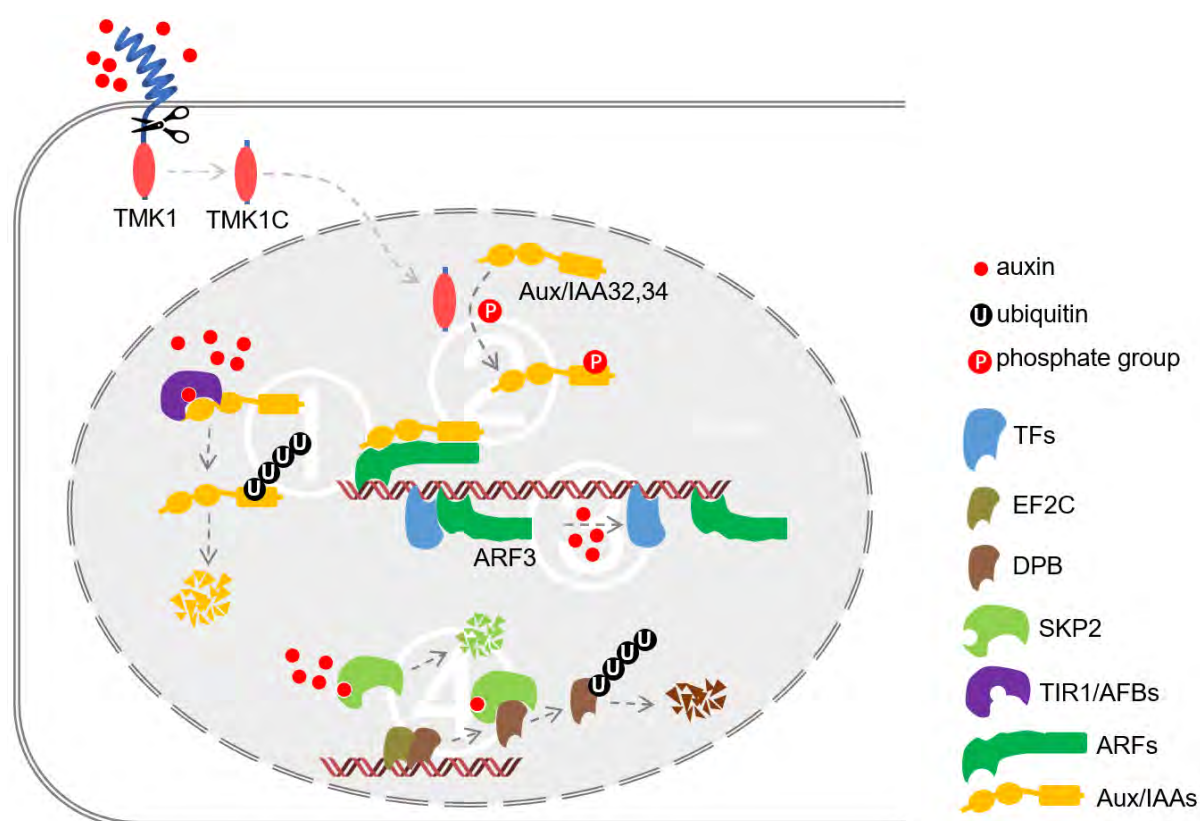


Figure 2. Overview of identified auxin signaling pathways. (1) Canonical TIR1/AFBs-Aux/IAA-ARFs-mediated gene transcription regulation. Auxin acts as “molecular glue” to enhance TIR1/AFBs-Aux/IAA interaction, thus promoting Aux/IAA degradation. Subsequently, ARFs are activated after release of Aux/IAA inhibition. TIR1/AFBs-Aux/IAA also controls a non-transcriptional pathway for rapid growth, which is not shown in this figure. (2) TMK1 mediated Aux/IAA phosphorylation. Auxin induces cleavage of TMK1 into TMK1C. TMK1C translocates from the PM into the nucleus to phosphorylate and stabilize Aux/IAA32 and Aux/IAA34, thereby increasing transcriptional inhibition. The auxin receptor for this pathway is still unknown. (3) ARF3/ETTIN-transcription factor (TF) complex-mediated direct transcription alteration. The interaction between ARF3 and different TFs is directly disrupted by auxin. ARF3 and the TFs and ARF3-TF complexes govern completely different transcriptomes. (4) SKP2-DPB-EF2C transcription factor degradation pathway. Auxin has a dual role in this pathway: on the one hand, auxin binding leads to SKP2 degradation; on the other hand, auxin binding enhances SKP2A-DPB interaction and the proteolysis of DPB and EF2C. For presentation purpose, EF2C proteolysis is not shown.

Apart from auxin perception and transcriptional regulation in the nucleus, AUXIN BINDING PROTEIN 1 (ABP1) was for a long time accepted as a receptor for fast, non-transcriptional auxin responses at the plasma membrane (PM). The mutant phenotype, signaling pathway, and even crystal structure of ABP1 have been well studied in the past decades (Woo et al., 2002; David et al., 2007; Braun et al., 2008; Xu et al., 2010; Xu T et al., 2014). However, Gao and coworkers identified two novel *abp1* null alleles, which surprisingly do not display any noticeable developmental defects or auxin insensitivity (Gao et al., 2015). The initially reported embryonic lethality of *abp1-1* mutant was found to be caused by knocking out the adjacent *BELAYA SMERT* (*BSM*) gene (Michalko et al., 2015; Dai et al., 2015). This has put the importance and role of ABP1 in auxin signaling into question. Nevertheless, research on the PM-localized transmembrane kinase (TMK) receptor-like kinase, which was reported to transduce the auxin signal from ABP1 to ROP GTPases to control the shape of leaf pavement cells, was continued (Xu T et al., 2014; Cao et al., 2019). The most recently obtained results show that the C-terminal kinase domain of TMK1 is cleaved off and translocated to the nucleus after auxin treatment, where it specifically phosphorylates and stabilizes the non-canonical transcriptional repressors Aux/IAA32 and Aux/IAA34 (Cao et al., 2019). It provides a new model explaining how high auxin levels inhibit growth originated at the cell surface. The falling-down of ABP1 as PM localized auxin receptor has made the mechanism of auxin-induced rapid growth a mystery again, with the acid growth theory still as original key model (Kutschera, 1994). Advanced research results have shown that auxin-induced cell wall/apoplast acidification is caused by local activation of H⁺-ATPases downstream of TIR1/AFBs-Aux/IAA and SAUR19 in aerial organs (Fendrych et al., 2016). The existence of a non-transcriptional branch of TIR1/AFBs-Aux/IAA signaling pathway in rapid root growth inhibition has also been proposed (Fendrych et al., 2018).

4 Polar auxin transport and the transporters

As mentioned above, auxin transport is a crucial step between auxin biosynthesis and -signaling. Auxin is redistributed from its sites of biosynthesis by fast nonpolar transport via the phloem or by slow polar cell-to-cell transport. Especially this slow polar auxin transport (PAT) contributes to many developmental processes, including tropic growth, apical dominance, and organ initiation, and depends on the cellular balance between auxin influx and -efflux (Petrásek and Friml, 2009). According to the chemiosmotic hypothesis proposed for PAT (Raven, 1975) (Rubery and Sheldrake, 1974; Raven, 1975), IAA can enter the plant cell with the help of influx carriers or by passive diffusion of the non-ionized protonated form (IAAH) that is abundantly present in the acidic apoplast environment (pH≈5.5) (Raven, 1975). In the more basic (pH≈7.0) cytoplasm, however, auxin becomes deprotonated (IAA⁻) and becomes trapped in the cell, as it is not able to cross the PM by passive diffusion any more. For export, auxin requires efflux carriers, and by asymmetric placement of these carriers at one side of the PM, auxin is mainly exported at this side, where it will be readily taken up by the neighboring cell to be exported again at the polar efflux carrier domain. This then results in a polar cell-to-cell flow of auxin.

Three classes of auxin influx carriers and two efflux carrier families have been discovered in Arabidopsis up to now. AUXIN1/LIKE-AUX1 (AUX1/LAX) proteins are influx carriers that are either polarly or symmetrically localized in different cell types for importing auxin. The nitrate

transporter NRT1.1 and two ATP-BINDING CASSETTE subfamily B (ABCB) family members (ABCB4 and ABCB21) have been shown also to contribute to auxin uptake (Krouk et al., 2010; Kubeš et al., 2012; Kamimoto et al., 2012). NRT1.1-mediated auxin uptake only happens at low nitrate availability, thereby providing a connection between nutrient- and auxin signaling (Krouk et al., 2010). The PIN-FORMED (PIN) proteins and ABCBs are two prominent families of transporters associated with auxin efflux. Besides these transporters on the PM for intercellular auxin transport, endoplasmic reticulum (ER)-localized PIN5/6/8 and PIN-LIKES (PILS) are assumed to be responsible for intracellular auxin transport and –homeostasis. In addition, vacuolar membrane-localized WALLS ARE THIN1 (WAT1) transporter facilitates auxin export from the vacuole to the cytoplasm (Ranocha et al., 2013). A detailed description of four prominent gene families will be discussed below.

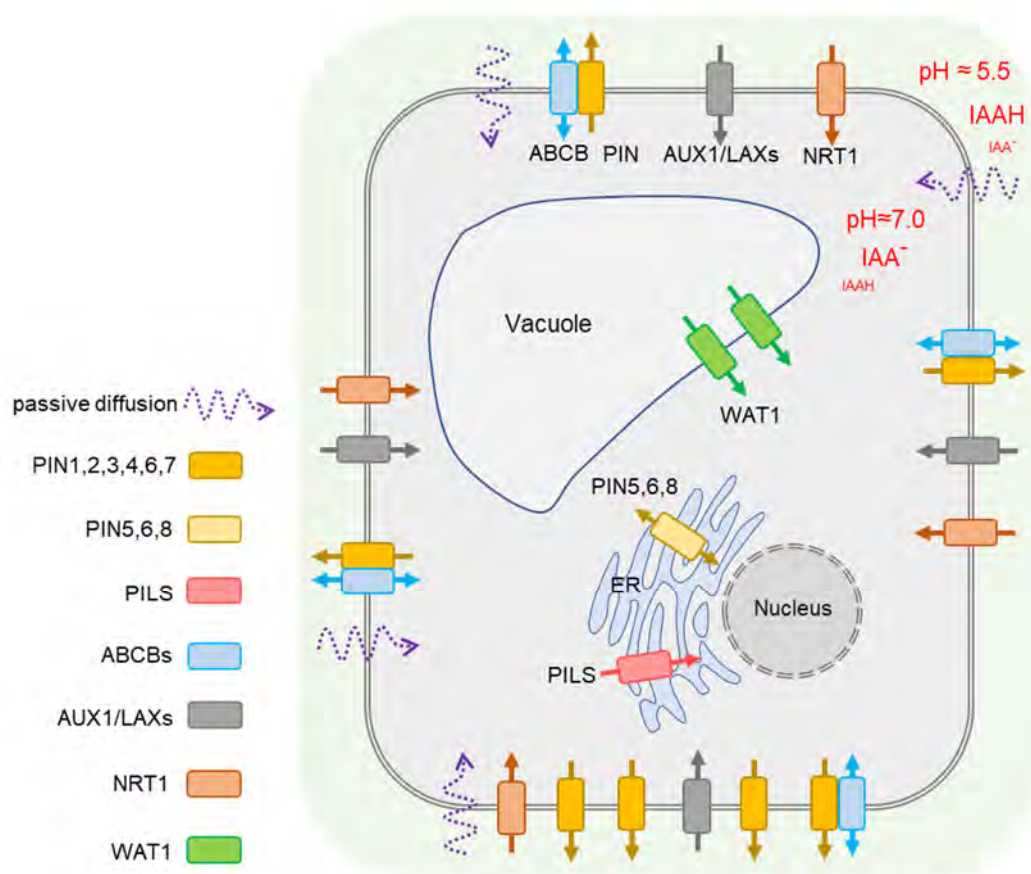


Figure 3. Schematic representation of auxin transport. Since IAA is a weak acid ($pK_a \approx 4.8$), it is preferentially in the protonated form in the apoplast ($pH \approx 5.5$) and in the ionized form in the cytosol ($pH \approx 7.0$). Therefore, IAA can enter the cell by passive diffusion, or by PM located AUX1/LAXs and NRT1.1 auxin influx carriers. NRT1.1 only works at limiting nitrate concentrations. ABCBs are PM-localized auxin carriers that export IAA in most conditions. However, ABCB4 and ABCB21 import IAA when triggered by low cytosolic IAA concentrations. Asymmetrical PM localized PIN proteins (PIN1,2,3,4,6,7) are the main drivers of PAT. They transport IAA out of the cell synergistically with ABCBs. Shorter PINs (PIN5, PIN6, PIN8) are present in the ER to mediate IAA exchange between the cytosol and the ER lumen. PIN5 transports IAA from the cytosol to the ER lumen, ER-localized PIN6 seems to work in the same way but PIN8 in the opposite way. PILS are newly identified putative auxin transporter on the ER that seem to perform a PIN5-like function. Vacuolar membrane-localized WAT1 exports IAA from the vacuole into the cytoplasm.

4.1 The PM-localized AUX1/LAXs auxin influx carriers

The Arabidopsis *aux1-1* mutant was initially identified by screening an ethyl methane sulfonate (EMS)-induced mutant population for 2,4-D resistant mutants (Maher and Martindale, 1980). Its defect in the auxin pathway was reconfirmed in a touch-stimulated root waving study (*aux1-1*), and allelic mutants were selected from a screen for resistance to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (*aux1-7*, *aux1-12*, *aux1-15*, and *aux1-19*) (Okada and Shimura, 1990; Pickett et al., 1990). The identification of the T-DNA mutant allele *aux1-100* eventually led to the cloning of the auxin influx carrier encoding gene *AUX1* (Bennett et al., 1996).

AUX1 belongs to a family of permease-like proteins that are responsible for amino acid transport in animals. The Arabidopsis genome encodes three highly conserved *AUX1* paralogs, *LAX1*, *LAX2* and *LAX3*, which are multi-spanning transmembrane proteins. The subcellular localization of *AUX1* is highly correlated with the direction of auxin unloading and varies in different cell types. It shows shootward (apical) localization in protophloem and rootward (basal) localization in lateral root cap cells, whereas it is more symmetrically distributed in columella and epidermis cells (Swarup et al., 2001; Kleine-Vehn et al., 2006; Liu et al., 2017). *AUX1* localization is dependent on the proper function of *AUXIN-RESISTANT4* (*AXR4*) and *RopGEF1* (Dharmasiri et al., 2006; Kleine-Vehn et al., 2006). High auxin affinity and auxin transport ability of *AUX1/LAX1/LAX3* have been confirmed in heterologous expression systems, whereas this could not be demonstrated for *LAX2* (Yang et al., 2006; Carrier et al., 2008; Swarup et al., 2008; Peret et al., 2012). The efficiency of *AUX1*-mediated IAA influx is 15 times higher than the diffusive contribution, implying the predominance of *AUX1* in auxin uptake (Swarup et al., 2005). Mutations in the *AUX1/LAX* genes cause many auxin-related growth defects in Arabidopsis. Due to their specific expression patterns, *aux1* and *lax* loss-of-function mutations cause distinct tissue-specific phenotypes in Arabidopsis. *aux1* displays agravitropic primary root growth and reduced lateral root initiation (Bennett et al., 1996; Bao et al., 2007; Peret et al., 2012). *lax2* fails to establish normal vascular patterning in cotyledons and leaves (Peret et al., 2012; Moreno-Piovanio et al., 2017). *lax3* is defective in lateral root emergence but not in lateral root initiation or primary root gravitropism (Swarup et al., 2008). However the *AUX1/LAX* genes also act redundantly, as shown by the reduced hook opening in etiolated *aux1 lax3* seedlings (Vandenbussche et al., 2010), embryo defects in the *aux1, lax1 lax2* triple mutant (Robert et al., 2015) and the irregular phyllotaxis of *aux1 lax1 lax2 lax3* quadruple mutant inflorescences (Bainbridge et al., 2008).

4.2 The PM- and ER-localized PIN auxin efflux carriers

The *PIN* gene family encoding auxin efflux carriers has eight members in Arabidopsis. All *PIN* proteins share a similar structure with five membrane-spanning domains at the amino- and carboxy-terminus, linked by a central hydrophilic loop (Gälweiler et al., 1998; Mravec et al., 2009). Based on the length of the hydrophilic loop (HL), *PIN* proteins can be classified into three subgroups: *PIN1* to 4 and *PIN7* (long HL), *PIN6* (intermediate HL), and *PIN5* and *PIN8* (short HL). Long *PIN*s localize at the PM where they show an asymmetric distribution in specific cell types. They have been shown to be the rate-limiting drivers of PAT (Petrásek et al., 2006), determining the direction of PAT through their polar localization (Wisniewska et al.,

2006). All PIN proteins have been functionally characterized, and their subcellular localization and functionality *in planta* will be briefly summarized below.

The *pin formed* mutant *pin1-1* was first isolated in the 1980s, and was named after its pin-like inflorescences. This phenotype was found to be caused by impaired auxin transport and cloning of the mutated gene has shown that *PIN1* encodes a PM-localized carrier protein (Okada et al., 1991; Gälweiler et al., 1998). The PIN1 protein is preferentially basally localized in cells of pre-vascular and vascular tissues, where it drives vascular patterning by canalizing PAT and vascular differentiation by controlling the auxin concentration in the cells (Scarpella et al., 2006). Conversely, PIN1 shows apically localization in the epidermis cells of post-globular stage embryos and of the shoot apical meristem (SAM), where it generates auxin maxima for organ initiation (Gälweiler et al., 1998; Benkova et al., 2003; Friml et al., 2003; Blilou et al., 2005; Guenot et al., 2012).

PIN2 is mainly expressed in the cortex and epidermal cells of the root tip, and in lateral root cap cells to control root tropic growth (including gravitropism and halotropism) (Utsuno et al., 1998; Wisniewska et al., 2006; Rahman et al., 2010; Galvan-Ampudia et al., 2013). The PIN2 protein shows a contrasting subcellular localization in these cells, with predominant apical localization in epidermis and lateral root cap cells, and basal and lateral localization in cortex cells. Therefore, PIN2-mediated basipetal auxin movement in root epidermal cells together with acropetal auxin movement in root cortex cells by PIN2 and in the stele by PIN1 generates a cyclic flow of auxin between the root tip and -elongation zone (Benkova et al., 2003; Blilou et al., 2005; Wisniewska et al., 2006).

PIN3 is expressed in the distal root stele, where PIN3 is basally localized, and in shoot endodermis cells and root columella cells, where the protein is apolarly localized in non-stimulated shoots or roots. After gravistimulation or photostimulation, however, PIN3 rapidly polarizes, causing redistribution of auxin to the lower or dark side of the root. As such, PIN3 is a crucial initiator of phototropic and gravitropic responses in the shoot and root (Friml et al., 2002b; Ding et al., 2011; Rakusová et al., 2011; Zhang et al., 2013; Haga and Sakai, 2012; Kleine-Vehn et al., 2010). PIN3 is also involved in lateral root development, apical hook opening, and red-far-red light induced shade avoidance (Keuskamp et al., 2010; Chen et al., 2015; Yu et al., 2016).

PIN4 is expressed in root meristem precursor cells at the embryo stage, and in quiescent center (QC) and the surrounding cells of the seedling root. It plays a role in focusing the auxin maximum below the QC, which functions as an organizing center for root meristem maintenance and patterning (Sabatini et al., 1999; Friml et al., 2002a).

PIN7 is expressed in embryonic suspensor cells, where its apical localization generates an auxin flow to the embryo proper (Friml et al., 2003), and in root columella cells where it participates with PIN3 in the root gravitropic response and in terminating radial root expansion (Kleine-Vehn et al., 2010; Rosquete et al., 2018).

As a result of the diversity in expression patterns and subcellular localizations of the PM localized PIN proteins the contribution of each PIN in controlling specific developmental or growth processes is dissimilar. At the same time, the overlap in expression between the *PIN* genes implies that they also partially act redundantly (Blilou et al., 2005; Vieten et al., 2005), such as *PIN3* and *PIN7* in the gravitropic and phototropic response (Christie et al., 2011; Ding

et al., 2011), and PIN3, PIN4 and PIN7 in the communication between axillary meristem and branch control (Bennett et al., 2016).

PIN5, as an atypical PIN with a short HL, localizes at the ER where it controls the intracellular free auxin levels by transporting auxin into the ER (Mravec et al., 2009). *pin5* null mutants shows a decrease in root and hypocotyl growth, and lateral root initiation (Mravec et al., 2009). PIN6 shows both ER and PM localization, and the *pin6* mutant phenotype indicates that the protein regulates auxin homeostasis during lateral and adventitious root organogenesis and stamen development (Simon et al., 2016; Ditengou et al., 2018; Cazzonelli et al., 2013; Bender et al., 2013). The PIN8 protein shows a similar subcellular localization as PIN5, but has been suggested to function antagonistically, transporting auxin from the ER back to the cytosol (Ding et al., 2012). PIN8 is mainly involved in gametophyte and sporophyte development (Dal Bosco et al., 2012; Ding et al., 2012). In addition, PIN1, PIN6 and PIN8 function in vein patterning antagonistically with PIN5 (Sawchuk et al., 2013).

4.3 The PM-localized ABCB auxin exporters and importers

The ATP-BINDING CASSETTE SUBFAMILY B/MULTIDRUG RESISTANCE/P-GLYCOPROTEIN (ABCB/MRD/PGP) transporters have twenty-one full-size family members in Arabidopsis. They are orthologs of the mammalian ABCB1 transporters, a class of multi-pass transmembrane proteins that are able to transport cytotoxic chemotherapeutic agents (Bailey et al., 2012). The first identified Arabidopsis ABCB gene, *ABCB1/AtPGP1*, was reported to be involved in light-dependent hypocotyl cell elongation, but its activity was not linked to auxin yet (Sidler et al., 1998). In 2001, its closest paralog *ABCB19/AtMDR1* was discovered in a mutant screen for loss of anion channel function, and two *abcb19* null alleles were isolated (Noh et al., 2001). These *abcb19* mutants display obvious auxin-related phenotypes with downward-folded cotyledons and curled rosette leaves, and *abcb1 abcb19* double mutants show even more severe phenotypes, such as twisted cotyledons and leaves in the light, wavy hypocotyls in the dark, wrinkled and curled organs at the mature plant stage, and reduced apical dominance (Noh et al., 2001). As these phenotypes can be mimicked by growing wild-type plants on the PAT inhibitor naphthylphthalamic acid (NPA), and PAT in *abcb1 abcb19* was found to be reduced by 70%, these ABCB transporters have been proposed to mediate PAT. In that research, ABCB1 and ABCB19 showed high binding affinity for NPA (Noh et al., 2001). In addition, the NPA-binding property of ABCB1, ABCB2 and ABCB19 was shown in an independent biochemical screen for NPA-binding proteins (Murphy et al., 2002). Subsequently, direct evidence for the auxin efflux ability of Arabidopsis ABCB1 and ABCB19 was obtained from auxin export assays in yeast and HeLa cells (Geisler et al., 2005; Bouchard et al., 2006). Until now, only four of the twenty-one Arabidopsis ABCBs (ABCB1, ABCB4, ABCB19 and ABCB21) have been shown to be involved in auxin transport (summarised in Kang et al., 2011; Kamimoto et al., 2012). Different from ABCB1 and ABCB19, ABCB4 and ABCB21 seem to play a dual role in PAT, both as importer and as exporter (Santelia et al., 2005; Terasaka et al., 2005; Cho et al., 2007; Kamimoto et al., 2012; Kubeš et al., 2012). The switch between these two roles seems to be dependent on the cytoplasmic auxin concentration: low auxin triggers ABCB-mediated influx, whereas high auxin promotes the exporter function (Kamimoto et al., 2012; Kubeš et al., 2012). The importer function was also found for OsABCB14 in *Oryza sativa* (Xu et al., 2014).

Although ABCBs contribute to PAT, they are mainly symmetrically distributed over the PM, except for polar localization in the mature root and the root transition zone (Geisler and Murphy, 2006). Several models have been raised for their possible roles in PAT. One proposes that ABCBs and PINs interact to increase the transport rate or specificity (Geisler et al., 2017). Based on transport measurements the PIN1-ABCB1 interaction was proposed to enhance export, whereas the ABCB-PIN2 interaction changes substrate specificity or the PIN-ABCB4 interaction affects PAT directionality (Blakeslee et al., 2007; Bandyopadhyay et al., 2007). Another model proposes that ABCBs determine the amount of auxin available in the cells for PIN-mediated PAT (Mravec et al., 2008).

4.4 ER-localized PILS control the intracellular free IAA levels

PIN-LIKES (PILS) proteins comprise a family of putative auxin transporters that were identified more recently in an *in-silico* screen for PIN homologs (Barbez et al., 2012). The predicted topology of PIN and PILS proteins are highly similar (Barbez et al., 2012). However, evolutionary analysis has shown that the PILS family is evolutionarily older, as members have been identified in unicellular algae, whereas PIN protein occurrence is restricted to land plants and their water dwelling close ancestors (Feraru et al., 2012b; Boot et al., 2016). Unlike the PINs, PILS protein localization is restricted to the ER (Barbez et al., 2012). Even though their transport ability has not been directly demonstrated, they have been shown to control auxin homeostasis in the ER, and thereby to regulate auxin availability for signaling in the nucleus (Barbez et al., 2012). Overexpression of *PILS* causes dwarfism and sterility due to floral organ defects (Barbez et al., 2012). Knocking out *PILS2* and *PILS5* increases hypocotyl growth, whereas *PILS5-GFP* overexpression has the opposite effect (Barbez et al., 2012). PILS proteins also mediate light-controlled apical hook opening by differentially depleting auxin for nuclear signaling (Béziat et al., 2017). PILS6 is an important crosstalk point between heat- and auxin responses in roots (Feraru et al., 2019).

5 Auxin transport regulation by PIN post-translational modification

Because of their polar localization, PIN proteins have been pioneer proteins for the study of intracellular trafficking and polarity establishment of PM proteins in plants. PIN subcellular localization is regulated by many environmental factors (gravity, light, salt and heat) and internal signal molecules (auxin itself, cytokinin, stigolactones, gibberellic acid and jasmonic acid) (Paciorek et al., 2005; Kleine-Vehn et al., 2008; Sun et al., 2009; Marhavý et al., 2011; Willige et al., 2011; Shinohara et al., 2013; Hanzawa et al., 2013; Galvan-Ampudia et al., 2013; Du et al., 2013; Blakeslee et al., 2004).

De novo synthesized PINs in the rough ER reach the PM by secretion from the trans-Golgi network/early endosome (TGN/EE). PM-localized PINs are constitutively endocytosed to the sorting endosome (component of the TGN/EE). From there, PINs are recycled back to the TGN/EE and PM via the recycling endosome (RE), or sorted to the lytic vacuole for degradation via late endosomes/multivesicular bodies (LE/MVBs) (Kleine-Vehn et al., 2010; Luschnig and Vert, 2014; Rakusová et al., 2015). The protein endocytic sorting mechanism is the central controller for PIN PM abundance and polarity establishment, and thus of auxin transport polarity and activity (Kleine-Vehn et al., 2011). More recently, an additional system

has been proposed to regulate auxin transport activity of PIN proteins (Barbosa et al., 2014, 2018).

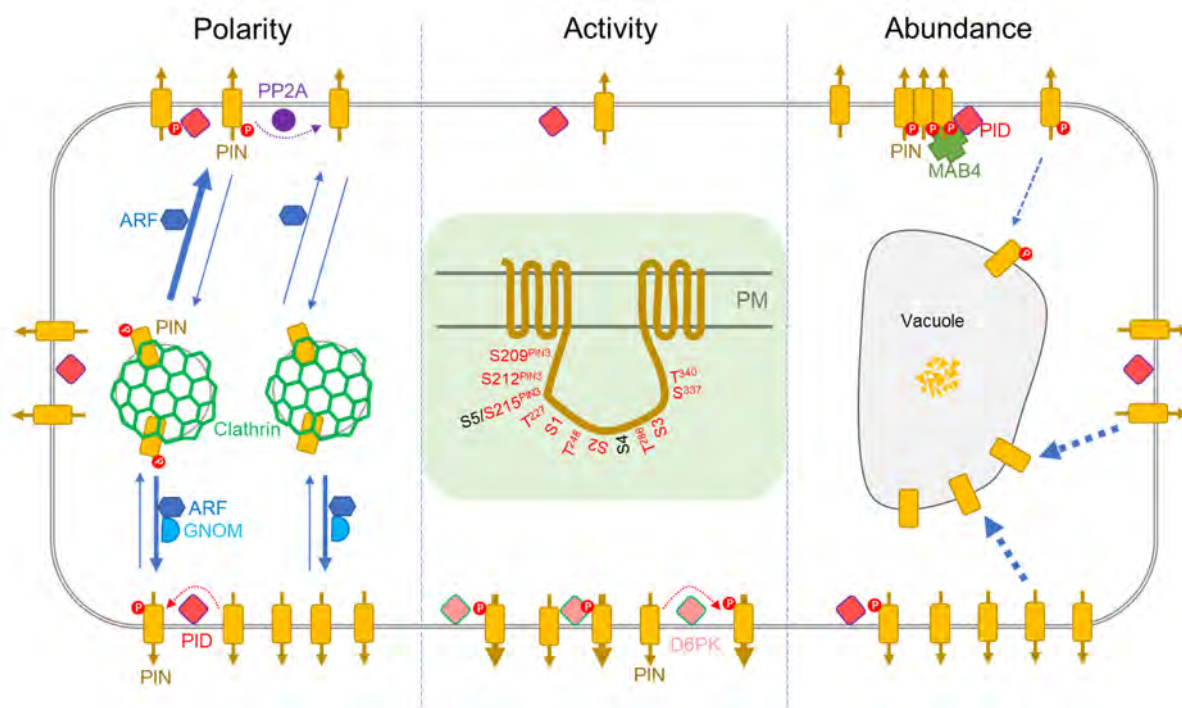


Figure 4. Schematic overview of regulatory systems controlling PIN polarity, activity and PM abundance. Left panel: PIN polarity is established by trafficking to different recycling pathways that deliver PIN from endomembrane compartments to the PM at different sides of the cell. This relies on clathrin-mediated endocytosis and also exocytosis components, including ARFs and ARF regulators (for example, GNOM). The antagonistically controlled PINHL phosphorylation by the AGC3 PID/WAG kinases and the PP2A phosphatases is a crucial signal for recruitment of PINs into the different recycling pathways. Phosphorylated PINs are preferentially recruited to the GNOM-independent apical recycling pathway, whereas non-phosphorylated PIN cargoes are sorted to the basal PM by the GNOM-dependent recycling pathway. Middle panel: PIN phosphorylation has also been reported to regulate auxin transport by modulating IAA export activity of PIN without changing protein polarity. This is mediated by AGC1 kinase subgroup members, such as D6PK and PAX. Right panel: PM abundance is the third regulatory system for PINs. Vacuole-targeted protein degradation is central to this process, it needs PIN ubiquitination and complexes for vacuolar trafficking. MAB4 interacts with and stabilizes PIN on the PM, which also enhances PIN PM abundance. PID is suggested to decrease PIN vacuole targeting and to stimulate its interaction between MAB4 and phosphorylated PIN to form a trimeric complex and microdomains. Features are labelled with protein names with the same colors. Thickness of the blue line with arrow represents relative preference. Thickness of the brown line with arrow represents relative auxin transport activity. In the left panel, only clathrin-coated vesicles are shown, non-clathrin-coated vesicles during the intermediate membrane trafficking are omitted for presentation purpose. In the right panel, ubiquitination and intermediate endosomes are represented by dashed blue lines. Identified functional phosphorylation sites are indicated in the PINHL in the middle panel. Phosphorylation sites which have been proposed to be related to PIN1 localization or activity are indicated in red or black, respectively. Sites identified in PIN3 are specially indicated. S5 in PIN1 is substituted by an aspartic acid (D).

5.1 Proper membrane trafficking is essential for PIN polarity

To make it easy to comprehend, we review the mechanisms of PIN intracellular trafficking following the sequential delivery steps of *de novo* synthesized PIN protein. Like all PM proteins, PINs are synthesized, modified and folded in the ER. The factors involved in this phase are mostly known from studies on other membrane proteins. Misfolded or mismodified membrane proteins will not pass the endoplasmic reticulum quality control (ERQC) to enter the secretory pathway. Instead, they will be retained at the ER, and undergo ER-associated protein degradation (ERAD) (Dancourt and Barlowe, 2010; Luschnig and Vert, 2014; Liu et al., 2015;

Liu and Li, 2014). To investigate the role of specific PIN amino acid residues in membrane trafficking, several PIN mutants have been created, some of which show accumulation in the ER, such as PIN1 NPNTY→NSLSL, PIN1 F165A and PIN1 Y480A (Mravec et al., 2009; Marcote et al., 2016; Sancho-Andrés et al., 2016). Researchers have explained this mislocalization by disturbed adaptor protein (AP) complex binding and protein sorting. However, whether these mutants cause misfolding and fail to pass the ERQC for secretory sorting has not been checked.

Like in other eukaryotes, sorting from the ER to the Golgi in *Arabidopsis* is also mediated by COPII-coated vesicles. Several components required for this process have been identified in *Arabidopsis*, such as Rab-D1/2 GTPases, AtRab1b GTPase, SECRETION-ASSOCIATED RAS 1 (SAR1), Sec24A, p24δ5/β2, and a few ADP ribosylation factor (ARF) GTPases, (Pinheiro et al., 2009; Batoko et al., 2000; Richter et al., 2007; Teh and Moore, 2007; Bar-Peled and Raikhel, 1997; Faso et al., 2009; Montesinos et al., 2012). However, direct evidence for the role of these common proteins in PIN trafficking is still limited. GNOM-LIKE1 (GNL1), an ARF GTP exchange factor (ARF-GEF), performs a role in COPI-coated vesicle-mediated Golgi to ER retrograde trafficking of PIN (Richter et al., 2007; Teh and Moore, 2007). In addition, GNL1 is also crucial for selective PIN endocytosis from the PM (Richter et al., 2007; Teh and Moore, 2007). A well-studied homolog of GNL1 in *Arabidopsis*, GNOM/EMB30 (Steinmann et al., 1999), locates to the Golgi and endosomes and it can functionally replace GNL1, but not vice versa (Richter et al., 2007; Teh and Moore, 2007; Steinmann et al., 1999; Naramoto et al., 2014b). Sorting of different PM proteins between the ER and the Golgi involves shared common components, but other components seem specific for certain PM proteins. For example, AUXIN RESPONSE 4 (AXR4) is required for AUX1 to exit the ER, whereas PIN1, PIN2 and H⁺-ATPase are not mislocalized in the *axr4* mutant background (Dharmasiri et al., 2006).

After translocation from the ER to the Golgi, the secretory sorting events proceed to the PM via the TGN/EE. Some vesicle-tethering-related proteins SEC6, SEC8 and EXO70A1 facilitate exocytosis of PM proteins (Cole et al., 2005; Kulich et al., 2010; Fendrych et al., 2013). ARF- and Rab-GTPases are still the key regulators of vesicular trafficking at this step. BFA-VISUALIZED EXOCYTIC TRAFFICKING DEFECTIVE (BEX5/RabA1b) is the first genetically identified GTPase in *Arabidopsis* that mediates PIN trafficking from the TGN/EE compartment to the PM (Feraru et al., 2012a). Later, BFA-VISUALIZED EXOCYTIC TRAFFICKING DEFECTIVE1 (BEX1/ARF1A1C) and many other ARF GTPase have been reported to regulate this process (Tanaka et al., 2014; Singh et al., 2018). Obviously, proteins that can adjust ARF- or Rab-type GTPase activity also participate in the regulation of membrane protein translocation at this phase. GNOM, GNL1, GNL2 and five BIG ARF-GEFs have all been shown to be functional during membrane trafficking (Steinmann et al., 1999; Richter et al., 2007; Teh and Moore, 2007; Anders and Jürgens, 2008; Singh et al., 2018). Among them, GNL1, GNL2 and BIG3 are resistant to the fungal toxin brefeldin A (BFA), while the others are not (Richter et al., 2007). GNOM/GNLs and BIGs work redundantly, but also specifically, due to their differential expression pattern and distinct subcellular localization: GNOM is required for PIN1 delivery from endosomes to the basal PM (Richter et al., 2010). PIN1 basal polarity is disrupted in *gnom* mutants, and treatment with high BFA concentrations leads to reversible intracellular PIN accumulation, whereas long term treatment with lower BFA concentrations

leads to a basal-to-apical PIN1 polarity shift (Geldner et al., 2003; Kleine-Vehn et al., 2009); GNL1 regulates the Golgi to the ER trafficking as discussed above; *GNL2* is pollen-specific expressed to control polar recycling facilitated polar tip growth (Richter et al., 2012); BIG1-4 are responsible for the TGN to PM transport (Richter et al., 2007; Kitakura et al., 2017); HOPM INTERACTOR 7/BFA-VISUALIZED ENDOCYTIC TRAFFICKING DEFECTIVE 1 (MIN7/BEN1/BIG5) mediates early endosomal trafficking (Tanaka et al., 2009).

Another regulator family of ARF-GTPase activity in Arabidopsis that stimulates ARF GTPase activity are the ARF GTPase-activating protein domain proteins (AGDs) (Sieburth et al., 2006). The central family member VASCULAR NETWORK 3/SCARFACE (VAN3/SFC) localized to the TGN/EE and controls PIN PM delivery (Koizumi et al., 2005; Sieburth et al., 2006). In addition, VASCULAR NETWORK DEFECTIVE 4 (VAN4) encodes a GEF for Rab-GTPase located at the TGN/EE. Like VAN3/SFC, VAN4 mediates PIN exocytosis from the TGN to the PM (Naramoto et al., 2014a), and *van4* mutants show similar vascular breaks as what is observed for the *gnom* or *van3/sfc* mutants (Geldner, 2003; Sieburth et al., 2006; Naramoto et al., 2014a).

PM-localized PIN proteins are constitutively endocytosed and delivered to various endosomes. AP-complex binding and the subsequent assembly of the clathrin coat are two essential steps for clathrin-mediated endocytosis (CME) of PIN. AP-complex binding is the selective step for PIN cargo recognition and recruitment into vesicles. The Arabidopsis genome encodes five different putative AP heterotetrametric complexes. The localization and function of each of these AP complexes are not yet so well characterized as in animals. Nevertheless, more and more studies describe their distinct functions: e.g. the AP2 complex facilitates endocytosis, whereas the AP1 complex mediates post-Golgi trafficking, mainly late secretory and vacuolar trafficking, and the AP3 and AP4 complexes are also required for vacuolar trafficking (Fan et al., 2013; Kim et al., 2013; Park et al., 2013; Fuji et al., 2016; Feng et al., 2017). Among the four subunits of the AP complex, the medium subunit (M, μ) is the one for recognition of the phenylalanine or the tyrosine-based motif (YXX Φ , Φ is a residue with bulky hydrophobic side chain) at respectively the beginning or the end of PIN hydrophilic loop as sorting signals (Kleine-Vehn et al., 2011; Sancho-Andrés et al., 2016). Subsequently, the membrane domains containing sorted PIN cargo will be coated with clathrin light chains (CLCs) and clathrin heavy chains (CHCs) to initiate endocytosis. An Arabidopsis CLC T-DNA null allele and a dominant-negative mutant both verified the importance of clathrin in PIN endocytosis and polarity establishment (Wang et al., 2013; Kitakura et al., 2011; Yu et al., 2016; Dhonukshe et al., 2007). In these clathrin mutants, PIN1 is evenly distributed on every side of the cell and fails to form intracellular aggregates after BFA treatment. By comparative analysis, other CME components have been identified in Arabidopsis, such as the dynamin-related proteins DRP2B and DRP1A that act as molecular scissors that bud off clathrin-coated vesicle (CCV) from the PM (Fujimoto et al., 2010). In addition, the TPLATE adaptor complex has been reported as a plant-specific CME-related complex that arrives on the PM at the endocytic site preceding DRP and AP2 to function as the earliest marker for CME (Gadeyne et al., 2014).

In addition to the structural variation between cellular compartments, their membranes are marked by different phospholipids to attract binding of different sorting-related proteins. Many proteins we mentioned above, such as VAN3/SFC, the AP complex and DRPs, contain lipid binding domains. They exhibit differential binding affinity to different phospholipids. The

pleckstrin homology (PH) domain of VAN3/SFC, for example, specifically binds to phosphatidylinositol-4-monophosphate [PI(4)P] (Carland and Nelson, 2009). This interesting phenomenon hints that enzymes involved in phospholipid biosynthesis may be able to regulate PIN intercellular trafficking as well. Several phospholipid enzymes such as phospholipase D (PLD ζ 2), phospholipase A₂ (PLA2), phosphatidylinositol 4-phosphate 5-kinases (PIP5K1 and PIP5K2), and phosphoinositide 5-phosphatases (COTYLEDON VASCULAR PATTERN 2/CVP2 and CVP2-LIKE1/CVL1), have been shown to be involved in the regulation of intracellular PIN trafficking (Li and Xue, 2007; Lee et al., 2010; Mei et al., 2012; Tejos et al., 2014). Also other lipids, such as sterols, have been shown to mark polar domains in the cell (Frescatada-Rosa et al., 2014). Enzymes in early sterol biosynthesis such as sterol methyltransferases (SMTs) and FORKED1/FOERKED-LIKES (FKD1/FLs), were shown to control vascular development by regulating PIN polarity (Willemssen et al., 2003; Carland et al., 2010; Steynen and Schultz, 2003; Mariyamma et al., 2018). Phospholipid-regulated auxin transport is relying on CME of PIN protein, and on the activity of membrane-associated AGC kinases, which we will discuss in the “post-translational regulation of AGC kinases” section.

5.2 PIN phosphorylation, polarity or activity?

The PM abundance and polarity of the PIN1-type PINs has been shown to be regulated by post-translational modification by phosphorylation of the PIN HL on the one hand (Friml et al., 2004; Michniewicz et al., 2007; Dhonukshe et al., 2010; Huang et al., 2010; van Gelderen, 2017) and by ubiquitination on the other hand (Abas et al., 2006; Leitner et al., 2012). In addition, phosphorylation has been reported to activate the PIN auxin efflux carriers (Barbosa et al., 2014; Haga et al., 2018; Marhava et al., 2018). Up to now, a large number of relatively conserved serine and threonine residues have been identified to be functional phosphorylation sites (Ganguly et al., 2012; Barbosa et al., 2018). Among them, seven serines (S209^{PIN3}, S212^{PIN3}, S215^{PIN3}, S1, S2, S3, S337^{PIN1}) and four threonines (T227^{PIN1}, T248^{PIN1}, T286^{PIN1} and T340^{PIN1}) are correlated to PIN subcellular localization, while the other two serines (S4 and S5) govern PIN activity (Ganguly et al., 2012; Ki et al., 2016; Barbosa et al., 2018) (Figure 4). In Arabidopsis, AGC kinases (named after the cyclic AMP dependent protein kinase A, cGMP-dependent protein kinase G, and the diacylglycerol- or calcium-dependent protein kinase C in animals), MITOGEN-ACTIVATED PROTEIN KINASES (MPKs) and Ca²⁺/calmodulin-dependent kinase-related kinase 5 (CRK5) have been identified to be involved in phosphorylation of these ten serine and threonine residues.

AGC kinases are the most well-studied serine/threonine kinases regulating PIN phosphorylation, as the AGC kinase PINOID (PID) was the first discovered PIN-related kinase. Loss-of-function *pid* mutants share the pin-like inflorescence phenotype with *pin1* loss-of-function mutants (Bennett et al., 1995; Christensen et al., 2000; Benjamins et al., 2001), and PID was shown to induce a basal-to-apical shift in PIN polarity by phosphorylating three conserved serine residues in the PINHL (Friml et al., 2004; Huang et al., 2010). The Arabidopsis genome encodes 39 AGC kinases, of which two distinct subgroups, the AGC3 kinases PID, WAG1 and WAG2 and the AGC1 kinases D6PK and the D6PK-LIKES (D6PKLs), have been genetically and biochemically verified to phosphorylate the PINHL (Rademacher and Offringa, 2012; Habets and Offringa, 2014; Barbosa et al., 2018).

The closest paralogs of *PID*, *WAG1* and *WAG2*, control auxin-dependent root waving (Santner and Watson, 2006). Furthermore, *PID/WAG1/WAG2* redundantly regulate cotyledon development, root growth and PIN3-mediated hypocotyl phototropism (Cheng et al., 2008; Dhonukshe et al., 2010; Ding et al., 2011; Haga et al., 2014); *PID/WAG2* control dehiscence in fruit development by modulating PIN3 PM abundance (van Gelderen et al., 2016); *WAG2* is involved in apical hook opening (Willige et al., 2012), whereas *PID* controls stigma and style formation (Benjamins et al., 2001). Moreover, recent research has shown that *OsPID* also controls stigma and style formation in rice (He et al., 2019; Xu et al., 2019). *PID/WAGs* are evenly associated with the PM and phosphorylate serine residues in conserved TPRXS motifs in the PIN HL (Huang et al., 2010; Dhonukshe et al., 2010). *PID*-directed phosphorylation is proposed to recruit PIN protein into GNOM-independent recycling pathway and to shift PIN from the basal side of the cell to the apical side (Friml et al., 2004; Kleine-Vehn et al., 2009; Huang et al., 2010).

Different from *PID/WAGs*, the AGC3 kinases D6PK/D6PKLs are basally localized on the PM, they phosphorylate the PINHL at the conserved serines S4 and S5, thereby causing local activation of PIN-mediated auxin transport (Figure 4) (Willige et al., 2013; Zourelidou et al., 2009; Barbosa et al., 2014). D6PK/D6PKLs participate in many developmental processes like shoot and lateral root differentiation, and response to environmental factors like hypocotyl phototropism and root gravitropism (Barbosa et al., 2014; Willige et al., 2013; Haga et al., 2018; Barbosa et al., 2016; Zourelidou et al., 2009). *PAX/AGC1-13* and *AGC1-12* are close orthologs of D6PK/D6PKLs, and they control respectively root protophloem differentiation and phototropism in a similar way as the D6PK/D6PKLs by PIN phosphorylation (Marhava et al., 2018; Haga et al., 2018).

The distinct PIN regulation mechanisms between *PID/WAGs* and D6PK/D6PKLs have been explained by differential phosphorylation site preference, although they both can phosphorylate S1-S5 *in vitro* (Zourelidou et al., 2014). Recently, this model has been challenged, based on the observation that phosphorylation of S1-S3 seemed to be insufficient to change PIN polarity, as phosphosite-specific antibodies show basal instead of apical accumulation of S1-S3 phosphorylation (Weller et al., 2017). *PID* interaction with other proteins but not PIN phosphorylation status itself are assumed to be responsible for PIN polarity shifting (Weller et al., 2017). However, the phosphosite immunolocalization cannot explain the previously observed non-phosphorylated (S1,2,3A) and phosphomimic (S1,2,3E) PIN distribution (Huang et al., 2010; Dhonukshe et al., 2010). Moreover, T227, T248 and T286 belonging to the TPRXS motifs are included in PIN1 peptides used for antibody production (Weller et al., 2017). Recently, phosphorylation of the three threonine residues by MPK4 and MPK6 was shown to result in changes in PIN localization (Dory et al., 2018), suggesting that the threonines and serines co-evolved in the same motif allowing polarity regulation by different signaling pathway. If phosphorylation of both serines and threonines are essential for the basal-to-apical polarity shift, this may explain why apical localized PINs are not detected by the phosphosite-specific antibodies. However, this hypothesis needs to be experimentally verified. We still hesitate to make a final conclusion about the mechanism of *PID/WAG*-directed PIN polarity shift. It is more likely that S1, S2 and S3 phosphorylation is sufficient to alter PIN subcellular localization, but that *PID* also interacts with other factors to make the

polarity shift more efficient. In the later section for AGC kinase regulation, we will mention some PID interactors possibly related to this process.

Apart from the AGC kinases and MPK4/6, the PINHL is also found to be a phosphorylation target of MPK6, which is downstream of MPK KINASE7 (MKK7). MPK6 phosphorylates PIN1 at S337 (Jia et al., 2016). The study on MKK7-MPK6-PIN cascade provided strong genetic and cytological evidence for an explanation of the phenotype of the *MKK7* gain-of-function mutant *bushy and dwarf1 (bud1)* and the earlier discovered PIN1 polarity alteration after mutating S337 (Zhang et al., 2010; Jia et al., 2016; Dai et al., 2006). In addition, CRK5 is a unique plasma membrane-associated kinase regulating PIN phosphorylation. The *crk5* knock-out mutant exhibits shoot and root gravitropic defects as a result of slower PIN2 recycling (Rigó et al., 2013). However, the particular phosphorylation site for CRK5 has not been identified.

Corresponding to these kinases, many phosphatases antagonistically dephosphorylate PIN proteins to adjust their intracellular trafficking or activity. Protein phosphatase 2A A (PP2AA), phytochrome-associated serine/threonine protein phosphatases (FyPP1 and FyPP3) and SAPS DOMAIN-LIKE (SAL) can form a heterotrimeric protein phosphatase 6 holoenzyme (PP6) that partially co-localizes with and dephosphorylates PINs at the PM (Dai et al., 2012). Mutation of any one of these three components causes PIN polarity shifts resulting in strong growth defects (Michniewicz et al., 2007; Li et al., 2011; Dai et al., 2012). For example, the *pp2aa1 pp2aa3* double mutant displays insensitivity to NPA, abnormal embryo development and short root phenotype at the seedling stage. The basal-to-apical shift in PIN polarity in the *pp2aa1 pp2aa3* mutant is reversed in the *pid* mutant background, and this partially rescues the root growth defects (Michniewicz et al., 2007). Another PP2A catalytic subunit mutant *pp2a-c3 pp2a-c4* shows an even more severe phenotype with roots being stunted or even absent (Ballesteros et al., 2013). In addition, type-one protein phosphatase 4 (TOPP4) dephosphorylates PIN1 to regulate the interdigitated pavement cell patterning via PIN polarity modulation (Guo et al., 2015).

5.3 PIN protein abundance on the membrane

Vacuole-targeted degradation and MAB4-mediated PM retaining are two reported mechanisms affecting PIN protein abundance on the PM. PIN vacuolar sorting and degradation are critical for root growth and gravitropism (Sassi et al., 2012; Leitner et al., 2012; Hsua and Jauh, 2017; Abas et al., 2006). The retromer complex, the endosomal sorting complexes required for transport (ESCRT)-related CHMP1A and CHMP1B proteins and ESCRT-II component VPS36, and PIN2 ubiquitination are all essential for this process (Spitzer et al., 2009; Kleine-Vehn et al., 2008; Wang et al., 2017; Leitner et al., 2012). Different from vacuolar sorting, ENHANCER OF PINOID/NAKED PINS IN YUC MUTANTS/MACCHI-BOU 4 (ENP/MAB4/NPY1) and its orthologs MAB4/ENP/NPY1-LIKE1 (MELs) enhance PIN protein membrane abundance by local interaction and inhibition of PIN endocytosis (Furutani et al., 2011; Cheng et al., 2008; Furutani et al., 2007; Cheng et al., 2007b). Recently, a connection between PID-directed PIN protein phosphorylation and PIN PM abundance has been found. MAB4 preferentially forms a trimeric complex with PID and phosphorylated PIN, leading to PIN microdomains and reducing PIN mobility on the PM (van Gelderen, 2017). This is in line

with the observation that PIN phosphorylation strongly reduces dark-induced PIN vacuole targeting (van Gelderen, 2017).

6 Transcriptional and post-translational regulation of AGC kinases

In recent years, more and more regulation mechanisms on AGC kinases have been reported in plants. For instance, the 3-phosphoinositide-dependent kinase-1 (PDK1) was shown to phosphorylate AGC1 kinases, AGC2-1 and S6Ks to modulate their activity *in vitro* and/or *in vivo* (Anthony et al., 2004; Devarenne et al., 2006; Otterhag et al., 2006; Zegzouti et al., 2006b; Matsui et al., 2010; Camehl et al., 2011; Gray et al., 2013). The subcellular localization of membrane-localized AGC kinases, which possess their own lipid-binding domain, is regulated by lipid biosynthesis pathway (Stanislas et al., 2015; Zegzouti et al., 2006b). For the reason that our research interest is on the regulation of PID/WAGs-related signaling, this section will mainly focus on the regulation on this AGC3 subgroup.

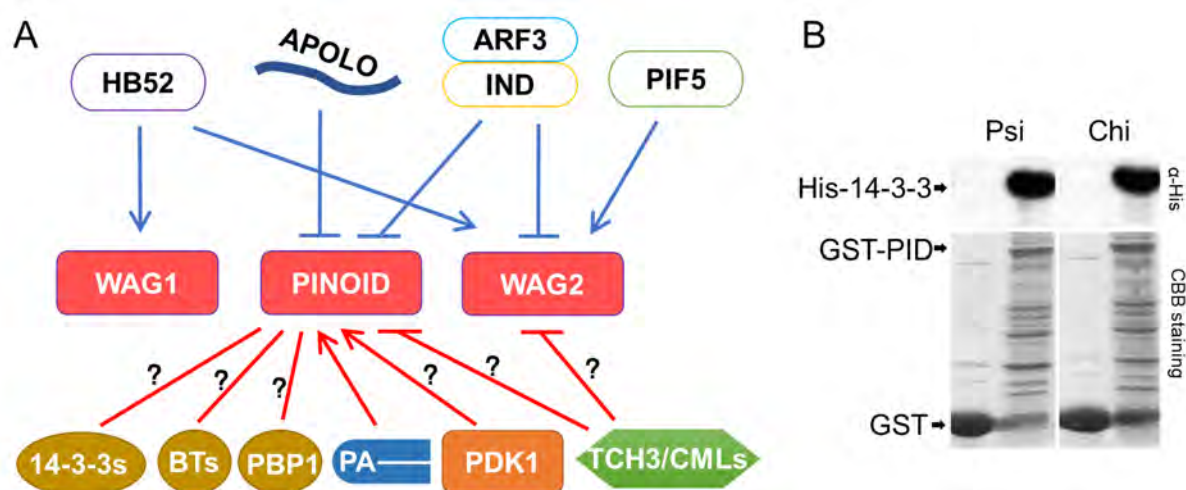


Figure 5. Regulation of AGC3 kinases. (A) PID/WAGs transcriptional and post-translational regulation model. Transcriptional interactions and protein interactions are linked with blue and red lines respectively. Lines with arrow and butt mean positive and inhibiting effect, respectively. Naked line means undefined effect. Interactions without *in planta* proof are noted with question marks. (B) Pull-down assay with GST-PID and His-14-3-3s.

PID and/or WAGs transcription is controlled by various signals such as auxin, light, ethylene and GA (Ding et al., 2011; Willige et al., 2012; Simonini et al., 2016). As mentioned previously, ARF3 directly binds to two auxin response elements (AuxREs) in *PID* promoter, by forming a complex with ETT, to repress *PID* expression (Simonini et al., 2016). The IND-mediated repression is also found for WAG2 in the indehiscence of siliques (van Gelderen et al., 2016). The downstream transcription factor of GA and light, PHYTOCHROME INTERACTING FACTOR 5 (PIF5) directly binds to the G-box in WAG2 promoter to activate WAG2 expression, thereby controlling apical hook opening via modulation of PIN-directed auxin transport (Willige et al., 2012). HOMEODOMAIN PROTEIN 52 (HB52) physically binds to WAG1 and WAG2 promoter to mediate crosstalk between ethylene and auxin transport (Miao et al., 2018). By analyzing auxin induced accumulation of long intergenic noncoding RNA (lincRNA) AUXIN REGULATED PROMOTER LOOP RNA (APOLO RNA), scientists found the co-repression and co-activation phenomenon of *APOLO* and *PID* transcripts. Further study suggests that

the *APOLO* transcript helps to form a chromatin loop in the promoter region of itself and its neighboring gene *PID* to repress their expression. Auxin induces demethylation and opening of the encompassing site of the chromatin loop, and leads to increased *PID* and *APOLO* transcription (Ariel et al., 2014). These transcription regulation mechanisms are all based on solid biochemical and genetic evidence, while the post-translation regulation PID/WAGs that we will discuss below is mostly supported by *in vitro* data.

At present, PID kinase activity has been proposed to be regulated by two factors, PDK1 and phosphatidic acid (PA). PDK1 has been reported to directly interact with the PDK1-interacting fragment (PIF) domain of PID and to phosphorylate S288 and S290 to increase PID kinase activity *in vitro* and in protoplast (Zegzouti et al., 2006a). PA has been shown to bind to the PID insertion domain (ID) in the catalytic domain in a dosage-dependent manner to activate PID phosphorylation on PIN2. This mechanism was shown to be crucial for salt stress tolerance in Arabidopsis (Wang et al., 2019). Another PID kinase activity controller identified in a yeast two-hybrid screen using PID as bait is the calmodulin-like protein TOUCH3 (TCH3) (Benjamins et al., 2003) (Fan, 2014). TCH3 has a dual role in PID regulation. On the one hand, TCH3 interacts with the PID ID and reduces its activity *in vitro*. On the other hand, TCH3 rapidly recruits PID from the PM to the cytosol, in an auxin-dependent manner, to disrupt PID-PIN interaction in protoplasts and root epidermis cells (Fan, 2014). The sequestration from the PM to the cytosol can also be observed between PID/WAG2 and CML10 (Fan, 2014). The same yeast two-hybrid screen also identified BTB and TAZ domain scaffold protein 1 (BT1/PBP2) and Ca²⁺ binding protein PINOID-BINDING-PROTEIN 1 (PBP1) as PID interaction proteins (Benjamins et al., 2003; Robert et al., 2009). The interactions have been confirmed in *in vitro* pull-down assays. Recently, we identified two 14-3-3 proteins (Psi and Chi) following co-immunoprecipitation-mass spectrometry (Co-IP-MS) from 35S::PID::YFP seedling extracts using anti-GFP antibody. Their direct interactions were verified in *in vitro* pull-down (Figure 5B) and semi-*in vivo* pull-down assays (data not shown, protocol from Cai et al., 2014). However, like PDK1, the *in vivo* evidence for all the interactors mentioned above (except for PA) is still not available yet, and the functional relevance unknown.

Thesis outline

The PID-directed shift in PIN polarity has been broadly accepted as one of the essential mechanisms for the regulation of auxin transport polarity. However, there are very limited reports about how PID is regulated at the post-translation level and how PINs can be differentially sorted to different sides of the cell after phosphorylation by PID.

To uncover the upstream signals, we first checked the functionality of PDK1, which has been proposed to activate PID *in vitro* (Zegzouti et al., 2006a). Neither *PDK1* overexpression lines nor newly created *pdk1 pdk2* null alleles phenocopied PID overexpression or mutant lines. In **chapter 2**, we verified that both PID functionality and its subcellular localization do not depend on PDK1 function. However, the *pdk1 pdk2* mutant did show many auxin-related growth defects. By detailed analysis on these phenotypes and the expression of the auxin response reporter, we found the *pdk1 pdk2* double mutant to be impaired in auxin transport in vascular tissues. Together with the mutant phenotypes in reproductive organs and other phenotypes in dark growth condition, we suspect that PDK1 may be the master regulator of AGC1 kinases.

A detailed description of *pdk1 pdk2* phenotypes and the correlation with auxin transport are presented in **chapter 2**. Based on the *pdk1 pdk2* short root phenotype caused by phloem differentiation defects that phenocopied the *pax* mutant, we suspected that PDK1 acts in part by regulating the AGC1 kinase PAX. To get further evidence, we introduced wild type and a phosphomimic version of PAX (AGC1.3) into the *pdk1 pdk2* background to rescue the root growth caused by protophloem development failure. **Chapter 3** shows that PDK1-dependent PAX phosphorylation and activation are essential for its full biological function. In **chapter 3**, we also explain the molecular basis of PDK1 basal localization and the unnecessary of this polarity for vascular development. **Chapter 4** is about the investigation of downstream action after PID phosphorylation. We mutated several conserved tyrosine residues close to serine phosphorylation sites in the PIN1 and PIN2 HLs. Two of these tyrosines redundantly affected PIN polarity. However, observation of PIN2 polarity and genetic evidence in *35S::PID* background state that PID-mediated phosphorylation and tyrosine-based PIN trafficking are independent processes.

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Chapter 2

PDK1 has a pleiotropic PINOID-independent role in Arabidopsis development

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Abstract

The 3-Phosphoinositide-Dependent Protein Kinase 1 (PDK1) is a conserved and important master regulator of AGC kinases in eukaryotic organisms. *pdk1* loss-of-function causes a lethal phenotype in animals and yeast. In contrast, only very mild phenotypic defects have been reported for the *pdk1* loss-of-function mutant of the model plant *Arabidopsis thaliana* (Arabidopsis). The Arabidopsis genome contains two *PDK1* genes, hereafter called *PDK1* and *PDK2*. Here we show that the previously reported Arabidopsis *pdk1* T-DNA insertion alleles are not true loss-of-function mutants. By using CRISPR/Cas9 technology, we created true loss-of-function *pdk1* alleles, and *pdk1 pdk2* double mutants carrying these alleles showed multiple growth and development defect, including fused cotyledons, a short primary root, dwarf stature, late flowering, and reduced seed production caused by defects in male fertility. Surprisingly, *pdk1 pdk2* mutants did not phenocopy *pid* mutants, and together with the observations that *PDK1* overexpression does not phenocopy the effect of *PID* overexpression, and that *pdk1 pdk2* loss-of-function does not change PID subcellular localization, we conclude that PDK1 is not essential for PID membrane localization or functionality *in planta*. Nonetheless, most *pdk1 pdk2* phenotypes could be correlated with impaired auxin transport. *PDK1* is highly expressed in vascular tissues and YFP:PDK1 is relatively abundant at the basal/rootward side of root stele cells, where it colocalizes with PIN auxin efflux carriers, and the AGC1 kinases PAX and D6PK/D6PKLs. Our genetic and phenotypic analysis suggests that PDK1 is likely to control auxin transport as a master regulator of these AGC1 kinases in Arabidopsis.

Introduction

Protein phosphorylation by protein kinases is a ubiquitous and crucial posttranslational modification in eukaryotic cells. It is involved in almost all cell activities, such as cell division, cell growth and environmental signaling. The AGC kinase family comprises some of the best-characterized protein serine/threonine kinases in eukaryotic cells, such as the founder members cyclic AMP-dependent protein kinase A (PKA) and calcium-dependent protein kinase C (PKC) (Pearce et al., 2010). These kinases play crucial roles in basal cellular functions in lower (yeast) and higher (human/mice) eukaryotes. For example, protein kinase B (PKB/c-Akt) is important in apoptosis inhibition and insulin signaling (Lawlor and Alessi, 2001), whereas p70 ribosomal protein S6 kinase (S6K) plays a vital role in mRNA translational control (Pearce et al., 2010; Bahrami-B et al., 2014). AGC kinases themselves are also phosphorylation substrates that can be activated by serine/threonine phosphorylation in the activation loop (T-loop) or in the C-terminal hydrophobic motif of the kinase domain (H-motif) (Chamoto et al., 2010). The 3-Phosphoinositide-Dependent Protein Kinase 1 (PDK1) is a well-established activator responsible for AGC kinase T-loop phosphorylation (Mora et al., 2004; Chamoto et al., 2010).

PDK1 itself is also a conserved member of AGC kinase family, and typically contains a kinase domain with a PDK1-Interacting Fragment (PIF)-binding pocket at its N-terminus and a PH domain at the C-terminus (Biondi et al., 2000; Frödin et al., 2002). Other AGC kinases have a C-terminal hydrophobic PIF motif, and the interaction with the PIF binding pocket in PDK1 enhances their activation by phosphorylation. The PH domain is essential for PDK1 plasma membrane recruitment and kinase activity in mammals. Binding of the PH domain to the phospholipid phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P3] at the plasma membrane triggers PDK1 dimer to monomer conversion and phospho-activation (Alessi et al., 1997; Ziemba et al., 2013). PDK1 was originally named PtdIns(3,4,5)P3-dependent protein kinase 1 (Alessi et al., 1997), but the name was changed when PtdIns(3,4)P2, PtdIns3P and PtdIns(4,5)P2 also appeared to bind its PH domain (Currie et al., 1999; Deak et al., 1999). *Arabidopsis thaliana* PDK1 (AtPDK1) binds to an even broader selection of phospholipids *in vitro* (Deak et al., 1999). Nevertheless, the two most important phospholipids for mammalian PDK1, PtdIns(3,4,5)P3 and PtdIns(3,4)P2, have not been identified in *Arabidopsis thaliana* (Arabidopsis) (Heilmann, 2016), and AtPDK1 activity has been reported to be controlled by PtdIns(4,5)P2 and phosphatidic acid (PA) (Anthony et al., 2004). Arabidopsis has two highly homologous *PDK1* genes, At5g04510 (*AtPDK1.1*) and At3g10540 (*AtPDK1.2*), and for convenience reasons we renamed them to respectively *PDK1* and *PDK2*.

The yeast PDK1 orthologs Pkh1 and -2, which lack a PH domain, still have the ability to phosphorylate AGC kinases (Casamayor et al., 1999; Niederberger and Schweingruber, 1999; Voordeckers et al., 2011). *Physcomitrella patens* PDK1 (PpPDK1), which also lacks a PH domain, is able to rescue the lethal phenotype of the yeast *pkh1 pkh2* double mutant. This indicates that the PH domain is not required in all eukaryotes or full PDK1 functionality (Dittrich and Devarenne, 2012a). Besides yeast, complete loss-of-function of *PDK1* is also lethal for fruit flies and mice (Lawlor et al., 2002; Rintelen et al., 2002). In plants, several methods have been employed in different species to analyze PDK1 function *in planta*. Virus-induced gene silencing (VIGS) has been used to knock down tomato *PDK1*, whereas *Tos17* transposon

mutagenesis or homologous recombination has been used in rice or *Physcomitrella patens*, respectively. However, in tomato the claimed cell death phenotype made *PDK1* knock-out expression unprovable (Devarenne et al., 2006), and in rice the *Tos17* insertion only led to a knock-down of *PDK1* expression (Matsui et al., 2010; Dittrich and Devarenne, 2012a). Deletion of *PDK1* in *Physcomitrella patens* was not lethal, but *pdk1* knock-out mutants showed strong developmental defects (Dittrich and Devarenne, 2012a). In Arabidopsis, three combinations of *pdk1 pdk2* T-DNA insertion alleles have been reported to show altered sensitivity to *Piriformospora indica* induced growth promotion, and a weak developmental defect resulting in reduced silique length (Camehl et al., 2011; Scholz et al., 2019). Inhibition of *PDK1* expression in Arabidopsis cell suspensions using RNAi technology delivered no mutant cell phenotype (Anthony et al., 2004).

In contrast to the lack of a clear *in planta* role for PDK1, all Arabidopsis AGC kinases phosphorylated by PDK1 *in vitro*, including PINOID (PID), Oxidative Signal-Inducible1(OXI1), UNICORN (UCN) and most AGC1 family members, do play key roles in plant development and defense (Anthony et al., 2004, 2006; Zegzouti et al., 2006a, 2006b; Devarenne et al., 2006; Camehl et al., 2011; Enugutti et al., 2012; Gray et al., 2013; Scholz et al., 2019). PID phosphorylates PIN auxin efflux carriers to control their polarity and thereby direct the auxin flux (Dhonukshe et al., 2010; Huang et al., 2010; Ding et al., 2011). OXI1 plays a dual role in regulating both root hair growth and the basal immune response against virulent pathogen infection (Anthony et al., 2004; Rentel et al., 2004; Anthony et al., 2006; Petersen et al., 2009; Matsui et al., 2010; Camehl et al., 2011). UCN was recently shown to be a phosphorylation target of PDK1 *in vitro*, but genetic evidence suggests that UCN negatively regulates PDK1 at the post-transcriptional level to control planar growth (Scholz et al., 2019). The other established PDK1 targets all belong to the AGC1 protein kinases family (Galván-Ampudia and Offringa, 2007; Rademacher and Offringa, 2012), which has been well-characterized during the past decade. The D6 protein kinases (D6PKs, including D6PK/AGC1.1, D6PKL1/AGC1.2, D6PKL2/PK5, D6PKL3/PK7), PROTEIN KINASE ASSOCIATED WITH BRX (PAX/AGC1.3), PAX LIKE (PAXL/AGC1.4) and AGC1-12 all have been shown to phosphorylate PIN proteins to enhance auxin transport activity (Zourelidou et al., 2009; Willige et al., 2013; Barbosa et al., 2014, 2018; Haga et al., 2018; Marhava et al., 2018). The tomato ortholog of PAX, also known as AvrPto-dependent Pto-interacting protein 3 (Adi3), negatively controls plant cell death caused by pathogen attack (Devarenne et al., 2006; Gray et al., 2013). AGC1.5 and AGC1.7 control the polar growth of pollen tubes by phosphorylating RopGEFs. (Zhang et al., 2009; Li et al., 2018), and ROOT HAIR SPECIFIC 3 (RSH3/AGC1.6) specifically regulates root hair morphology (Won et al., 2009). The disproportion between the *in vivo* data on the functions of the different Arabidopsis AGC kinases that are established *in vitro* phosphorylation targets of AtPDK1, and the small role that AtPDK1 itself seems to play in development based on the *pdk1 pdk2* double mutant phenotype, made us reinvestigate the published data on *AtPDK1*. PID has been reported as one of the prime targets of PDK1 (Zegzouti et al., 2006a), but the *pdk1 pdk2* double mutant lacks the typical *pid* loss-of-function phenotypes. We therefore generated Arabidopsis lines overexpressing *PDK1* (*PDK1ox*), and found that seedlings of these lines lacked the strong phenotypes observed in seedlings overexpressing *PID* (*PIDox*). These results suggest that either PDK1 requires activation and that this is not triggered in the *PDK1ox* seedlings, or that it is not rate-limiting for PID activity. Next, we re-analyzed the

published *pdk1* and *pdk2* T-DNA insertion alleles. Based on RT-PCR experiments, the two *pdk2* alleles appeared to represent true loss-of-function mutants. However, functional *PDK1* mRNA was still detectable in the three *pdk1* alleles, explaining the lack of strong phenotypes in the *pdk1 pdk2* double mutant combinations. Using CRISPR/Cas9, we generated several true *pdk1* loss-of-function mutant alleles, which when combined with the *pdk2* T-DNA insertion allele did display strong growth and developmental defects. The mutant phenotypes indicate a pleiotropic, but PID-independent role for PDK1 in plant development as a regulator of auxin transport.

Results

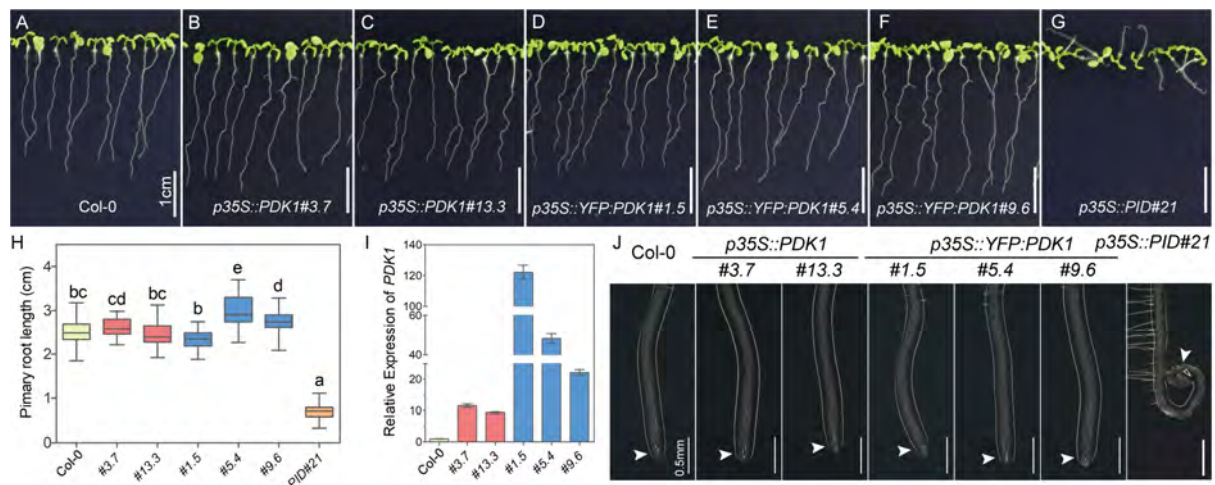


Figure 1. Seedling and root phenotype of *PDK1* and *PID* overexpression lines. A-G, Representative 7-day-old seedlings for indicated lines. Please note that only *p35S::PID#21* seedlings show agravitropic growth. Scale bars represent 1cm. H, Box plot with Min/Max whiskers showing the quantification of the primary root length of 7-day-old seedlings of Arabidopsis wild type (Col-0), *p35S::PDK1* lines #3.7 and 13.3 (red box), *p35S::YFP:PDK1* lines #1.5, 5.4 and 9.6 (blue box), and *p35S::PID* line #21. The results are from a single experiment ($n > 36$ per line), but similar results were obtained in 3 experimental repeats. Lower case letters indicate statistically different groups ($p < 0.05$), as determined by a one-way ANOVA followed by Tukey's test. I, *PDK1* expression levels in Col-0 and in the *p35S::PDK1* and *p35S::YFP:PDK1* lines used in H. The bar graph shows the mean value $\pm$ SEM. J, Representative images showing a detail of the root tip phenotype of seedlings in A-H. White arrow heads point out collapsed (*p35S::PID#21*) or normal root meristems (all other lines). Scale bar indicates 0.5 mm.

PDK1ox and *PIDox* seedlings do not share phenotypes

The key defects caused by *PIDox* in Arabidopsis are agravitropic seedling growth and collapse of the main root meristems as a result of redirected polarity of PIN-mediated auxin transport (Figure 1G, H, J) (Benjamins et al., 2001; Friml et al., 2004). In view of the model that *PDK1* regulates *PID* kinase activity (Zegzouti et al., 2006a), we expected *PDK1ox* to cause similar phenotypes as *PIDox*. More than thirty independent *p35S::YFP:PDK1* or *p35S::PDK1* transgenic lines were selected and T2 seedlings grown on vertical agar plates showed normal gravitropic growth. Five single locus homozygous T3 lines with different *PDK1* overexpression levels were subsequently selected for further phenotype observation and quantification (Figure 1I). All of the representative *PDK1ox* lines showed normal gravitropic seedling growth and no collapse of the main root meristem was observed (Figure 1). Roots of *p35S::YFP:PDK1#5.4* and *p35S::YFP:PDK1#9.6* seedlings were even slightly longer than wild-type roots (Figure 1H), however, this phenotype did not clearly correlate with the *PDK1*

overexpression level (Figure 1I). Also mature *PDK1ox* plants developed and flowered like wild-type *Arabidopsis* plants.

The above results suggest that PDK1 is not rate-limiting for endogenous PID activity. However, we cannot exclude that the PDK1 kinase itself requires signaling to be activated, and that therefore its overexpression does not lead to additional phenotypes under normal growth conditions.

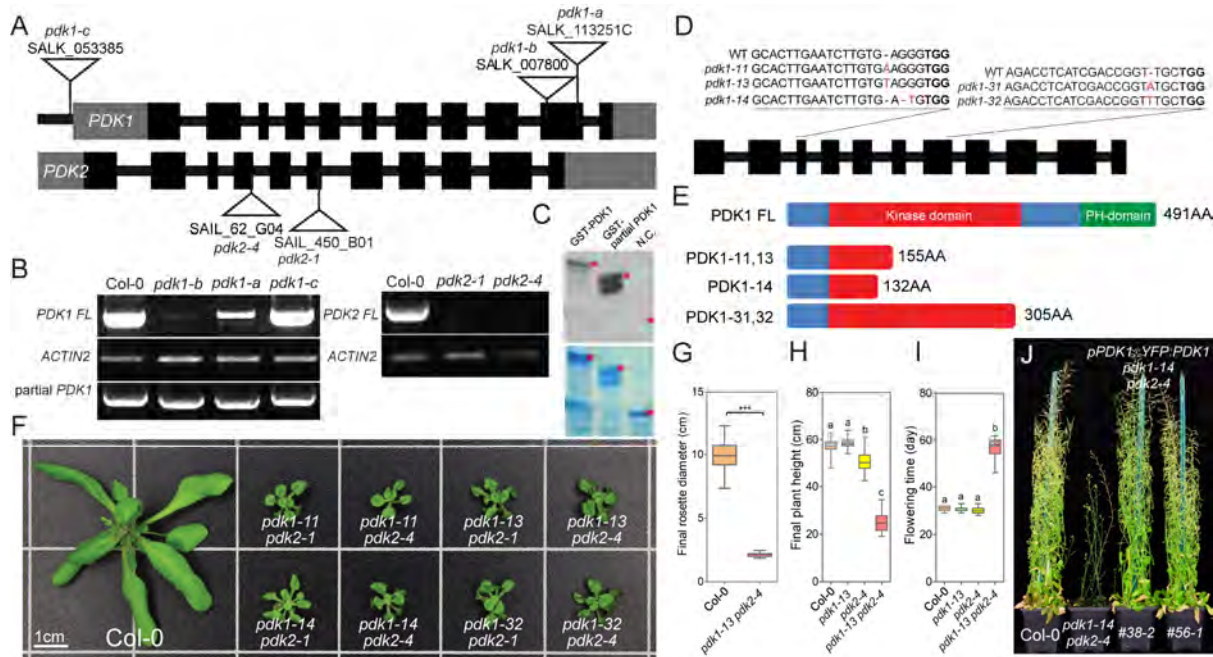


Figure 2. *Arabidopsis* double mutants that combine the new CRISPR/Cas9-generated *pdk1* loss-of-function alleles with one of the *pdk2* loss-of-function T-DNA alleles exhibit a dwarf stature. A, Schematic representation of the *PDK1* and *PDK2* gene structure, with the T-DNA insertion positions and names of the available mutant alleles indicated. Wide black boxes represent exons, gray boxes represent 5' and 3' untranslated regions (UTRs), and narrow black boxes represent introns and promoter sequence upstream of *PDK1* 5' UTR. B, Semiquantitative RT-PCR to detect *PDK1* or *PDK2* expression in the different *pdk1* or *pdk2* T-DNA insertion mutant alleles, respectively. C, Autophosphorylation activity of GST-PDK1 and GST-PDK1S0 (lacking PH domain, similar size as partial PDK1 in *pdk1-b*). N.C.: Negative Control, GST-PDK1 with the incomplete kinase domain. The bands of these GST tagged proteins are marked with red asterisks. D, Schematic representation of part of the *PDK1* gene with the guide RNA target sites and the resulting mutations in the newly obtained CRISPR/Cas9-generated alleles. The PAM sequence for Cas9 is highlighted in bold. Inserted or replaced nucleotides in the new mutant alleles are highlighted in red. Mutant alleles obtained at editing site 1 (3rd exon) and site 3 (7th exon) are named *pdk1-1n* and *pdk1-3n*, respectively. Since *pdk1-12* and *pdk1-13* have the same "T" insertion, only *pdk1-13* is shown. E, Schematic linear representation of the full length (FL, 491 amino acids) PDK1 protein (protein kinase domain: amino acids 44 to 311, PH domain: amino acids 386 to 491, <https://www.uniprot.org>), and the shorter versions produced in the *pdk1-11*, -13, -14, -31 and -32 alleles. F, Rosette phenotype of 30-day-old wild-type *Arabidopsis* (Col-0) and eight different *pdk1 pdk2* loss-of-function allele combinations. G, Quantification of the rosette diameter of the 30-day-old *Arabidopsis* wild-type (Col-0, n=30) and *pdk1-13 pdk2-4* (n=19) plants shown in F. Asterisks indicate significant differences (t-test, P<0.0001). H, Final plant height of the indicated lines (n>14). I, Flowering time of the indicated lines (n>14). Letters a, b, and c in H and I indicate statistical differences, as determined by one-way ANOVA followed by Tukey's test (p<0.05). J, Introduction of *pPDK1::YFP:PDK1* in the *pdk1-14 pdk2-4* background completely rescues the mutant phenotype.

CRISPR/Cas9-generated mutant alleles indicate a central role for PDK1 in development

To obtain further indications for the proposed role of PDK1 as an upstream regulator of PID, we re-assessed the previously described *pdk* loss-of-function mutant alleles. Three *pdk1* and two *pdk2* T-DNA insertion alleles have been reported to be loss-of-function mutants (Camehl et al., 2011; Scholz et al., 2019). Neither *pdk1* nor *pdk2* single mutants showed any noticeable

phenotype, and different double mutant combinations of the *pdk1* and *pdk2* alleles only showed a mild reduction in silique length and plant height (Camehl et al., 2011; Scholz et al., 2019). Two *pdk2* alleles, *pdk2-1* and *pdk2-4*, were confirmed to be true knock-out mutants by RT-PCR analysis (Figure 2A, B). However, in contrast to published data, the *pdk1-c* allele appeared to produce a full length mRNA (Figure 2A, B) (Camehl et al., 2011; Scholz et al., 2019), whereas the *pdk1-a* and *pdk1-b* alleles produced a partial or mutated mRNA (Figure 2A, B, Figure S1), leading to the production of a PDK1 kinase lacking its PH domain (Figure S1). Previous studies have suggested that a PH domain may not be essential for PDK1 function in plants (Dittrich and Devarenne, 2012b). When we tested the kinase activity of PDK1 lacking a PH domain *in vitro*, it showed very high autophosphorylation activity (Figure 2C). Based on these findings, we concluded that the three published *pdk1* T-DNA insertion alleles are not likely to be true loss-of-function mutants.

In order to obtain true *pdk1* alleles for studying PDK1 biological function, we designed guide RNAs against the 3rd and 7th exon, and were able to obtain five CRISPR/Cas9-generated mutants with frame shifts in the *PDK1* open-reading frame (Figure 2D, E). Like the *pdk1* T-DNA insertion alleles, these new *pdk1* mutant alleles did not result in significant morphological differences from wild type. However, when combined with the *pdk2-1* or *pdk2-4* alleles, all double mutant combinations showed the same striking dwarf phenotype (Figure 2F-J). Complementation analysis using either *p35S::PDK1*, *p35S::YFP:PDK1* or *pPDK1::YFP:PDK1* showed that the dwarf phenotype was caused by *pdk* loss-of-function (Figure 2J, Figure S2). For all three constructs, several lines were obtained that showed complete rescue of the *pdk1-13 pdk2-4* double mutant phenotype (Figure S2, Figure S3A). The results show that *PDK1* and *PDK2* act redundantly and have a much more important role in plant growth and development than was previously reported.

***pdk* loss-of-function leads to many developmental defects, but not to a *pid* phenocopy**

Besides the decreased rosette diameter and reduced final plant height (Figure 2G, H), *pdk1 pdk2* double mutant plants flowered much later and showed strong reproductive defects (Figure 2I, J; Figure 3). The number of double homozygous F2 progeny obtained was much lower (1 in 47.7 ± 2.6) than the Mendelian ratio (1 in 16). Also F2 plants with the *pdk1*(-/-) *pdk2*(+/-) or *pdk1*(+/-) *pdk2*(-/-) genotype produced homozygous progeny at a much lower frequency than the expected 1 in 4 ratio (Table S1). Seed production of the homozygous *pdk1 pdk2* mutants (1.5 ± 0.21 per silique for *pdk1-13 pdk2-4*) was significantly reduced compared to wild type (65.9 ± 0.61 per silique). Mutant plants developed very short siliques (Figure 3A), a phenotype that has previously been reported for Arabidopsis plants that are both male and female sterile (Huang et al., 2016). These results implied that *pdk1 pdk2* loss-of-function causes gametophyte and/or embryo development defects in Arabidopsis. Reciprocal crosses between wild-type and *pdk1-13 pdk2-4* double mutant plants revealed both male-related and female-related reduced fertility. However, since the cross Col-0♀ x *pdk1-13 pdk2-4*♂ produced fewer seeds than the reciprocal cross *pdk1-13 pdk2-4*♀ x Col-0♂ (Figure S4A), it is likely that male gametophyte development is more strongly impaired by *pdk* loss-of-function than female gametophyte development. Alexander staining showed that pollen grain development in the *pdk1 pdk2* double mutant was not aborted, but that anther dehiscence was the major cause of the male fertility problems (Figure 3B-E, I). In addition, *in vitro* germination of *pdk1 pdk2*

pollen resulted in strangely shaped pollen tubes as a result of aberrant tip growth (Figure 3F, G). After 18-hour incubation on pollen germination medium, *pdk1-13 pdk2-4* pollen tube growth arrested with a bulb-like structure, and as a result, they remained much shorter than wild type pollen tubes (Figure 3G, H). The ovules of double mutant plants did not show noticeable morphological alterations (Figure S4B, C), which is in line with the predominant effect of *pdk* loss-of-function on male fertility.

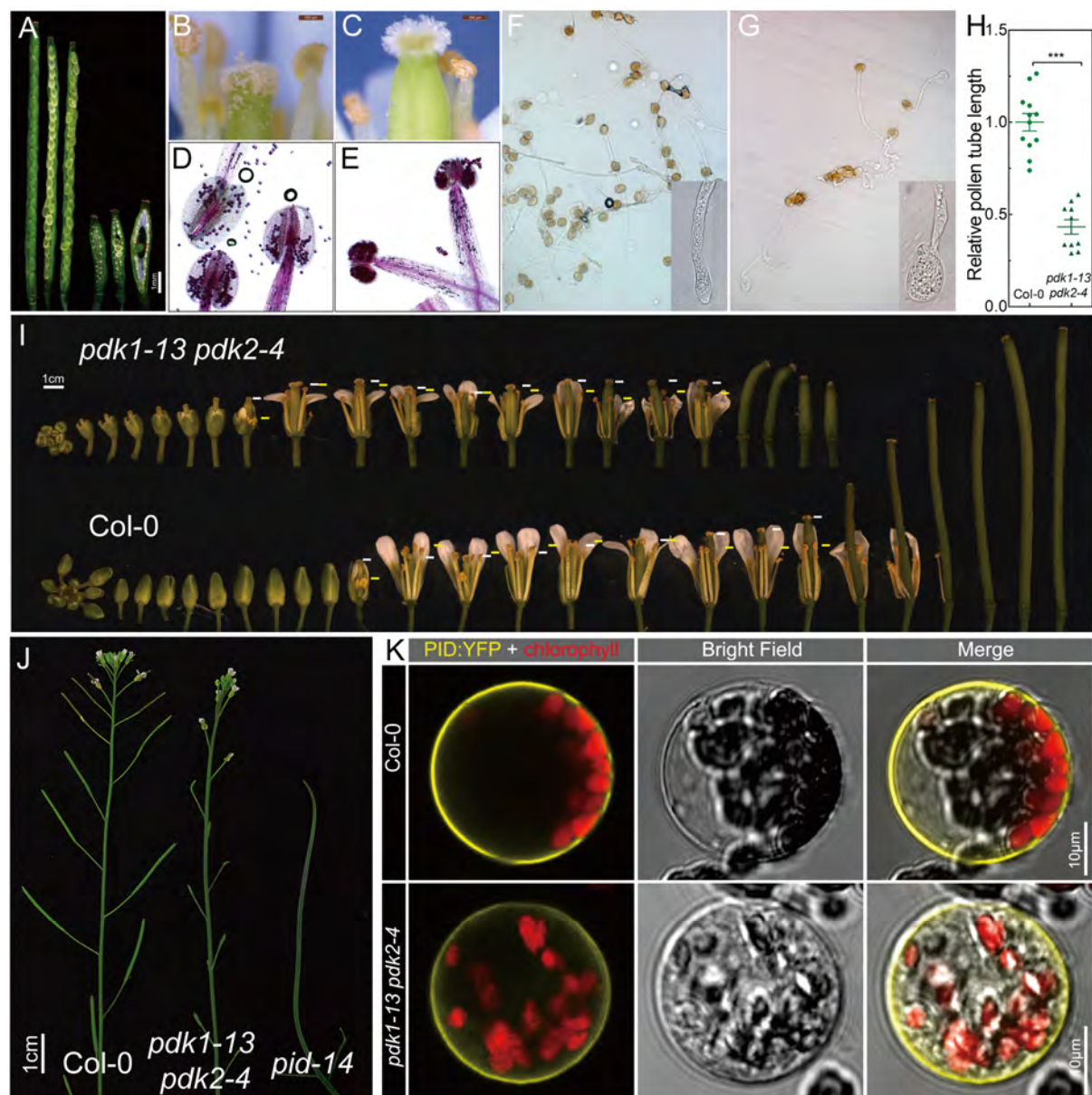


Figure 3. Flowering *pdk1 pdk2* mutant plants show clear developmental defects, but do not phenocopy *pid* mutant plants. A, *pdk1 pdk2* siliques (three on the right) are much shorter than wild-type siliques (three on the left), and contain many unfertilized ovules. B and C, Difference in pollen grain deposition on the stigma of a wild-type (B) or a *pdk1-13 pdk2-4* mutant (C) flower. D and E, Mature wild-type (D) or *pdk1-13 pdk2-4* mutant (E) anthers stained with Alexander's showing that pollen grains are viable, but that mutant anthers do not sufficiently dehisce. F and G, *In vitro* germination of wild-type (F) and *pdk1-13 pdk2-4* mutant (G) pollen. A detail of pollen tube tip is shown in the inset. H, Relative pollen tube length after 18 hours incubation. The average length of wild-type (Col-0) pollen tubes is put at 1.0. Asterisks indicate a significant difference (Student's *t*-test, *p* < 0.001). I, Developmental series of *pdk1-13 pdk2-4* mutant and wild-type (Col-0) flowers. The white bars indicate the position of the gynoecium apex, the yellow bars indicate the position of the anthers. J, Inflorescence phenotype of wild type (Col-0), *pdk1-13 pdk2-4* and *pid-14*. K, Representative images of PID:YFP subcellular localization in Col-0 or *pdk1-13 pdk2-4* protoplasts. More than ten observed protoplasts for each line showed the same localization.

In contrast to the fertility problems, *pdk1 pdk2* double mutants developed relatively normal flowers that showed no clear patterning defects. Flowers did show early stigma exposure due to impaired sepal growth, and slightly reduced filament elongation (Figure 3I). The short inflorescences seemed not the result of reduced internode elongation, but were most likely caused by early inflorescence meristem arrest (Hensel et al., 1994) (Figure 2J). The lack of phenotypic resemblance between *pdk1-13 pdk2-4* and *pid-14* inflorescences and flowers (Figure 2J) suggests that PDK1 is not essential for full PID function during inflorescence development. Moreover, expression of a PID:YFP fusion in *pdk1-13 pdk2-4* protoplasts showed that PDK1 activity is not necessary for the predominant localization of PID at the plasma membrane (Figure 2K). Based on these results and the overexpression data we conclude that, in contrast to what has previously been suggested (Zegzouti et al., 2006a, 2006b), PDK1 is not a key regulator of PID activity.

Alternative splicing produces a functional cytosolic PDK1 isoform lacking a PH domain

The *PDK2* gene produces a single transcript, whereas transcription of *PDK1* results in at least six different mature transcripts, due to alternative splicing events at the 5th, 7th and 9th intron (<https://www.araport.org/>). These transcripts can be translated into five different protein isoforms, which we named respectively PDK1S0, PDK1S1, PDK1S2 and PDK1S3 (Figure 4A). We checked the abundance of each mature transcript using semi-quantitative RT-PCR followed by restriction digestion. The full-length *PDK1* transcript was most abundant, and the short *PDK1* transcripts producing isoforms lacking part of the kinase domain (PDK1S1, PDK1S2, and PDK1S3) were also present at high levels, while the transcript producing the PDK1S0 isoform with a complete kinase domain, was the least abundant (Figure 4B).

In order to test the functionality of the different isoforms, we expressed the corresponding cDNAs in yeast (*S. cerevisiae*) strain INA106-3B, in which the *PKH2* gene copy has been replaced by *LEU2*, and the *PKH1* gene copy has been mutated so that strain INA106-3B is able to grow normally at 25°C but not at 35°C. As expected based on previous experiments, expression of the full length *PDK1* or *PDK2* cDNAs allowed this strain to grow at 35°C (Dittrich and Devarenne, 2012a) (Figure 4C). In contrast, expression of the cDNAs producing the PDK1S1, PDK1S2 or PDK1S3 isoforms did not allow growth at the restrictive temperature, suggesting that any deletion of the conserved kinase domain renders PDK1 non-functional (Figure 4C). This is in line with loss-of-function observed for the new *Arabidopsis* alleles *pdk1-11*, *-13*, *-14*, *-31* and *-32*, which all express partial PDK1 proteins having a small or larger deletion of the C-terminal part of the kinase domain (Figure 2D, E, F). However, expression of the PDK1S0 did permit INA106-3B to grow at 35°C (Figure 4C). The yeast data were confirmed by 35S promoter-driven expression in the *Arabidopsis pdk1 pdk2* loss-of-function mutant background. *p35S::PDK1* provided full rescue of the vegetative growth phenotypes of the *Arabidopsis pdk1 pdk2* mutant, and some *p35S::PDK1S0* lines showed the same level of rescue (Figure S3A). In contrast, expression of PDK1S1 and PDK1S2 did not result in any rescue (Figure S3A). Expression of a *YFP:PDK1S0* fusion under control of the *PDK1* promoter in the *pdk1-13 pdk2-4* mutant background also completely rescued the mutant vegetative growth phenotypes (Figure 4D). However, *pPDK1::YFP:PDK1S0 pdk1-13 pdk2-4* plants developed shorter siliques carrying fewer seeds compared to wild-type or *pPDK1::YFP:PDK1 pdk1-14 pdk2-4* plants (Figure 4E). Interestingly, a similar silique phenotype has also been

described for the *Arabidopsis pdk1-b pdk2-1* double mutant, and according to our own analysis the T-DNA insertion in the *pdk1-b* allele leads to the production of a shorter PDK1 protein with an intact kinase domain but lacking the PH domain (Camehl et al., 2011) (Figure 2A, B).

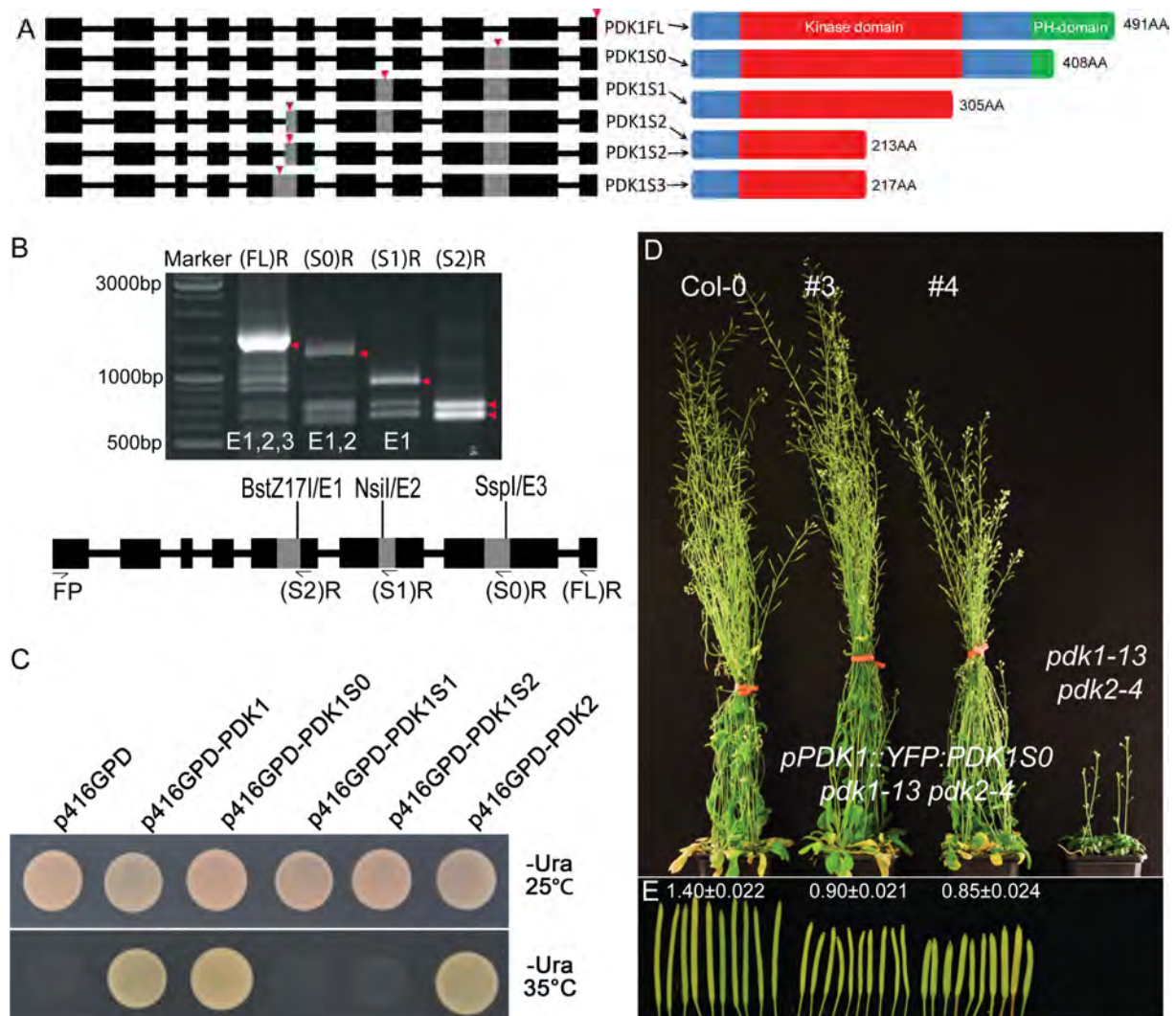


Figure 4. Alternative splicing produces a *PDK1* transcript encoding a functional PDK1 variant lacking the PH domain. A, Schematic representation of the *PDK1* gene indicating the alternative splice events (left) and the respective protein isoforms produced by the splice variants (right). On the left: wide black boxes represent exons, gray boxes represent unspliced introns, black lines represent spliced introns, red arrows point out the locations of stop codons. Please note that *PDK1S1* is lacking six amino acids of the kinase domain. B, Expression level of the different splice variants, as detected by RT-PCR. Primer binding and restriction enzyme recognition site locations are shown in the schematic representation below (see detailed description in the materials and methods section). Red arrowheads point out the *PDK1FL*, *PDK1S0*, *PDK1S1* and *PDK1S2/3* transcripts (from left to right) detected using the reverse primers and restriction enzymes indicated above and below the gel image, respectively. C, Rescue of the temperature-sensitive growth of the yeast *pkh1 pkh2* mutant strain by expression of the *PDK1* or *PDK2* full-length cDNA or the *PDK1S0* splice variant cDNA. Three biological repeats showed the same result. D and E, The *pPDK1::YFP:PDK1S0* construct rescues the delay in flowering time, short plant height and dwarf rosette leaves of the *pdk1-13 pdk2-4* mutant (D, 14 independent lines were observed), but plants develop shorter siliques carrying less seeds (E). Shown in (D) are 65-day-old plants. Numbers above siliques in (E) represent average length $\pm$ SEM (n=10).

These results corroborate the conclusions from the complementation experiments in yeast that a full-length kinase domain is essential for PDK1 function, but that surprisingly the PH domain is not essential for PDK1 function during *Arabidopsis* vegetative growth. Since the PH

domain is responsible for lipid binding, we checked the *PDK1* promoter-driven YFP:PDK1 and YFP:PDK1S0 localization in root columella cells, where *PDK1* is highly expressed. YFP:PDK1 localized both on the plasma membrane and in the cytoplasm, whereas YFP:PDK1S0 was only found in the cytoplasm (Figure S3B, C). The functional relevance of the latter low abundant cytosolic isoform remains unclear. Our findings do suggest, however, that PH domain-dependent plasma membrane association of PDK1 is only essential during specific developmental processes.

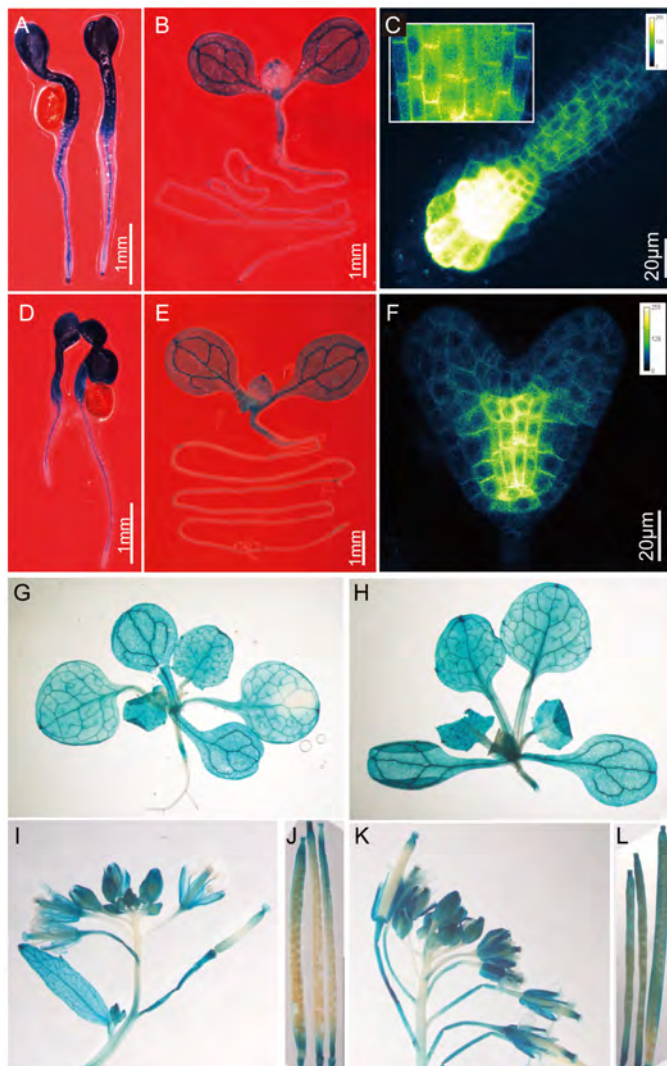


Figure 5. *PDK1* and *PDK2* are predominantly expressed in (pro) vascular tissues, where *PDK1* associates with the basal plasma membrane. Spatio-temporal expression pattern of *PDK1* (A, B, G, I, J) and *PDK2* (D, E, H, K, L) as reported by histochemical staining of 3-day-old seedlings (A, D), 7-day-old seedlings (B, E), 16-day-old plants (G, H) and inflorescences and siliques from 40-day-old plants (I-L) of representative *pPDK1*-GG and *pPDK2*-GG lines, respectively. C and F, Confocal images of a 4-day-old *pPDK1*::YFP:PDK1 root tip (C) or a heart stage embryo of the same transgenic line (F). The inset in (C) shows a detail of YFP:PDK1 localization in root stele cells.

***PDK1* and *PDK2* are broadly expressed during development**

Since the *pdk1 pdk2* mutant shows many defects in development and growth, we analyzed the spatio-temporal expression pattern of the two *PDK* genes to uncover their tissue-specific functions. For this purpose we generated Arabidopsis (Col-0) lines carrying the *pPDK1*::*turboGFP*:*GUS* (*pPDK1*-GG) or *pPDK2*::*turboGFP*:*GUS* (*pPDK2*-GG) construct and used a *pdk1-14 pdk2-4* mutant line carrying the complementing *pPDK1*::YFP:PDK1 construct. *PDK1* appeared to be strongly expressed in (pro)vascular tissues from the early globular embryo stage on, and in the columella root cap (Figure 5, Figure S5). The gene also showed more general expression in young hypocotyls, cotyledons, leaves and floral organs, and in

growing siliques. The expression pattern of *PDK2* was very comparable to that of *PDK1* (Figure 5 D, E, H, K, L), except that no expression was observed in the root apex (Figure 5D) or in embryos (data not shown).

As previously observed for *PDK2:EGFP* (Scholz et al., 2019), *YFP:PDK1* did not localize in the nucleus, but was mainly found in the cytoplasm or associated with the plasma membrane (Figure 5C, F; Figure S3B;). In (pro)vascular cells in heart stage embryos and roots, *YFP:PDK1* showed predominant basal (rootward) localization (Figure 5C, F; Figure S5), just like *PIN1* and the *PDK1* targets *PAX* and *D6PK* (Gälweiler et al., 1998; Zourelidou et al., 2009; Marhava et al., 2018).

Auxin transport is impaired in *pdk1-13 pdk2-4* mutant

Even though the *PDK1* overexpression and loss-of-function phenotypes did not point to an important role for *PDK1* in PID function, the *pdk1-13 pdk2-4* mutant seedling phenotypes did suggest the involvement of *PDK1* in the regulation of auxin response or -transport (Figure 6A-E). Mutant primary roots elongated normally up to two days after germination, but after that their growth rate declined (Figure 6 A, B), and roots started to oscillate randomly with a large amplitude, resulting in curved short roots (Figure S6A). Of 199 7-day-old seedlings, 18.1% of the primary roots grew into the air. On a total of 460 *pdk1-13 pdk2-4* mutant seedlings, 58% showed fused or single dark green cotyledons, and the remaining 42% developed two cotyledons with short petioles positioned at an abnormal angle ($< 180^\circ$) (Figure 6C-E). The cotyledon phenotypes and short agravitropic roots are usually observed in auxin response or -transport mutants or transport inhibitor-treated seedlings. By combining the *pdk1-13 pdk2-4* double mutant with the or *pDR5::GUS* auxin response reporter, we observed that auxin response was absent or strongly decreased in the root stele and confined to the root tip, while an enhanced auxin response was observed at the mutant cotyledon edges and in the fragmented cotyledon veins (Figure 6F, G, I, K). This highly resembled the *pDR5::GUS* expression of 7-day-old seedlings grown on medium supplemented with the auxin transport inhibitor naphthylphthalamic acid (NPA) (Figure 6H, J) (Sabatini et al., 1999; Bao et al., 2004). Moreover, the increase in *pDR5::GUS* expression in cotyledons corroborated that *pdk1-13 pdk2-4* mutants are defective in auxin transport, rather than in auxin biosynthesis or -signaling. Short time treatment of wild-type and *pdk1-13 pdk2-4* seedlings with IAA and subsequent qPCR analysis showed that the auxin inducible expression of the *IAA5*, *GH3.3* and *SAUR16* genes was not impaired, confirming that the mutants are not defective in auxin response (Figure 6L). Instead, *pdk1-13 pdk2-4* mutant seedlings were hypersensitive to NPA treatment compared to wild type (Figure 6M). Moreover, the auxin transport capability of *pdk1-13 pdk2-4* inflorescence stems was significantly reduced compared to that of wild-type stems (Figure 6N). Together, the above data point toward a role for *PDK1* in enhancing polar auxin transport.

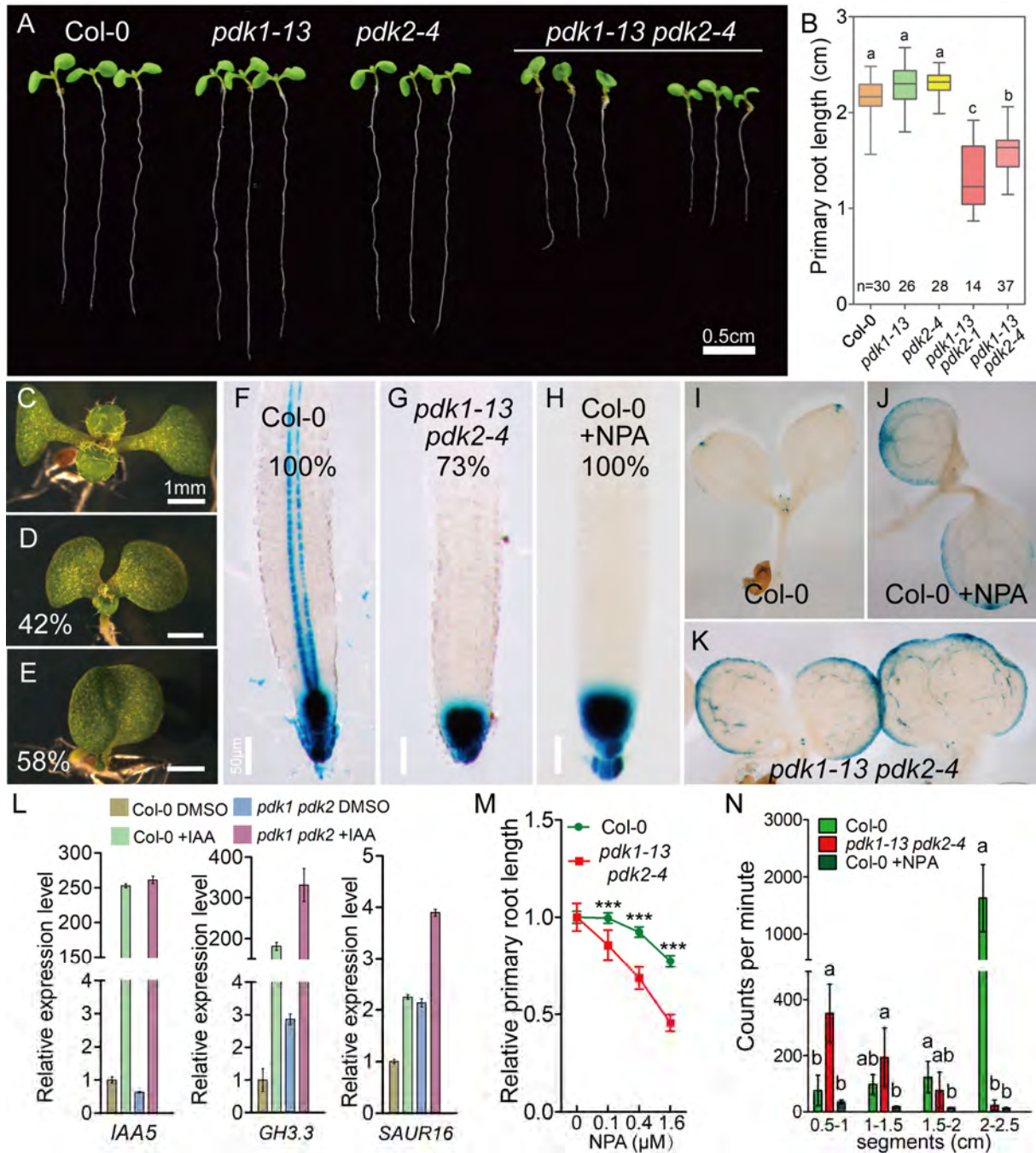


Figure 6. The phenotype and auxin response pattern of *pdk1pdk2* mutant seedlings resembles that of auxin transport inhibitor treated seedlings. A, The phenotype of aligned 7-day-old wild-type (Col-0) and *pdk1 pdk2* mutant seedlings. B, Primary root length of 7-day-old seedlings. Lowercase letters indicate averages that are significantly different, as tested by a one-way ANOVA followed by Tukey's test ($p < 0.05$). C-E, Cotyledon phenotype of wild-type (Col-0) seedlings (C), showing two symmetrically distributed cotyledons with extended petioles, or *pdk1-13 pdk2-4* seedlings, of which 42% position at an angle with short petioles (D, $n=460$) and 58% show fused cotyledons (E, $n=460$). F-K, Histochemical GUS staining of 7 day old wild-type seedlings (Col-0, F, I), wild-type seedlings grown on 0.5μM NPA (Col-0 + NPA, H, J), or *pdk1-13 pdk2-4* seedlings (G, K), all three containing the *pDR5::GUS* auxin response reporter. Percentages in F-H indicate the ratio of representative image out of the observed seedlings ($n=15$). The rest 27% of *pdk1-13 pdk2-4* show strongly decreased but not absent *pDR5::GUS* signal in the stele. L, Quantitative RT-PCR analysis of the auxin-induced expression of *IAA5*, *GH3.3* and *SAUR16* in 5-day-old Arabidopsis wild-type (Col-0) and *pdk1-13 pdk2-4* mutant seedlings. The values displayed in the graph are means $\pm$ SEMs. M, NPA sensitivity of wild-type (Col-0) and *pdk1-13 pdk2-4* based on the primary root length of seedlings grown on medium with an increasing NPA concentration ($n > 22$, Student's *t*-test was used for analysis between groups from the same NPA concentration, $p < 0.001$). Error bar = 95% confidence interval. N, Transport of ^3H -IAA in 2.5 cm wild-type (Col-0), wild-type with NPA and *pdk1 pdk2* inflorescence stem pieces. Bars represent the average number of counts per segment $\pm$ 95% confidence interval. Data were analyzed using a one-way ANOVA followed

by Tukey's test. Significant differences are indicated with different letters in each segment group. A representative experiment of three biological repeats displaying similar results is shown.

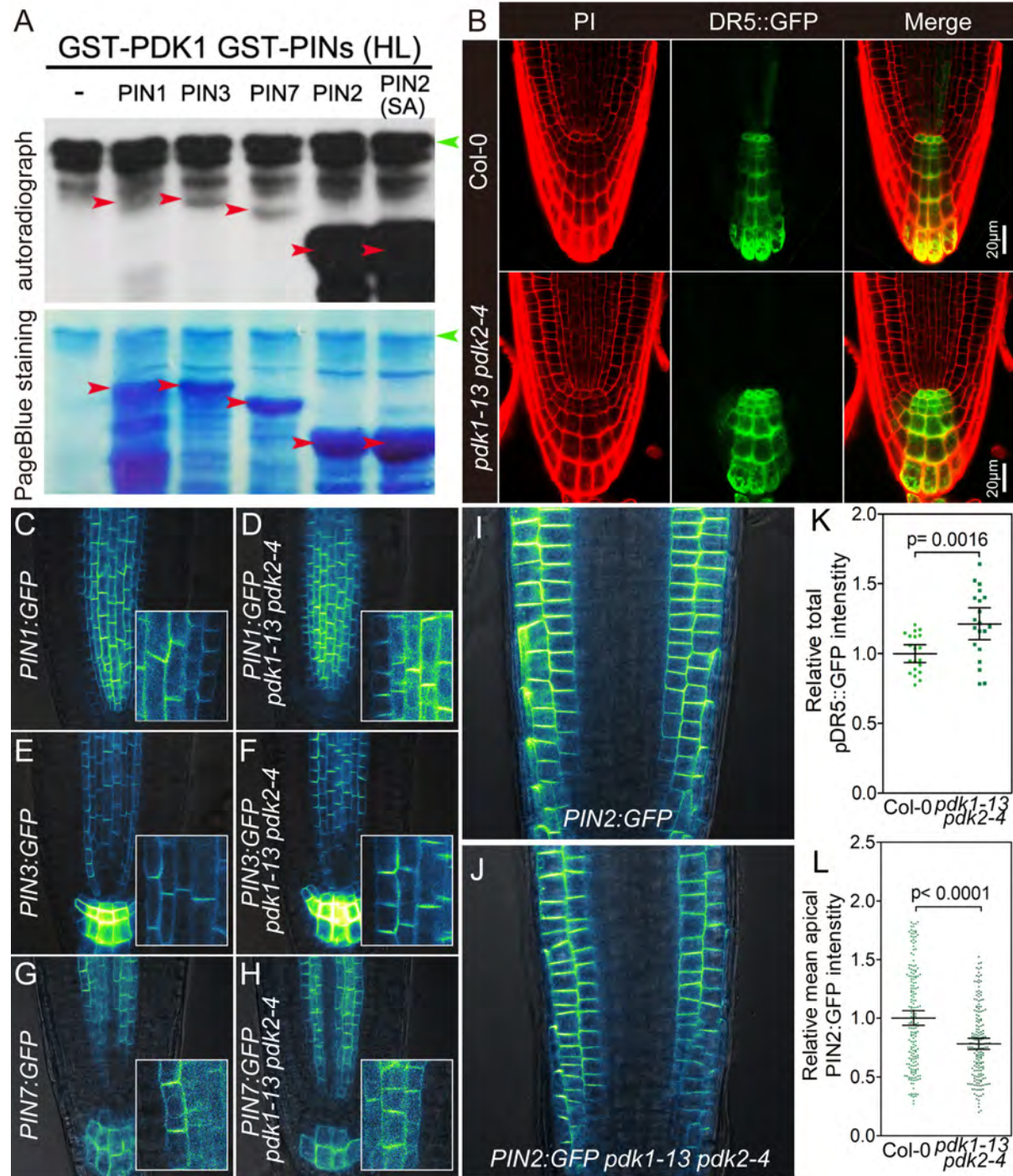


Figure 7. PDK1 is not involved in PIN polarity control. A, PDK1 strongly phosphorylates the PIN2HL, but not the PIN1HL, PIN3HL or PIN7HL, in a S1, S2, S3-independent manner *in vitro*. Red or green arrows point out the position of the GST-PINHL or GST-PDK1, respectively. B, Confocal images of *pDR5::GFP* expression in a wild-type (Col-0, upper panel) or a *pdk1-13 pdk2-4* mutant root tip (lower panel). Left: propidium iodide (PI) staining; middle: GFP signal; right: merged image. C-J, Confocal images showing the subcellular localization of PIN1:GFP (C, D), PIN3:GFP (E, F), PIN7:GFP (G, H), and PIN2:GFP (I, J) in wild-type (Col-0, C, E, G, I) or *pdk1-13 pdk2-4* mutant (D, F, H, J) root tips. Insets in C-H show the details of PIN polarity in stele cells. K and L, Relative total GFP intensity produced by *pDR5::GFP* expression in columella-QC cells (K, $n=20$) or representing PIN2:GFP at the apical side of the epidermal cells (L, $n=8$), in wild-type (Col) or *pdk1-13 pdk2-4* mutant roots. *pDR5::GFP* and PIN2:GFP intensities are shown relative to Col-0 control.

PDK1 regulates PIN-mediated auxin efflux probably through the AGC1 kinases

Several *in vitro* PDK1 phosphorylation substrates are AGC kinases that have been reported to regulate auxin transport by direct phosphorylation of PIN auxin efflux carriers (Zegzouti et al., 2006b; Willige et al., 2013; Marhava et al., 2018; Haga et al., 2018). The fact that their loss-of-function mutants share phenotypic defects with the *pdk1-13 pdk2-4* mutant, such as the short root of the *pax* mutant (Marhava et al., 2018), or inhibited hypocotyl tropic response and the fused cotyledons of the *d6pk012* triple mutant (Zourelidou et al., 2009)(Figure S6), hinted that these AGC kinases might indeed be regulated by PDK1 *in planta*. The stronger *pdk1-13 pdk2-4* mutant phenotype suggested that PDK1 has many more phosphorylation substrates.

In order to investigate whether PIN proteins themselves are PDK1 phosphorylation substrates, we deduced based on published *in vitro* phosphorylation data, that PDK1 prefers to phosphorylate the second serine residue in the RSXS_FVVG motif (X represents any amino acids) that is part of the activation segment of the AGC kinases (Zegzouti et al., 2006a, 2006b). Analysis of the large central hydrophilic loop (HL) of the 5 Arabidopsis PIN1-type PIN proteins identified several RXXS motifs. However, *in vitro* phosphorylation assays using GST-tagged PDK1 (GST-PDK1) and GST-tagged versions of the HL of PIN1, PIN2, PIN3 or PIN7 (GST-PIN1/2/3/7HL) only showed strong phosphorylation of the PIN2HL (Figure 7A). PIN2HL S1,2,3A, in which the PID phosphorylation sites are substituted by alanines, was also phosphorylated by PDK1 at the same level as the wild-type PIN2HL (Figure 7A). PDK1 must therefore phosphorylate one or more other serine residues that are unique to the PIN2HL. However, PIN2 is not co-expressed with PDK1, and the PIN proteins that are co-expressed with PDK1 in the root stele or columella cells (PIN1, PIN3 and PIN7) are very weakly phosphorylated by PDK1 *in vitro*. Moreover, no noticeable alteration in PIN1/3/7 protein polarity was observed in *pdk1-13 pdk2-4* mutant roots (Figure 7C-J). The PIN2:GFP abundance was slightly decreased in *pdk1-13 pdk2-4* mutant root tips (Figure 7I, J, L), but this might be an indirect effect of *pdk* loss-of-function on auxin distribution in the root tip, as we measured a slight increase in GFP intensity in *pDR5::GFP pdk1-13 pdk2-4* mutant versus wild-type root tips (Figure 7B, K). Our results suggest that PDK1 regulates auxin transport, most likely mainly by activating one or more AGC kinases, such as PAX and D6PK, which subsequently regulate auxin efflux activity by direct phosphorylation of the PINs.

Discussion

PDK1 is a well-established key regulator of AGC kinases in animals and yeast, and its importance in these organisms is demonstrated by the lethality caused by loss-of-function mutations in the genes encoding for this protein kinase (Casamayor et al., 1999; Rintelen et al., 2002; Lawlor et al., 2002; Mora et al., 2004). Also in the model plant Arabidopsis, PDK1 has been shown to phosphorylate several AGC kinases *in vitro* (Zegzouti et al., 2006a, 2006b). However, the previously reported impact of loss-of-function of the two gene copies *PDK1* and *PDK2* on Arabidopsis development was only limited (Camehl et al., 2011; Scholz et al., 2019). In this study, we found that the published T-DNA insertion alleles of the Arabidopsis *PDK1* gene copy are not loss-of-function mutants. Here we generated several CRISPR/Cas9-based true loss-of-function *pdk1* alleles that, when combined with the available *pdk2* loss-of-function

mutant alleles, did lead to strong developmental defects. Different from animals and yeast though, and more similar to the situation in *Physcomitrella Patens*, Arabidopsis *pdk1 pdk2* loss-of-function mutants are viable, indicating that the substrate preference of plant PDK1 has changed from that in other eukaryotes, and that it has lost its involvement in signaling pathways that are essential for cell survival.

PDK1 is not essential for PID activity controlling inflorescence and cotyledon development

By carefully recording the *pdk1 pdk2* mutant phenotypes, we analyzed the genetic relation between PDK1 and its reported *in vitro* substrates, of which PID was the key candidate (Zegzouti et al., 2006a). Loss-of-function of both *PDK* genes leads to fused cotyledons, short wavy roots, dwarf stature and reduced fertility resulting in short siliques. To our surprise, *pdk1 pdk2* does not share the three cotyledon, pin inflorescence and aberrant flower phenotypes that are typical for *pid* loss-of-function mutants, implying that PDK1 is not essential for PID function in these tissues. PID is an auto-activating kinase *in vitro* and might act independent of upstream activating kinases (Christensen et al., 2000; Benjamins et al., 2001), or other kinases than PDK1 might be involved in hyper-activating PID during embryo, inflorescence and flower development. The latter seems most likely based on the observation that flower, leaf and shoot extracts can hyperactivate PID *in vitro* (Zegzouti et al., 2006a). A physical interaction between PID and PDK1 through the PIF domain, as suggested by Zegzouti et al. (Zegzouti et al., 2006a), has never been proven, and was purely based on *in vitro* phosphorylation data. Here we show unequivocally that PID does not require PDK1 for its association with the PM, which corroborates the finding that this is mediated by an arginine-rich loop in the kinase domain of PID (Simon et al., 2016). All data is in line with the observation that a PID:GUS fusion lacking the PIF domain can still complement *pid* loss-of-function mutants (Benjamins et al., 2001). Although we cannot fully exclude that PDK1 and PID do have a functional interaction, our results at least indicate that this interaction is not essential for the majority of the PID activities in plant development.

Is PDK1 a master regulator of AGC kinases in Arabidopsis?

If not PID, which and how many other Arabidopsis AGC kinases are potential phosphorylation substrates of PDK1? The developmental defects of the *pdk1 pdk2* double mutant together with the altered *DR5* expression pattern and reduction in auxin transport all point toward a role for PDK1 in promoting polar auxin transport. Interestingly, several *pdk1 pdk2* mutant phenotypes are also observed for loss-of-function mutants of members of the AGC1 kinase sub-family, for some of which a role as regulator of polar auxin transport is now well established (Zourelidou et al., 2009; Willige et al., 2013; Barbosa et al., 2014; Haga et al., 2018; Marhava et al., 2018). For example, the fused cotyledons, deficient lateral root emergence and agravitropic primary root growth closely resemble phenotypes observed for the *d6pk012* triple mutant (Zourelidou et al., 2009). And a short primary root is also observed for the *pax* mutant (Marhava et al., 2018), and like *pdk1 pdk2*, the *agc1.5 agc1.7* double mutant is defective in pollen tube growth (Zhang et al., 2009). Since the corresponding AGC1 kinases are strongly dependent on PDK1 for their *in vitro* activation (Zegzouti et al., 2006b), it seems possible that PDK1 might act as a

master regulator of these AGC1 kinases. Further experimentation is required, however, for each of these kinases to prove this hypothesis.

Recently, a genetic interaction with *PDK1* was reported for a kinase of the AGC2 clade, UNICORN (UCN, AGC2-3), in controlling integument growth (Scholz et al., 2019). According to Scholz and coworkers, UCN acts as a repressor of PDK1 function. The absence of UCN activity or PDK1 overexpression leads to uncontrolled integument and petal growth, and the *pdk1-c pdk2* T-DNA insertion mutant combination can rescue the *ucn* loss-of-function mutant defects. Like Scholtz et al., we did not observe defects in ovule development for the new *pdk1 pdk2* allelic combinations (Figure S4B, C). It will be interesting to see if our new *pdk1-13 pdk2-4* double mutant combination will also lead to restoration of the *ucn-1* flower and ovule phenotypes.

Alternative splicing provides possible functional differentiation for PDK1

By studying *pdk1* T-DNA insertion alleles and splice variants produced by the *PDK1* gene, we revealed that the PH domain is not essential for the general PDK1 function in Arabidopsis. The alternative splicing product PDK1S0, which lacks phospholipid binding ability and membrane localization but still has kinase activity, is able to rescue the thermosensitive growth of the yeast *pkh1 pkh2* double mutant strain INA106-3B, and most of the developmental defects of the Arabidopsis *pdk1 pdk2* loss-of-function mutant. A similar PDK1 protein variant appeared to be produced in the Arabidopsis *pdk1-a* and *pdk1-b* T-DNA insertion alleles, which were initially thought to be complete loss-of-function alleles. This explains the relatively mild flower phenotypes observed for the Arabidopsis *pdk1-a pdk2* and *pdk1-b pdk2* double mutants (Camehl et al., 2011; Scholz et al., 2019). The reduced growth response of these mutants induced by phosphatidic acid downstream of *Piriformospora indica* infection (Camehl et al., 2011) suggests a differential function for PDK1S0 and PDK1 in development and stress response, respectively. This is in line with the observation that phosphatidic acid, an important second messenger for stress response, can directly bind and stimulate the activity of full-length Arabidopsis PDK1 (Deak et al., 1999; Anthony et al., 2004). In animals, phospholipids are known to bind to PDK1 to induce PDK1 dimer to monomer conversion and activation (Alessi et al., 1997; Ziemba et al., 2013). The functionality of PDK1S0 in most developmental processes questions the importance of the clear basal polarity of full-length PDK1 in (pro) vascular cells in the embryo and root tip. Apparently, PDK1 binding to the AGC kinase PIF domain is sufficiently efficient, and does not require prior co-localization at the PM. In conclusion, alternative splicing of *PDK1* transcripts may provide a novel and unique regulation mechanism for balancing growth and defense in Arabidopsis, which differs from animals and yeast.

Materials and methods

Plant lines and growth condition

Arabidopsis thaliana (L.) ecotype Columbia 0 (Col-0) was used as wild-type control for all experiments, since all mutant and transgenic lines are in the Col-0 background. Previously

described T-DNA insertion lines SALK_053385 (*pdk1.1-1*, renamed to *pdk1-c*), SALK_113251C (*pdk1.1a*, renamed to *pdk1-a*), SALK_007800 (*pdk1.1b*, renamed to *pdk1-b*), SAIL_62_G04 (*pdk1.2-2*, renamed to *pdk2-4*) and SAIL_450_B01 (*pdk1.2-3*, renamed to *pdk2-1*) were ordered from the Nottingham Arabidopsis Stock Centre (Camehl et al., 2011; Scholz et al., 2019). The following Arabidopsis lines are also described elsewhere: *pDR5::GFP* (Ottenschlager et al., 2003), *pDR5::GUS* (Benjamins et al., 2001), *pPIN1::PIN1:GFP* (Benkova et al., 2003), *pPIN2::PIN2:GFP* (Xu and Scheres, 2005), *pPIN3::PIN3:GFP* (Zádníková et al., 2010), *pPIN7::PIN7:GFP* (Blilou et al., 2005) and *p35S::PID#21* (Benjamins et al., 2001). For lines created in this study, the T-DNA constructs *p35S::YFP:PDK1*, *p35S::PDK1*, *pPDK1/2::turboGFP:GUS*, and *pYAO-Cas9-gRNA1/2/3* were transformed into Col-0 using *Agrobacterium*-mediated floral dip transformation (Clough and Bent, 1998). Homozygous lines with a single T-DNA insertion were selected for further analysis. Of the 80 CRISPR/Cas9 transgenic alleles obtained, 7 appeared to contain loss-of-function mutations in the 3rd or 7th exon of *PDK1*. The CRISPR/Cas9 T-DNA construct in the new *pdk1* mutant alleles was segregated out during the generation of the *pdk1 pdk2* double mutant. Five mutant alleles with open reading frame shifts were used for further analysis (Figure 2C).

For complementation analysis of PDK1 isoforms, the T-DNA constructs *p35S::YFP:PDK1* or *p35S::PDK1FL/S1/S2* were transformed into the *pdk1-13(+/-) pdk2-4(-/-)* mutant background, *p35S::YFP:PDKS0* or *p35S::PDK1S0* were transformed into the *pdk1-13(-/-) pdk2-1(+/-)* mutant background, or *pPDK1::YFP:PDK1FL* and *S0* were transformed into the *pdk1-14(-/-) pdk2-4(+/-)* or *pdk1-13(+/-) pdk2-4(-/-)* mutant background, respectively. The genotype of the *pdk1 pdk2* mutant background was confirmed by PCR before floral dip transformation. All genotyping primers are summarized in Table S2.

Plants were grown on soil at 21 °C, 16 hr photoperiod, and 70% relative humidity. For seedling growth, seeds were surface-sterilized by 1 minute in 70% ethanol, 10 minutes in 1% chlorine followed by five washes with sterile water. Sterilized seeds were kept in the dark at 4 °C for 2 days for vernalization and germinated on vertical plates with 0.5× Murashige and Skoog (1/2 MS) medium (Duchefa) containing 0.05% MES, 0.8% agar and 1% sucrose at 22 °C and 16 hr photoperiod.

RNA extraction and (q)RT-PCR

Total RNA was extracted from 5-day-old seedlings using a NucleoSpin RNA Plant kit (Macherey Nagel, #740949). Reverse transcription (RT) was performed using a RevertAid Reverse Transcription Kit (Thermo Scientific™, #K1691). For qRT-PCR on auxin induced genes, RNA was isolated from 5-day-old Col-0 and *pdk1-13 pdk2-4* seedlings treated for 1 hour with 10 μM IAA. Gene expression was normalized to the reference gene *PP2A-3* (AT2G42500) using the $\Delta\Delta C_t$ method. For analysis of the *pdk1* and *pdk2* T-DNA alleles, RT-PCR was performed with DreamTaq DNA Polymerases (Thermo Scientific™). (q)RT-PCR primers are listed in Table S2. For detection of the *PDK1* splice variants, RT-PCR was performed for 40 cycles using the forward (FP) and reverse (FL, S0, S1, and S2) primers (Figure 5B), as listed in Table S2. PCR reactions with primer pair FP and (FL)R were digested with *Bst*Z17I, *Nsi*I and *Ssp*I to detect PDK1FL, with primer pair FP and (S0)R with *Bst*Z17I and *Nsi*I to detect PDK1S0, and with primer pair FP and (S1)R with *Bst*Z17I to detect PDK1S1. 0.1 μL of the enzymes *Bst*Z17I, *Nsi*I and/or *Ssp*I (Thermo Scientific™) was directly added to

the 20 μ L PCR reaction and reactions were incubated at 37°C overnight before gel electrophoresis. Detection of PDK1S2 and PDK1S3 with primer pair FP and (S2)R did not require restriction enzyme digestion. qRT-PCR was performed in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using TB Green Premix Ex Taq II (Tli RNase H Plus) (Takara, #RR820B).

Cloning procedures

To generate the *Promoter::turboGFP:GUS* fusions, a *SacI-TurboGFP-PacI* fragment was cloned from *pICSL80005* into *pMDC163*, resulting in *pMDC163(gateway)-TurboGFP:GUS*. *PDK1* and *PDK2* promoter regions of approximately 2.0 kb including the first six codons were amplified from Col-0 genomic DNA using the primers listed in Table S2, and cloned in *pDONR207* by LR recombination. The resulting fragments were subsequently fused in-frame with the *turboGFP:gusA* reporter gene in *pMDC163(gateway)-TurboGFP:GUS* by BP recombination. (Invitrogen, Gateway BP/LR Clonase II Enzyme Mix, #11789020 and #12538120).

PDK1 splice variants were amplified from cDNA of 5-day-old seedlings using the respect primers (Table S2), after which restriction enzymes described in the RT-PCR section were employed. Fragments were cloned in *pDONR207* by BP recombination, and subsequently transferred to *pART7-35S::YFP:gateway* by LR recombination, resulting in *pART7-35S::YFP:PDK1FL/S0/S1/S2*. Expression cassettes were excised with *NotI* and cloned into *NotI* digested *pART27*, resulting in *pART27-35S::YFP:PDK1FL/S0*. The same entry vectors and LR recombination were used to generate *pMDC32-35S::PDK1FL/S0/S1/S2* and *pGEX-PDK1FL/S0*. *pGEX-PIN1HL*, *pGEX-PIN2HL* and *p35S::PID:YFP* have been described previously (Galván-Ampudia and Offringa, 2007; Huang et al., 2010; Dhonukshe et al., 2010). *PIN3HL* and *PIN7HL* were amplified from Col-0 cDNA using primers listed in Table S2 and cloned into *pGEX* also using Gateway cloning technology to obtain *pGEX-PIN3HL* and *pGEX-PIN7HL*.

To generate *pPDK1::YFP:PDK1FL/S0* fusions, the 2.0Kb *PDK1* promoter region was introduced into *pART27-35S::YFP:PDK1S0* by replacing the 35S sequence using restriction enzymes *BstXI* and *KpnI*. *pART27-pPDK1::YFP:PDK1S0* and *pDONR207* were mixed with BP clonase to obtain *pART27-pPDK1::YFP:gateway*. *PDK1FL* was then recombined into *pART27-pPDK1::YFP:gateway* by LR reaction to obtain *pART27-pPDK1::YFP:PDK1FL*.

To obtain the *p416GPD-PDK* constructs for expression in yeast, *BamHI-PDK1FL/S0/S1/S2-EcoRI* and *BamHI-PDK2-XhoI* fragments were amplified from *pDONR207-PDK1FL/S0/S1/S2* and 5-day-old seedling cDNA, respectively, using primers listed in Table S2. Fragments were digested with the appropriate restriction enzymes and ligated into vector *p416GPD*.

The *pCambia-pYAO-Cas9-gRNA1/2/3* plasmids for CRISPR/Cas9 mediated mutagenesis were obtained by ligating the *EcoRI*-(Cas9+terminator)-*AvrII* fragment from *pDE-Cas9* (Fauser et al., 2014) into *pCambia1300* digested with *EcoRI* and *XbaI*. The *EcoRI* and *SaI* sites in the resulting *pCambia-Cas9* plasmid were used to clone the *EcoRI*-YAO promoter-*EcoRI* (Yan et al., 2015) and *XhoI*-gateway-*XhoI* fragments amplified from respectively *Arabidopsis* Col-0 genomic DNA and the *pART7-35S::YFP:gateway* plasmid. Regions producing guide RNAs (Table S2) designed to target respectively the 3rd, 6th or 7th exon of *PDK1* were ligated into *pEn-Chimera* (Fauser et al., 2014), and introduced behind the YAO promoter in *pCambia*-

pYAO-Cas9-gateway by LR recombination.

All primers used for cloning are summarized in Table S2.

General phenotypic analysis and physiological experiments

NPA treated (stock in DMSO, 1/10⁴ dilution), dark-grown or normally grown seedlings, potted plants, siliques and inflorescences were photographed with a Nikon D5300 camera at the indicated time. For imaging of inflorescences, the top part of the inflorescence was cut from 15 cm high plants. For Figure 6A, seedlings were transferred to and aligned on a black plate before imaging. Primary root length, rosette diameter, silique length and length and hypocotyl curvature under directional light treatment were measured with ImageJ (Fiji). Plant height was measured directly using a ruler. Root tips, opened siliques, flowers, details of floral organs and cotyledons were imaged using a Leica MZ16FA stereomicroscope equipped with a Leica DFC420C camera. All measurements based on photos were performed in ImageJ and analyzed and plotted into graphs in GraphPad Prism 5.

Phenotypic analysis of reproductive organs

To examine pollen vitality, anthers were collected from flowers just before opening into 70 µL Alexander staining buffer [10% ethanol, 0.01% (w/v) Malachite green, 25% glycerol, 5% (w/v) phenol, 5% (w/v) chloral hydrate, 0.05% (w/v) fuchsin acid, 0.005% (w/v) OrangeG and 1.5% glacial acetic acid] on a microscopy slide, covered with cover slip, and incubated at 55 °C for 1 hr before imaging. For pollen tube growth, pollen of just opened flowers were transferred to a dialysis membrane placed on solid pollen germination medium [18% Sucrose, 0.01% Boric acid, 1 mM CaCl₂, 1 mM Ca (NO₃)₂, 1 mM MgSO₄ and 0.5% agarose] and incubated at 22 °C for 18 hrs. Ovules were cleared in chloral hydrate solution (chloral hydrate: glycerol: water = 4:2:1 by weight) for 4 hrs. Stained or germinated pollen and cleared ovules were imaged using a Zeiss AxioPlan 2 microscope with DIC optics and Zeiss AxioCam MRc 5 digital color camera. Pollen tube length was measured with ImageJ (Fiji).

Protoplast isolation and transformation

Protoplasts were isolated and transformed as previously described, but with some modifications to the protocol (Schirawski et al., 2000). Protoplasts were isolated from 4-week-old rosette leaves instead of from cell suspensions, and we used a 40% PEG4000 solution and 15 µg *pART7-35S::PID::YFP* for each transformation.

Auxin transport measurements

Auxin transport assays were carried out as previously reported, with some modifications (Zourelidou et al., 2009). Four 2.5 cm inflorescence stem segments from the basal part of 15cm inflorescence stems were placed in inverted orientation into 30 µL auxin transport buffer (0.5 nM IAA, 1% sucrose, 5 mM MES, pH 5.5) with or without 50 µM NPA for 1 hour, then transferred to 30 µL auxin transport buffer with or without 50 µM NPA containing 200 nM radiolabeled [³H]IAA (Scopus Research BV, Veenendaal, The Netherlands), allowed to

incubate for 30 minutes and subsequently transferred to 30 μ L auxin transport buffer without radiolabeled [^{3} H]IAA and incubated for another 4 hrs. Segments were cut into 5 mm pieces, the bottom piece (0-0.5 cm) was discarded and the remaining pieces were placed separately into 5 mL Ultima Gold™ (PerkinElmer, # 6013329) for overnight maceration. The [^{3} H]IAA was quantified using a PerkinElmer Tri-Carb 2810TR low activity liquid scintillation analyzer.

GUS staining and microscopy

Fresh seedlings and plant organs were directly soaked into GUS staining buffer [10 mM EDTA, 50 mM sodium phosphate (pH 7.0), 0.1% (v/v) Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-glucuronide] under vacuum for 15 min and incubated at 37 °C for 18 hrs. Subsequently, samples were cleared in 70% (v/v) ethanol at room temperature before imaging with Leica MZ16FA or Leica MZ12 equipped with Leica DFC420C or DC500 camera respectively.

To visualize YFP:PDK1 in embryos and roots or PID:YFP in protoplasts, a Zeiss LSM5 Exciter/Axiolmager equipped with a 514 nm laser and a 530-560 nm band pass filter was used on. GFP signals in roots of 5-day-old seedlings were visualized by optionally staining with 10 μ g/mL propidium iodide (PI) for 5 min on slides, and observing the samples with a Zeiss LSM5 Exciter/Axiolmager equipped with a 488 nm laser and a 505–530 nm band pass filter to detect GFP fluorescence, or a 650 nm long pass filter to detect PI fluorescence. All images were captured with a 40 $\times$ oil immersion objective (NA = 1.2). Images were optimized in Adobe Photoshop cc2018 and assembled into figures using Adobe Illustrator cc2017. pDR5::GFP total intensity was measured from three-dimensional reconstruction of the root tips with ImageJ (Fiji). Apical PIN2:GFP abundance was also measured with ImageJ (Fiji) by drawing a free-hand line along the center of the apical PM of epidermal cells.

***in vitro* phosphorylation and yeast complementation**

In vitro phosphorylation and yeast complementation experiments were performed as previously described (Huang et al., 2010; Dittrich and Devarenne, 2012a)

Acknowledgments

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Supplementary data



Figure S1. The DNA sequences of T-DNA insertion loci in the Arabidopsis *pdk1-a* (SALK_113251C) and *pdk1-b* (SALK_007800) T-DNA insertion mutant alleles from NASC. Letters with different colors represent indicated DNA features as indicated in the orange box on the top. Two colored boxes at the bottom show the alignments of part of the PDK1 transcript (green) and the corresponding protein sequence (yellow) in wild-type Arabidopsis (WT) and the *pdk1-a* allele (SALK_113251C). The differences are highlighted in red.

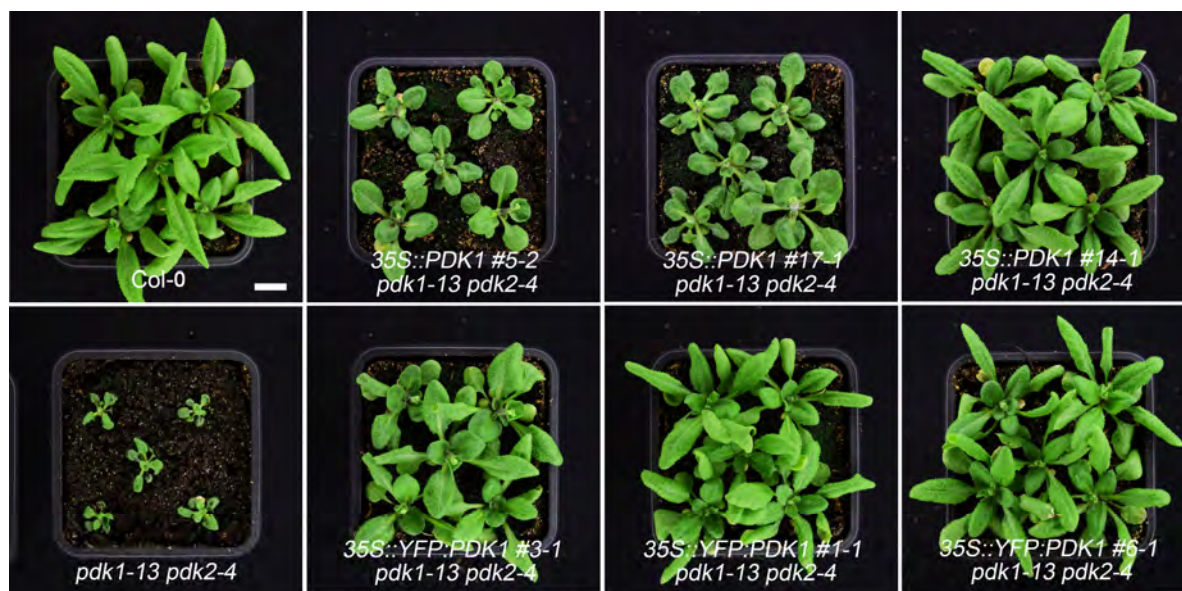


Figure S2. Complementation of *pdk1-13 pdk2-4* by 35S::PDK1 or 35S::YFP:PDK1. Plants of wild type (Col-0), *pdk1-13 pdk2-4* and representative complementation lines were grown on plates for 10 days then in soil for 20 days before photographing.

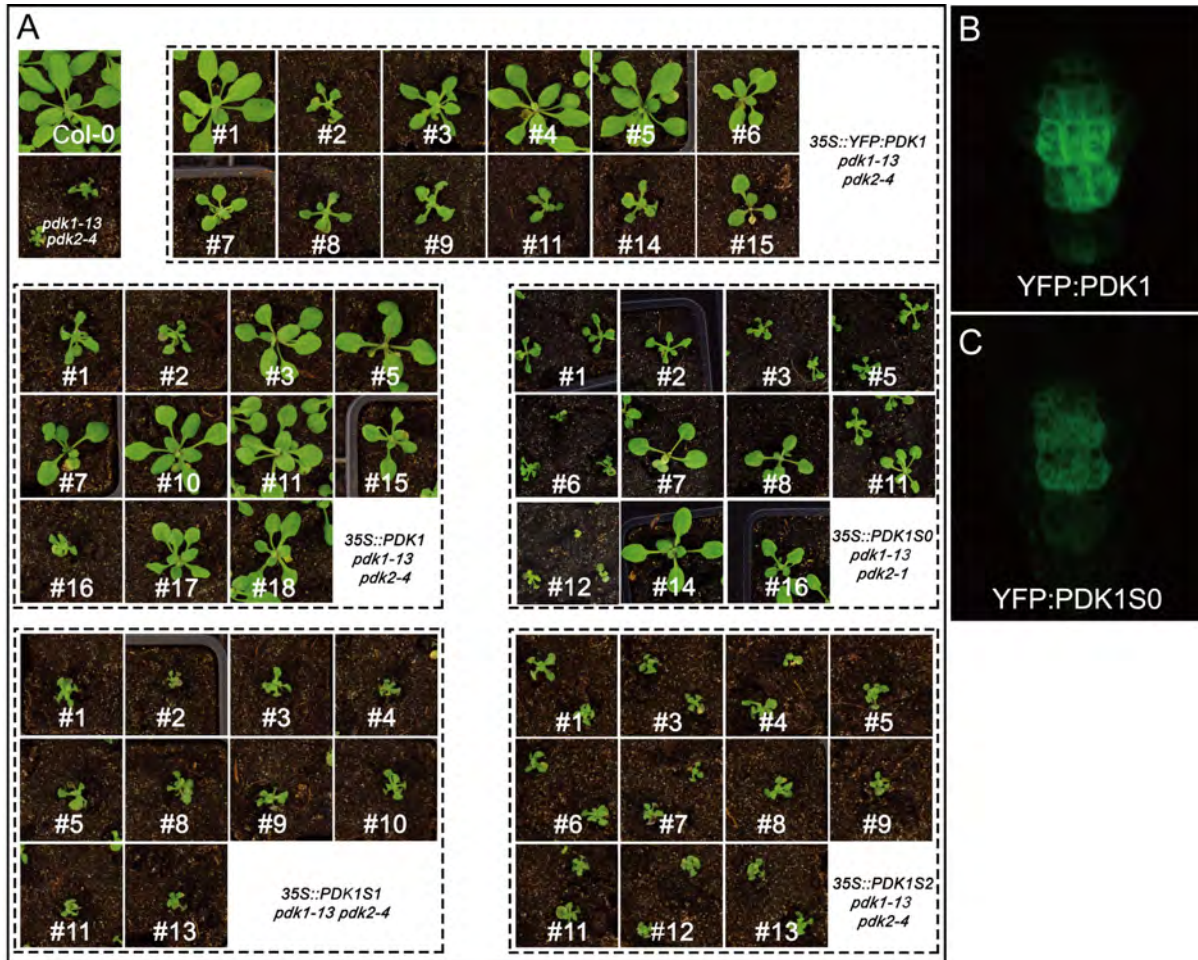


Figure S3. Splice variants. A, Complementation assay for overexpression of cDNAs representing the different *PDK1* splice variants in the *pdk1 pdk2* mutant background. Plants were grown on plates for 10 days, and subsequently transferred to and grown in soil for 10 days. B and C, Subcellular localization of YFP:PDK1 (B) and YFP:PDK1S0 (C) in root columella cells. YFP:PDK1S0 did not show any plasma membrane localization.

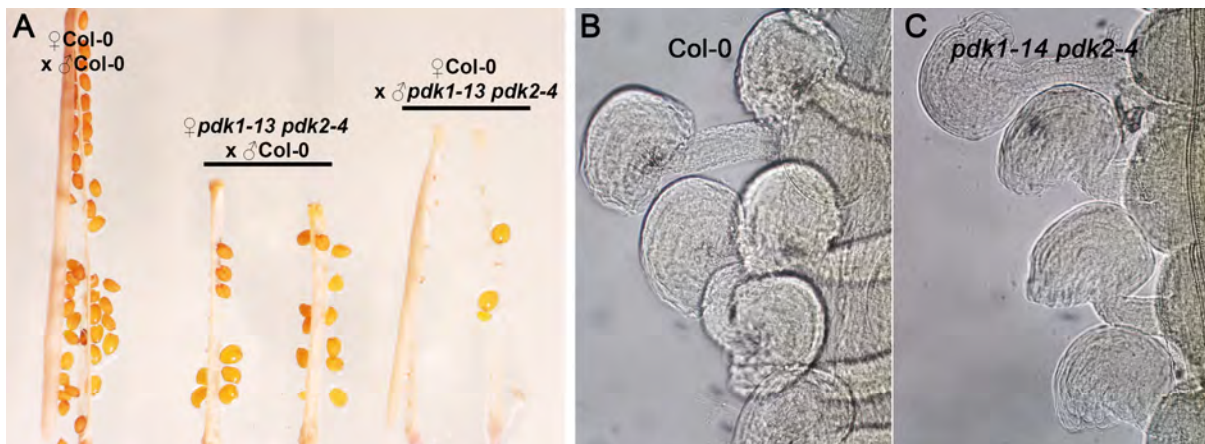


Figure S4. *pdk1 pdk2* mutants are strongly defective in male gametophyte development and show normal ovule development. A, Ripe siliques with the valves removed, derived from reciprocal crosses between wild-type Arabidopsis (Col-0) and the *pdk1-13 pdk2-4* loss-of-function mutant. B and C, Representative DIC images showing the phenotype of wild-type (B, Col-0, (n>300) and *pdk1-14 pdk2-4* mutant (C, n>300) ovules.

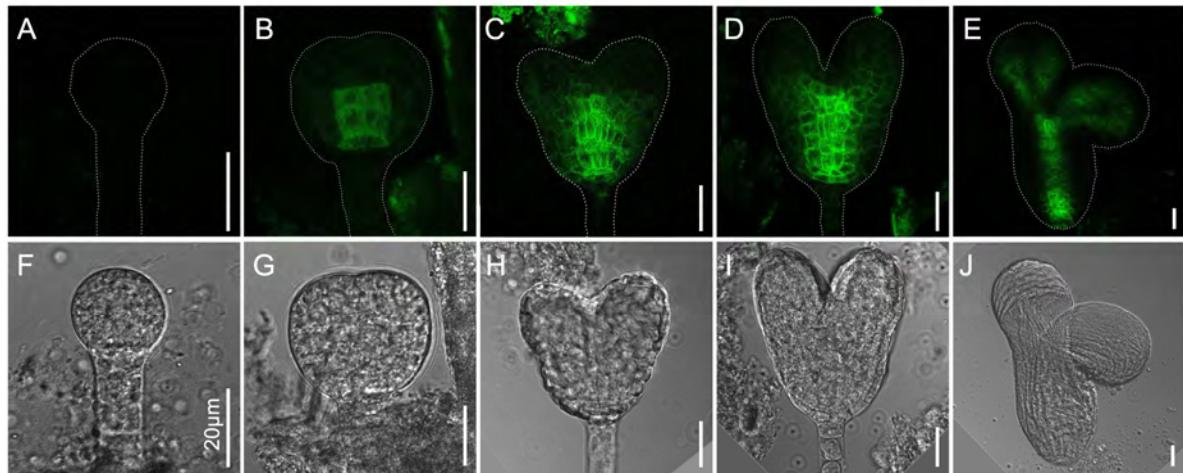


Figure S5. *PDK1* expression is confined to the provascular tissue during Arabidopsis embryo development. A-E, Confocal (A-E), and bright field images (F-J) of Arabidopsis *pPDK1::YFP:PDK1* 16-cell (A, F), globular (B, G), heart (C, H), late heart (D, I), and torpedo (E, J) stage embryos.

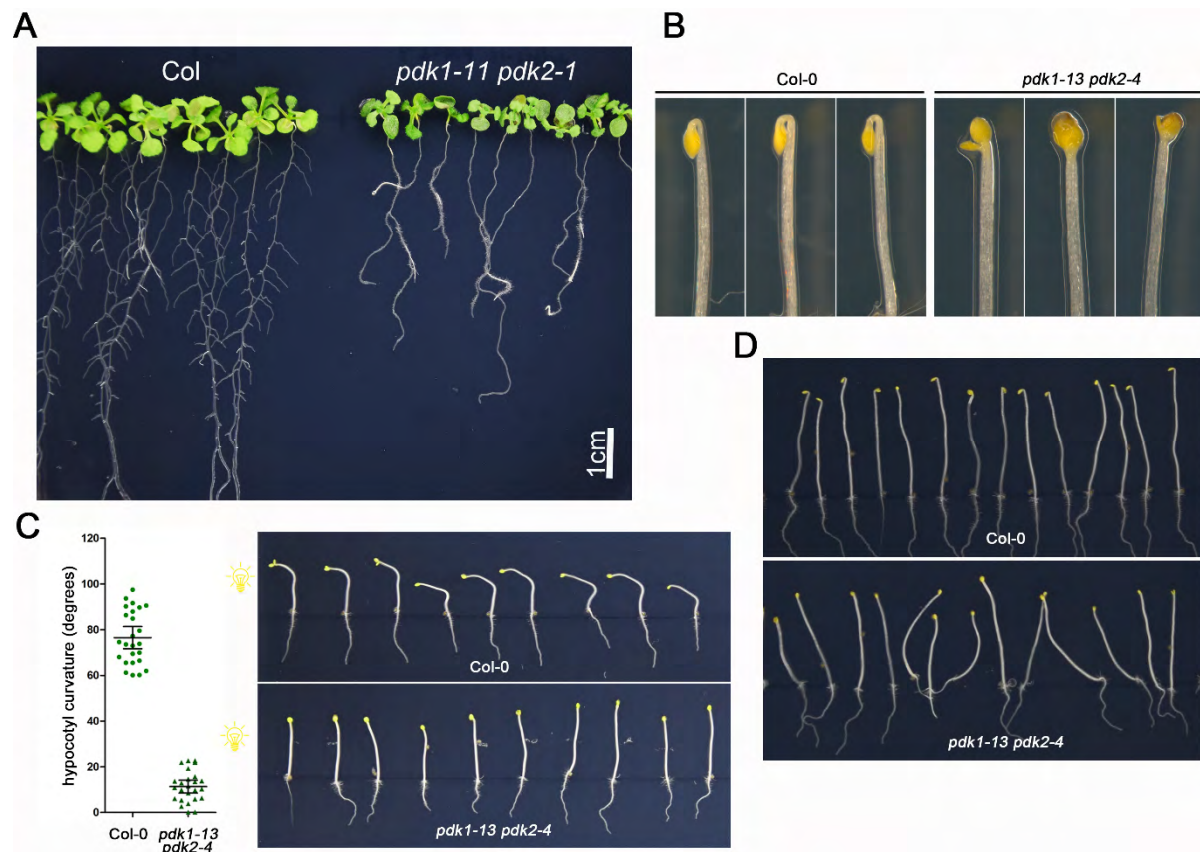


Figure S6. *pdk1 pdk2* mutant seedlings phenocopy *d6pk012* triple mutants. A, Phenotype of 15-day-old wild-type (Col-0) and *pdk1-11 pdk2-1* seedlings grown on vertical plates. B, Apical hook phenotype of 4-day-old wild-type (Col-0) and *pdk1-13 pdk2-4* etiolated seedlings. C, Hypocotyl curvature after 18h directional white light treatment on 4-day-old etiolated wild-type (Col-0) and *pdk1-13 pdk2-4* seedlings. D, 5-day-old wild-type (Col-0) and *pdk1-13 pdk2-4* seedlings grown in dark. Note the agravitropic hypocotyl growth of *pdk1-13 pdk2-4* mutant seedlings.

Table S1. Frequency of *pdk1 pdk2* double homozygous progeny obtained from *pdk1* (-/-) *pdk2* (+/-) or *pdk1* (+/-) *pdk2* (-/-) (gray) parent plants. n>130. n. a.: not analyzed.

	<i>pdk2-1</i>	<i>pdk2-4</i>
<i>pdk1-11</i>	1:5.8	1:9.6
<i>pdk1-13</i>	1:7.3	1:13.3
<i>pdk1-14</i>	1:26.6	1:12.3
<i>pdk1-31</i>	n. a.	1:9.3
<i>pdk1-32</i>	1:7.5	1:5.7

Table S2. Primers used in this study.

usage	primer name	sequence	target	note
genotyping	LBb1.3	ATTTTGCCGATTTCGGAAC	SALK lines	
genotyping	LB1	GCC TTTTCAGAAATGGATAAATAGCCTTGCTTCC	SALK lines	
genotyping	g-B01/G04-LP	CTTACCATGATTTCGAGCTCG	<i>pdk2</i> mutants	
genotyping	g-B01-RP	AACCTTCTGATCCAGCTCCTG	SALK 450 B01	
genotyping	g-G04-RP	ITCAGGAGCAACATATGCAGC	SALK 62 G04	
genotyping	g-3385C-LP	TGGAAGTTTGGTTGATCGAAG	SALK 053385	
genotyping	g-3385C-RP	ACATTAGCACCCTGGATGAG	SALK 053385	
genotyping	11325C LP	TGTTAAGATGCATCAAAAGCC	SALK 11325C	
genotyping	11325C RP	TTACCACGGTTTGTGAAAGG	SALK 11325C	
genotyping	007800 LP	TGAATGGCTGATTTCCTCAAAG	SALK 007800	
genotyping	007800 RP	AAAGACAAAACACCAACGAGG	SALK 007800	
genotyping	g-PDK1m1-MnlI-FP	ACTATgtaggtatttcaatc	pdk1-1n mutant	followed by MnlI digestion
genotyping	g-PDK1m1-MnlI-RP	ggaaccatgattgtatgcaaa	pdk1-1n mutant	followed by MnlI digestion
genotyping	PDK1-M3dCAPs-FP	ATCATTTTTCAGAGCAGCAAGAGACCTCACCAAC	pdk1-3n mutant	followed by XcmI digestion
genotyping	PDK1-M3dCAPs-RP	ATGTCCTTAAAGAGCAACATAACCT	pdk1-3n mutant	followed by XcmI digestion
cloning	SacI-TurboGFP-FP	CCGAGCTCAGAGGATCTGGATCTGAGTCT		
cloning	PacI-TurboGFP-RP	GGTTAATTAACTATTCTCTCACCAGCATCAGC		
cloning	proPDK1-attB1	GGGGACCAAGTTTGTACAAAAAGCAGGCTTCTTGAACCGAGTACCGCCTTTTC		
cloning	proPDK1-attB2	GGGGACCAAGTTTGTACAAAAAGCAGGCTTCTTCCATTGCCAAGATtcc		
cloning	proPDK2-attB1	GGGGACCAAGTTTGTACAAAAAGCAGGCTTCTGGTCATCTCATGGCTACATCC		
cloning	proPDK2-attB2	GGGGACCAAGTTTGTACAAAAAGCAGGCTTCTTGTCATTGTCAACATctcc		
cloning	BstXI-proPDK1-FP	AaccaacggttggTTGAACCGAGTACCGCCTTTTC		
cloning	KpnI-proPDK1-RP	AAGGTACCttccctttctctaaatctoga		
cloning	PDK1-attB1	GGGGACCAAGTTTGTACAAAAAGCAGGCTTCTATGTTGGCAATGGAGAAAGAATTG		
cloning	PDK1FL-attB2	GGGGACCAAGTTTGTACAAAAAGCAGGCTTCTCGGTTCTGAAGAGTCTCGATTG		
cloning	PDK1S0-attB2	GGGGACCAAGTTTGTACAAAAAGCAGGCTTtaggagcttgaacaaggttgc		
cloning	PDK1S1-attB2	GGGGACCAAGTTTGTACAAAAAGCAGGCTTtaggagcttgaacaaggttgc		
cloning	PDK1S2-attB2	GGGGACCAAGTTTGTACAAAAAGCAGGCTTtaggagcttgaacaaggttgc		
cloning	PIN3HL-attB1	GGGGACCAAGTTTGTACAAAAAGCAGGCTTAGGCGCCAAAGATGCTCATCATGG		
cloning	PIN7HL-attB1	GGGGACCAAGTTTGTACAAAAAGCAGGCTTAGGAGCTAAGATCTTGATCATGG		
cloning	PIN3/7HL-attB2	GGGGACCAAGTTTGTACAAAAAGCAGGCTTAAGAGTAAAGTGTGGGTTTC		
cloning/RT	PDK1FL-FP-BamHI	CGGGATCCCGATGTTGGCAATGGAGAAAGAATTG		
cloning/RT	PDK1FL-RP-EcoRI	GGGAATTCTCAGCGGTTCTGAAGAGTCTCGA		
cloning/RT	PDK1S0-RP-EcoRI	CGGAATTCCGtaggagcttgaacaaggttgc		
cloning/RT	PDK1S1-RP-EcoRI	GGGAATTCTtaAAGATTACTGCCATATC		
cloning/RT	PDK1S2-RP-EcoRI	GGGAATTCTTACAGAGACAGGCTTAAAGC		
cloning/RT	PDK2-FP-BamHI	CGGGATCCCGATGTTGACAATGGACAAGGAATT		
cloning/RT	PDK2-RP-XhoI	CCGCTCGAGCGGTCAACGGTTTGAAGAGTTTCG		
cloning	pYAO-EcoRI-FP	cgGAATTCgatgggaattcattgaaacc		
cloning	pYAO-EcoRI-RP	ccGAATTCCTCTTCTTCTTCTCGTTGTG		
cloning	GWR-XhoI-FP	CCGCTCGAGgagctccctagggctgttacccta		
cloning	GWR-XhoI-RP	CCGCTCGAGctagaggatccactagtcgggtccggac		
cloning	PDK1-CC-M1-FP	ATTGCACTTGAATCTTGTGAGGG	3rd exon of <i>PDK1</i>	gRNA
cloning	PDK1-CC-M1-RP	AAACCCCTCACAAGATTCAAGTGC	3rd exon of <i>PDK1</i>	gRNA
cloning	PDK1-CC-M2-FP	ATTGACTCCTCTCCCGCAACTTTC	6th exon of <i>PDK1</i>	gRNA
cloning	PDK1-CC-M2-RP	AAACGAAAGTTGCGGGAGAGGAGT	6th exon of <i>PDK1</i>	gRNA
cloning	PDK1-CC-M3-FP	ATTGAGACCTCATCGACCGTTTGC	7th exon of <i>PDK1</i>	gRNA
cloning	PDK1-CC-M3-RP	AAACGCAACCGGTCTGATGAGTCT	7th exon of <i>PDK1</i>	gRNA
RT	ACTIN2-RT-FP	TGAGACCTTTAACTCTCCCGCTA		reference gene
RT	ACTIN2-RT-RP	TGATTTCCTTGTCTCATACGGTCA		reference gene
qRT	PP2A-3-qRT-FP	GATGGATACAACCTGGGCTCACG		reference gene
qRT	PP3A-3-qRT-RP	TCCGGTGTGTTTCAAACTGG		reference gene
qRT	GH3.3-qRT-FP	TTCGGTGTACGGATGTTTAC		
qRT	GH3.3-qRT-RP	TTCGTTTATCTTCGGTTTGA		
qRT	SAUR16-qRT-FP	ACAATGCTACGACGAGGAAG		
qRT	SAUR16-qRT-RP	CTTCTTCAACAGGGATGGTG		
qRT	IAA5-qRT-FP	ACCGAATACGGCTAGGTCT		
qRT	IAA5-qRT-RP	CTGTTCTTCTCCGGTACGA		

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Chapter 3

PDK1 is part of an auxin-sensing rheostat controlling vascular differentiation

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Abstract

BREVIS RADIX (BRX) and PROTEIN KINASE ASSOCIATED WITH BRX (PAX) operate as a molecular rheostat in regulating PIN-FORMED (PIN) auxin efflux carrier activity during root protophloem differentiation. Elevated auxin levels decrease the plasma membrane abundance of BRX, thereby releasing PAX and allowing its activation leading to PIN phosphorylation and enhanced auxin efflux. However, how PAX itself is activated by phosphorylation is still unknown. Here we identified the 3'-*PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE 1* genes *PDK1* and *PDK2* as novel protophloem regulatory genes. The *pdk1 pdk2* double loss-of-function mutant shows severe protophloem differentiation defects in the root and fragmented vein patterning in the cotyledons. By complementation analysis we prove that PDK1 is responsible for PAX activation by phosphorylation at Serine596 during vascular development, and that it does not require its phospholipid binding Pleckstrin Homology (PH) domain for this process. Our data indicates an important role for PDK1 in auxin-directed feedback signaling during vascular differentiation.

Introduction

Vascular tissues in land plants consist of phloem and xylem, two functionally distinct domains that provide highways interconnecting highly differentiated plant organs and tissues, allowing them to benefit from each other so that plants can efficiently grow and survive on land. Xylem is composed of dead cells at maturity and transports water and minerals taken up from the soil from the root to stems and leaves. In contrast, phloem consists of living cells that transport sap containing sugars, amino acids, hormones and other messenger molecules from source to sink organs (De Rybel et al., 2016).

During the past decades, numerous genes have been identified that regulate phloem differentiation and development (summarized in Anne and Hardtke, 2018). Apart from several downstream nuclear-localized transcriptional regulators, such as ALTERED PHLOEM DEVELOPMENT (APL) (Durner et al., 2003), AUXIN RESPONSE FACTORS (ARFs) (Marhava et al., 2018), SMAX1-LIKEs (SMXLs) (Wallner et al., 2017), NAC45/86 (Furuta et al., 2014) and PHLOEM EARLY DOFs (PEARs) (Miyashima et al., 2019), the upstream signaling pathways can be classified into five different groups: 1) the brassinosteroid (BR) signaling pathway involving the BRASSINOSTEROID INSENSITIVE 1/ -LIKE1/ -LIKE3 (BRI1/BRL1/BRL3) receptor kinases and the downstream BRASSINOSTEROID-INSENSITIVE 2 (BIN2) kinase (Anne et al., 2015; Kang et al., 2017); 2) the OCTOPUS (OPS) and OPS-LIKE2 pathway that promotes BR signaling by inhibiting BIN2 (Truernit et al., 2012; Anne et al., 2015; Ruiz Sola et al., 2017); 3) a signaling cascade involving CLAVATA3/ENDOSPERM SURROUNDING REGION 45 (CLE45), the receptor-like kinase BARELY ANY MERISTEM 3 (BAM3) and the MEMBRANE-ASSOCIATED KINASE REGULATOR 5 (MAKR5) (Kang and Hardtke, 2016; Hazak et al., 2017); 4) the auxin-dependent pathway involving BREVIS RADIX (BRX), PROTEIN KINASE ASSOCIATED WITH BRX (PAX) and the PIN FORMED (PIN) auxin efflux carriers (Scacchi et al., 2010; Marhava et al., 2018); 5) enzymes involved in phosphoinositide homeostasis, including the PHOSPHATIDYLINOSITOL-4-PHOSPHATE 5-KINASEs (PIP5Ks) and the inositol polyphosphate 5'-phosphatases COTYLEDON VASCULAR PATTERN 2 and CVP2-LIKE1 (CVP2/CVL1) (Mouchel et al., 2004; Carland and Nelson, 2009; Rodriguez-Villalon et al., 2015). These pathways play parallel roles in controlling vascular differentiation, but there is clear cross-talk between them and most mutants in the corresponding genes display defects in root protophloem development.

The Arabidopsis primary root has been widely used as a model for studying phloem function and development at the molecular level, because of its easy experimental accessibility (Anne and Hardtke, 2018). In the root meristem zone, the stem-cell niche is maintained by high auxin levels, whereas relatively lower auxin levels keep the stem cell daughter cells in the dividing state before cell specification. The subsequent differentiation of the specified cells is accompanied by a gradual increase in auxin concentration. Expression of a constitutively active version of the ARF5/MONOPTEROS (MP) in protophloem sieve elements (PPSEs) using the CVP2 promoter results in accelerated PPSE differentiation, emphasizing that accurate modulation of the auxin response is a critical step in the protophloem differentiation process (Bauby et al., 2007; Marhava et al., 2018). This modulation of the auxin response is mainly achieved through the activity of the PIN auxin efflux carriers PIN1, PIN3, and PIN7, which are predominantly located at the rootward (basal) side of root stele cells, and control the

auxin concentration in the individual cell types by directing the auxin flux toward the root tip (Feraru and Friml, 2008; Marhava et al., 2018).

The auxin transport activity and polarity of these PIN1-type carriers are controlled by AGC kinase family members through direct phosphorylation of specific serine residues in their large central hydrophilic loop (PIN-HL). Phosphorylation by the AGC kinases PINOID (PID), WAG1 or WAG2 regulates PIN polarity (Friml et al., 2004; Dhonukshe et al., 2010; Huang et al., 2010), whereas phosphorylation by the PID, D6PK or D6PK-LIKE (D6PKL) AGC kinases has been reported to enhance PIN-mediated auxin efflux capacity (Barbosa et al., 2014; Zourelidou et al., 2014). Recently, the D6PK/D6PKL-related kinase PAX was identified from a screen for BRX interactors by co-immunoprecipitation mass spectrometry (Marhava et al., 2018). PAX is specifically expressed in the developing protophloem and xylem axis of the root, where it co-localizes with PIN1 and BRX at the basal side of cells. At lower auxin levels, BRX interacts with PAX at the plasma membrane and inhibits its activation, resulting in reduced PIN-mediated auxin efflux and leading to an increase in the cellular auxin concentration. These higher auxin levels induce movement of BRX from the PM to the nucleus, thereby releasing PAX and allowing it to be activated by phosphorylation at Serine596 (S⁵⁹⁶). Activated PAX subsequently enhances auxin efflux by phosphorylating PIN proteins, again leading to reduced cellular auxin levels. Loss-of-function mutants in the *PAX* gene have a shorter primary root. The root phenotype is much weaker compared to that of *brx* and *ops* mutants, but *pax* roots have similar gaps in the PPSE differentiation zone, which is caused by the failure of cell differentiation and characteristic cell-wall thickening (Marhava et al., 2018).

In Chapter 2 we reported on the strong developmental defects of the Arabidopsis double loss-of-function mutant in the 3-PHOSPHOINOSITIDE-DEPENDENT KINASE-1 (PDK1) encoding genes *PDK1* and *PDK2*. Interestingly, the root defects of this mutant strongly resemble those observed for the *pax*, *brx*, and *ops* loss-of-function mutants. Moreover, PAX has previously been shown to be activated by PDK1 by phosphorylation at serine⁵⁹⁶ (S⁵⁹⁶) (Zegzouti et al., 2006; Gray et al., 2013). Here we show that PDK1 functions directly upstream of PAX as an activator in the auxin-sensing rheostat during root protophloem differentiation. Unexpectedly, PDK1 does not require its phospholipid binding PH domain for this activator function.

Results

Vascular defects underly the short root phenotype of the *pdk1 pdk2* mutant

Seedlings of the *pdk1 pdk2* loss-of-function mutant develop fused or aberrantly positioned cotyledons and a significantly shorter primary root that starts to show agravitropic growth around 7 days after germination (Figure 1A, B) (Chapter 2). The specific expression pattern of *PDK1* and *PDK2* in (pro)vascular tissues from early embryo development suggested a role for these genes in vascular development (Figure 1C, D) (Chapter 2). PDK1 has previously been shown to be an activator of the AGC kinase PAX *in vitro* (Zegzouti et al., 2006; Gray et al., 2013). Since *pax* mutants also have a short root because of defects in protophloem differentiation (Marhava et al., 2018), we investigated *pdk1 pdk2* roots for vascular defects. Protophloem differentiation in *pdk1 pdk2* roots was indeed disturbed, leading to a high frequency of gaps in PPSEs (Figure 1E, F, G). These gaps were absent and root growth was restored to wild-type levels in *pPDK1::YFP:PDK1* (*PDK1*) complemented double mutant lines

(Figure 2A, B), indicating that defects in protoxylem differentiation are caused by *pdk1 pdk2* loss-of-function and that they underlie the short root phenotype of mutant seedlings.

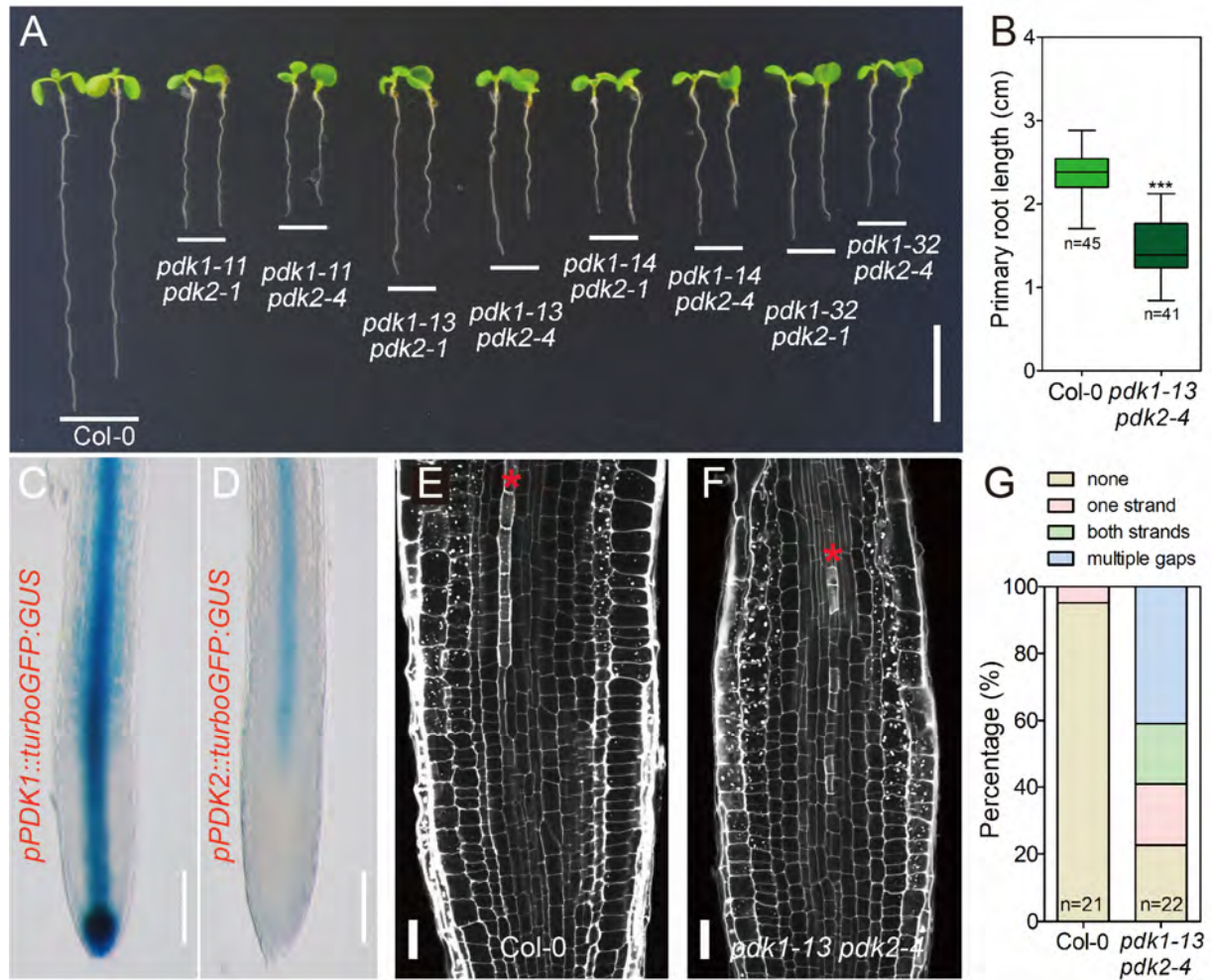


Figure 1. *PDK1* and *PDK2* are expressed in primary root vascular tissue to control phloem development-dependent root elongation. A, 7-day-old seedling phenotype of wild type (Col-0) and different *pdk1 pdk2* mutant combinations. Bar = 1 cm. B, primary root length of 7-day-old wild type (Col-0) and *pdk1-13 pdk2-4* (t-test, *** $p < 0.001$), whiskers: Min to Max. C and D, DIC microscopy images of roots of 5-day-old *PDK1* (C) and *PDK2* (D) *promoter::turboGFP-GUS* seedlings stained for GUS activity. Bar = 0.1 mm. E and F, Confocal sections of mPS-PI stained wild-type (Col-0, E) and *pdk1-13 pdk2-4* (F) roots. The protoxylem cell layer is indicated with a red asterisk. Bar = 10 μ m. G, Quantification of gaps in PPSEs in wild-type and *pdk1-13 pdk2-4* roots. The number of observed roots is indicated in each bar.

PDK1 is the PAX activator in the auxin-sensing rheostat regulating PPSE differentiation

Phosphorylation of PAX at S⁵⁹⁶, which is a PDK1 *in vitro* target (Zegzouti et al., 2006; Gray et al., 2013), has been reported to be induced by auxin to fine tune auxin concentrations during PPSE differentiation (Zegzouti et al., 2006; Gray et al., 2013; Marhava et al., 2018). Introduction of a construct containing a *PDK1* promoter driven *YFP:PAX(S596D)* fusion gene (*PAX(SD)*) in the *pdk1 pdk2* mutant background completely restored protoxylem differentiation and primary root growth (Figure 2A, B, C). Root gravitropic growth was also partially restored in *PAX(SD) pdk1 pdk2* seedlings (Figure S1M-O). *PDK1* promoter-driven expression of a *YFP*- wild-type *PAX* fusion (*PAX*) only rescued primary root growth when *PAX* was expressed at a very high level (Figure 2A, D). In comparison, the *PAX(SD)* construct only

required low expression for complementation. These results indicate that PDK1 is an activating kinase in the rheostat and also suggest that either PAX, like PID, is able to sufficiently auto-activate when expressed at high levels, or that another less efficient kinase can activate PAX when it is abundantly expressed. *PAX(SD) pdk1 pdk2* or *PAX pdk1 pdk2* seedlings still exhibited a strongly decreased lateral root development, similar to the *pdk1 pdk2* mutant, either with a shorter (#9-7 and #13-3) or comparable (#4-7) primary root (Figure S1J-L). The reduced lateral root initiation is likely to be caused by a lack of activation of other PDK1 substrates, most likely the D6PK/D6PKLs, and indicates that PAX has a specific role in vascular development, but cannot completely take over the role of these other AGC kinases.

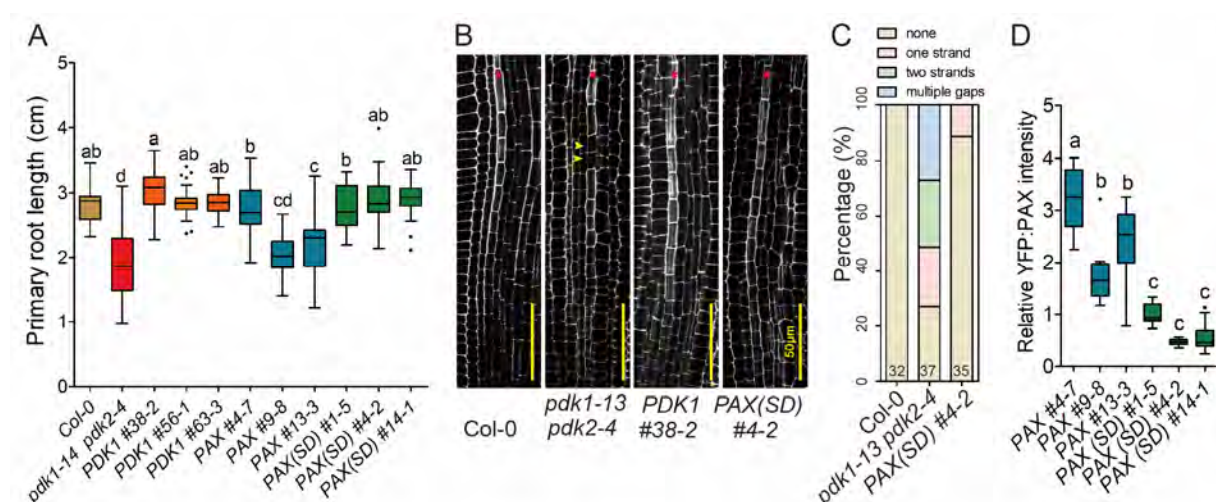


Figure 2. PDK1-dependent activation of PAX directs protophloem differentiation. A-C, *pdk1 pdk2* primary root length of 8-day-old- (A) and PPSE defects of 5-day-old (B, C) wild-type, *pdk1 pdk2*, *pdk1 pdk2 pPDK1::YFP:PDK1* (*PDK1*), *pdk1 pdk2 pPDK1::YFP:PAX* (*PAX*) or *pdk1 pdk2 pPDK1::YFP:PAX(SD)* (*PAX(SD)*) seedlings. 22 to 36 seedlings were measured for each line in (A). The red asterisks mark the protophloem cell layer, yellow arrowheads point out gap locations in (B). Numbers in columns of (C) represent the number of observed roots. D, Relative *PAX* expression level in the indicated lines, as determined by the YFP:*PAX* fluorescence intensity in columella cells. The average intensity in line *YFP:PAX(SD)#1-5* was put at 1. Statistically different groups in (A, D) are indicated with lower case letters, as determined by a One-way ANOVA followed by Tukey's test ($p < 0.05$). The whiskers are plotted with Tukey's method.

***pdk1 pdk2* cotyledon defects uncover a more general role for PDK1 in vascular development**

The fragmented pattern of *pDR5::GUS* expression in *pdk1 pdk2* mutant cotyledons (Chapter 2) suggested that *PDK1* and *PDK2* are also involved in vascular development in cotyledons. Compared to other known mutants with cotyledon vein defects, *pdk1 pdk2* cotyledons exhibited a unique, highly fragmented vein pattern similar to that in *scarface* (*scf-9*) or *gnom/emb30-1* mutants (Figure 3) (Richter et al., 2010). Fused *pdk1 pdk2* cotyledons showed even stronger defects with both fusion and fragmentation (Figure 3C). Even though D6PK/D6PKLs are PDK1 *in vitro* phosphorylation targets and fused cotyledons are also observed for *d6pk012* triple mutant seedlings, dissimilar vein patterns between these two mutants suggested that PDK1 controlled vascular development in cotyledons may not be predominantly dependent on D6PK/D6PKLs (Figure 3C, I) (Zegzouti et al., 2006). Meanwhile, vein patterning was largely restored in *PAX(SD) pdk1 pdk2* cotyledons, although some cotyledons showed few fragments and discontinued loops (Figure 3M). We also observed that

PAX(SD) pdk1 pdk2 plants developed relatively expanded rosette leaves when grown in the soil, compared to *pdk1 pdk2* plants, while *PAX pdk1 pdk2* did not (Figure S2). These results imply that PDK1-mediated PAX phosphorylation signaling is not limited to root (pro)vascular tissues, but occurs more globally during vascular development.

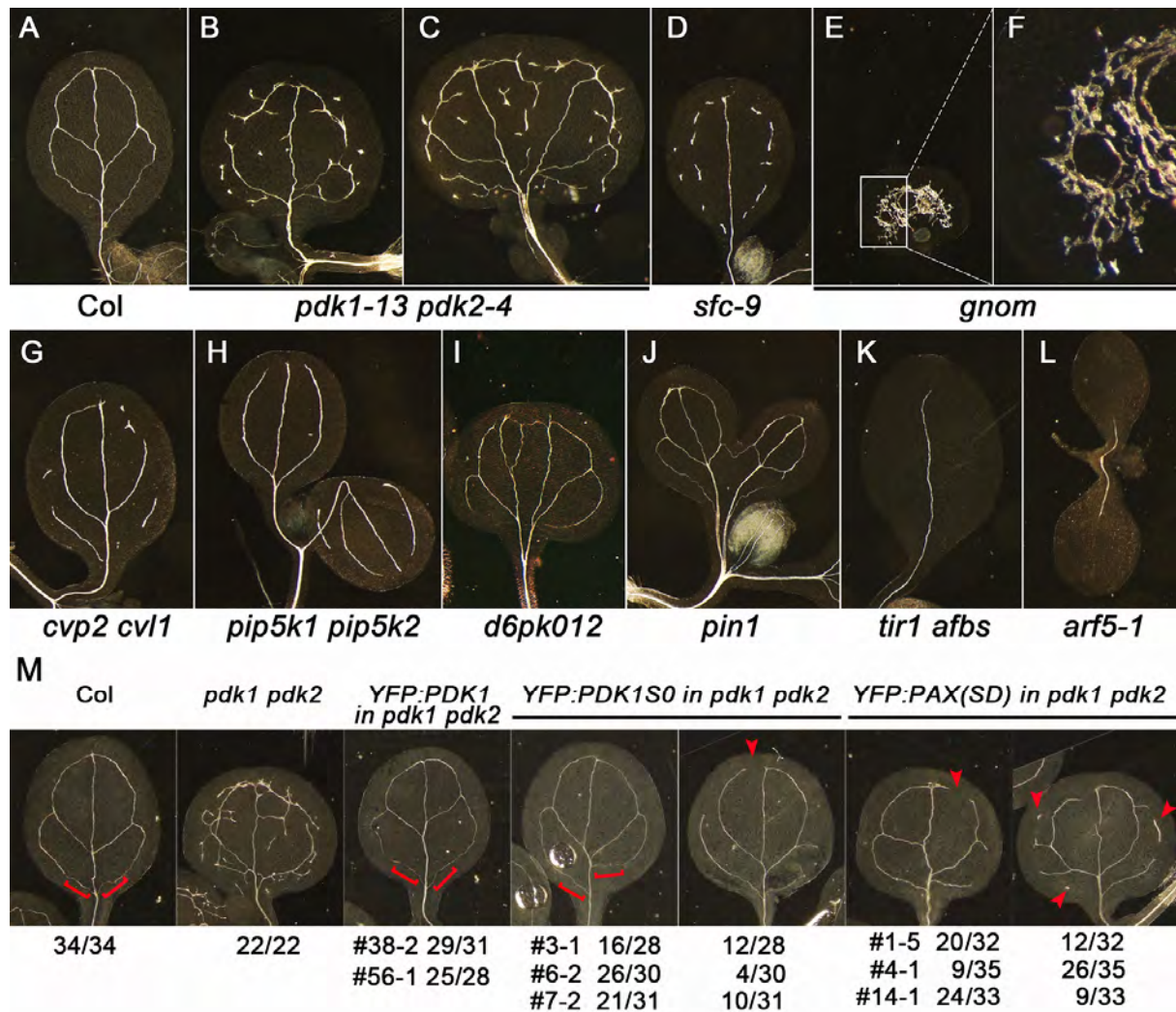


Figure 3. The unique *pdk1-13 pdk2-4* mutant cotyledon vein pattern can be partially rescued by *PDK1S0* and *PAX(SD)*. A-L, Dark-field images showing the vein pattern of cleared cotyledons of 7-day-old seedlings of wild-type Arabidopsis (Col-0) or the indicated mutants (below image). For each line a representative image was selected from >50 observed cotyledons. *tir1 afbs*: *tir1-1 afb1-3 afb2-3 afb3-4* (K). M, *pPDK1::YFP:PDK1S0* and *pPDK1::YFP:PAX(SD)* partially rescued cotyledon vein breaks in *pdk1 pdk2*. The fraction of the total number of observed cotyledons showing a similar phenotype is indicated below each image. Cotyledons showing vein pattern breaks at positions indicated with red brackets are classified into the same group as cotyledons with continuous veins. The red arrow heads point out break locations and isolated islands.

PDK1 PH domain and polar membrane localization are not essential for its role in vascular development

PDK1 co-localizes with PAX at the basal side of the root protophloem cells, thereby providing the opportunity for the two proteins to interact (Figure 4D). However, PAX basal localization does not seem to be dependent on its interaction with PDK1, nor on its phosphorylation status, as PAX and PAX(SD) show the same polar localization at the PM (Figure 4D). To study the contribution of PDK1 basalization on this signaling pathway, we expressed a YFP tagged

PDK1 splice variant product *PDK1S0* lacking the PH domain, under the *PDK1* promoter in the *pdk1 pdk2* background. The fragmented vein pattern in the cotyledon of *pdk1 pdk2* was rescued to wild-type-like or random-break loops by *PDK1S0* (Figure 3M). Moreover, a complete rescue of primary root elongation and PPSE differentiation and a partial restoration of the lateral root emergence was also observed in *YFP:PDK1S0 pdk1 pdk2* seedlings (Figure 4A, B, C; Figure S1). This indicates that PH domain-directed basal *PDK1* localization is not essential for vascular development and primary root growth. Most likely, PAX provides a co-localization and interaction site for *PDK1S0* at the basal side of root protophloem cells, and possibly the higher kinase activity of the *PDK1S0* version (Figure 5B) can compensate for the lack of immediate co-localization with the PAX substrate. The PH domain has been proposed to be characteristic for *PDK1* in vascular plant species (Dittrich and Devarenne, 2012). Based on our results, however, we reexamined the *PDK1* protein sequences in several non-vascular and vascular plants. Although all flowering plant *PDK1* proteins contain a C-terminal PH domain, the *PDK1* proteins in some ferns (*Sceptridium dissectum* and *Plagiogyria japonica*) do not, while these ferns do develop vascular tissue. The *PDK1* ortholog of the green algae *Prasinococcus capsulatus* does have a PH domain (Figure 4E), suggesting that the ancestral plant *PDK1* did have a PH domain, and that this domain has been lost in some early non-seed plant classes, but is maintained in flowering plants. In conclusion, this evolutionary analysis shows that the PH domain in *PDK1* is not specific for vascular plants.

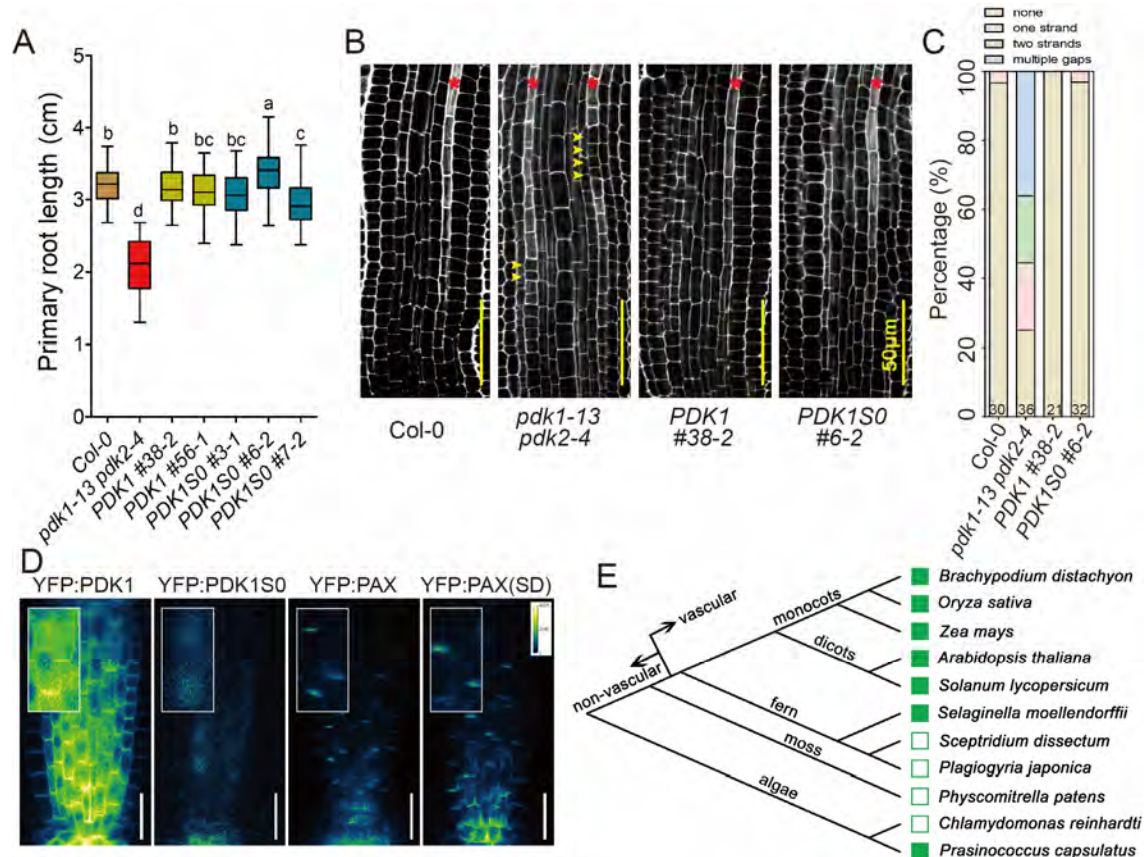


Figure 4. The PH domain is not essential for *PDK1* function in the auxin sensing rheostat during vascular development. A-C, *pdk1 pdk2* primary root length (A) and PPSE (B, C) defects are complemented by *PDK1S0*. 34 to 42 8-day-old seedlings were measured for each line (A). The red asterisks mark the protophloem cell layer, yellow arrowheads point out gap locations in (B). Numbers in columns of (C) represent total counts of observed roots. D, Subcellular localization of YFP:*PDK1*, YFP:*PDK1S0*, YFP:*PAX* and YFP:*PAX(SD)* in *pdk1 pdk2* mutant stele cells. Bar = 20 μ m. E, Phylogenetic analysis of the *PDK1* protein sequences from different plant species. The solid or hollow green box means *PDK1* encompasses or lacks a PH domain, respectively.

PDK1 is strictly regulated at the post-transcriptional level

Our results show that PDK1 is part of an auxin-sensing rheostat that modulates polar auxin transport during vascular development. This raised the question whether PDK1 is regulated by auxin. Quantitative RT-PCR result did not show any effect on *PDK1* transcription up to 4 hours after auxin treatment (Figure 5A). Also, no significant change in the production of the splice variant coding for the shorter but more active PDK1S0 protein version (lacking the PH domain) was detected after auxin treatment (Figure 5B, C). The phospholipids PI(4,5)P₂ and phosphatidic acid (PA) have been reported to bind PDK1 and allow its association with the PM (Deak et al., 1999; Anthony et al., 2004). Applying phosphoinositide biosynthesis inhibitors wortmannin (WM), phenylarsine oxide (PAO), but not the PA biosynthesis inhibitor 1-butanol, strikingly disrupted YFP:PDK1 basal PM localization, suggesting that phosphoinositide may play a more dominant role than PA in YFP:PDK1 PM targeting (Figure 5D, G-I). Treatment with the fungal toxin Brefeldin A (BFA), a specific inhibitor of the ARF-GEF GNOM, also recruited YFP:PDK1 from the PM to the cytosol, suggesting that GNOM-mediated recycling is crucial for the basal localization of YFP:PDK1 in root stele cells (Figure 5D, F). Phospholipid biosynthesis and GNOM-mediated membrane trafficking have been reported to be enhanced by auxin (Geldner et al., 2003; Tejos et al., 2014). However, when treated with IAA, YFP:PDK1 protein abundance on the PM was not noticeably changed (Figure 5D, E).

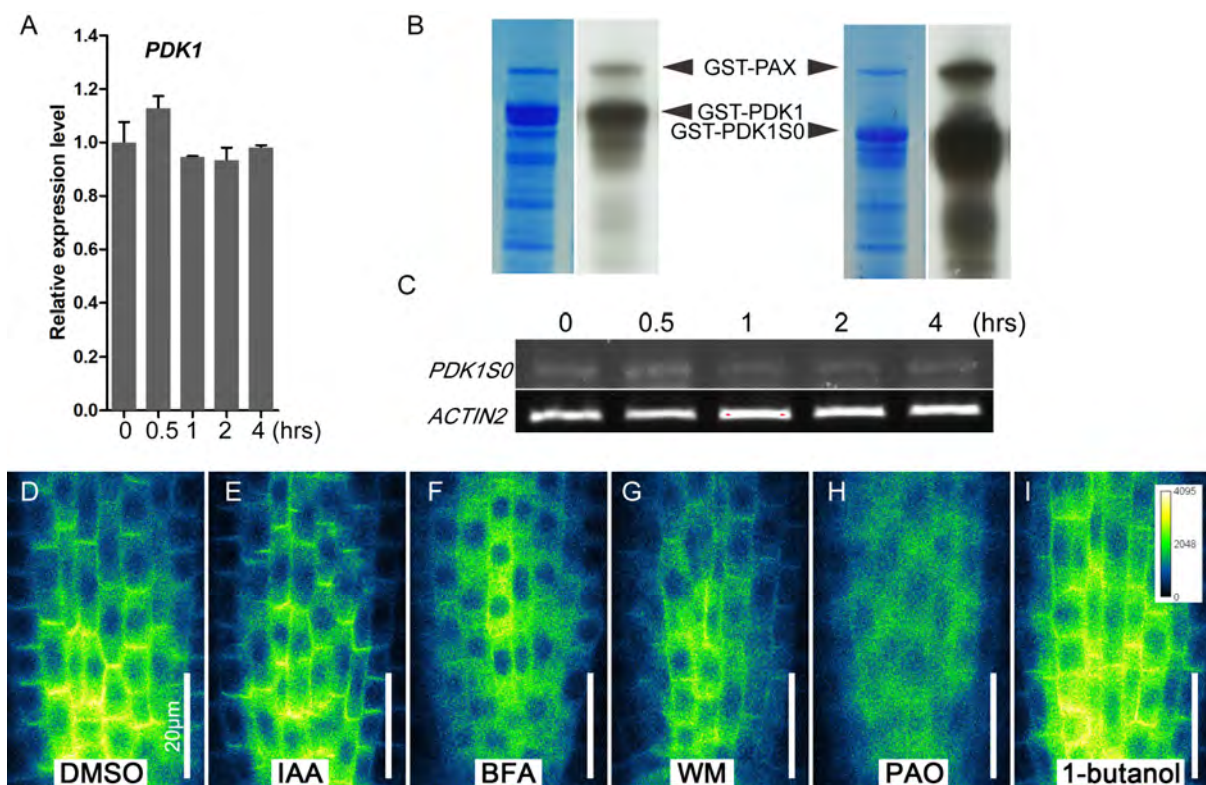


Figure 5. PDK1 is regulated at the post-transcriptional level. A, Relative *PDK1* transcript levels (including the different splicer variants) is not significantly changed in 5-day-old seedlings following 0.5 to 4 hours treatment with 1 μM IAA. B, *In vitro* phosphorylation assay showing GST-PDK1/PDK1S0 autophosphorylation and GST-PAX transphosphorylation. PageBlue stained gel (left) and autoradiograph (right) are shown, and positions of the GST-tagged PAX, -PDK1 and -PDK1S0 are indicated. C, Abundance of the *PDK1S0* splice variant is not strikingly altered in 5 day-old-seedlings after 0.5 to 4 hours treatment with 1μM IAA. D-I, Confocal images of *pdk1 pdk2 pPDK1::YFP-PDK1* roots showing YFP-PDK1 localization after 1 hour treatment with DMSO (D), 1 μM indole-3-acetic acid (IAA)(E), 50 mM brefeldin A (BFA)(F), 33 μM wortmannin (WM)(G) or 0.1% 1-butanol (I), or 30 min treatment with 30 μM phenylarsine oxide (PAO)(H).

Discussion

The development of vascular tissue has been a very helpful innovation for plants in their endeavors to colonize the land. By providing connections between different organs and tissues, vascular tissues allow these organs to communicate with each other and to exchange nutrients and functionally diversify, and in the end, plants to become bigger and more versatile. By using reverse genetics, we verified that two highly conserved protein kinases in eukaryotes *Arabidopsis* *PDK1* and *PDK2* are involved in the vascular development in cotyledons and roots. Our data does point to a role for *PDK1* in phosphorylating and activating AGC1 kinase *PAX*, as part of an auxin sensing molecular rheostat that controls polar auxin transport during vascular development, in roots and cotyledons. *PAX* is central to this rheostat as it enhances auxin efflux by phosphorylating PIN proteins in their large hydrophilic loop (Figure 6A, B). At low auxin levels, *PAX* activation by *PDK1* is prevented by *BRX* binding, resulting in limited auxin efflux (Figure 6A). As a consequence, auxin levels in the cell rise, leading to dissociation of *BRX*, thereby allowing *PDK1* to promote auxin efflux by activating *PAX* by phosphorylation (Figure 6B). Auxin treatment did not significantly enhance *PDK1* basal PM localization in root stele cells. This indirectly suggests that either auxin-controlled phospholipid biosynthesis might be rate-limiting for *PAX* polarity or for *PDK1* kinase activity (Anthony et al., 2004). The similarities in cotyledon vein fragmentation between *pdk1 pdk2*, *cvp2 cvl1* and *pip5k1 pip5k2* mutants do suggest that phospholipids are part of the auxin transport controlling regulatory pathway. Most likely they are necessary for the PM association of *PAX* and of other AGC1 clade kinases downstream of *PDK1*. Moreover, since the *PDK1S0* isoform is clearly more active than full length *PDK1* in auto- and transphosphorylation *in vitro*, alternative splicing of *PDK1* transcripts might provide a mechanism for balancing the phospholipid requirement of *PDK1* activity under specific growth conditions or in certain developmental processes. Polar localization does not contribute too much to proper *PDK1* function in root protophloem differentiation. Similarly, apical localization of *OPS* is not crucial for its function in vascular differentiation (Breda et al., 2017). Evolutional analysis of *PDK1* also excluded the co-relationship between PH-domain and the occurrence of vascular tissue in land plants, which is different from the co-emergence of the *OPS* gene and sieve elements (Breda et al., 2017). Auxin-transport dependent canalization has since long been accepted as a hypothesis for vascular pattern formation. But direct disturbance of auxin transport (*pin1* or NPA treatment) or -signaling (*tir1 afbs* or *arf5*) leads to very different vein pattern defects compared to what is observed for *pdk1 pdk2*, *cvp2 cvl1*, *pip5k1 pip5k2*, *sfc* or *gnom* mutants, in which auxin transport is affected indirectly (Mattsson et al., 1999; Aida et al., 2002; Sieburth et al., 2006; Carland and Nelson, 2009; Naramoto et al., 2009; Richter et al., 2010; Tejos et al., 2014). Here we explain these two types of disturbances in vein pattern formation with a direction-independent or a direction-dependent reduction of auxin flow. In the direction-dependent reduction of auxin transport in the *cvp2 cvl1* mutant, for example, altered accumulation of polarly localized phosphoinositide affects the proper polar localization of PM-associated regulatory proteins, leading to a decrease in auxin transport only in that polar direction. This causes the “mainstream” of auxin to be randomly divided into local auxin sinks, where rapid differentiation of provascular cells leads to vein fragments that are still arranged in a similar pattern as in wild type. In contrast, in direction-independent interference, e.g. by NPA treatment or loss of PIN function, auxin transport is evenly decreased in all directions, leading to reduced

canalization and thus to a more diffuse auxin stream, causing major changes in the vascular pattern and fused veins. Similarly, in auxin signaling mutants auxin sensitivity is evenly decreased in all cells of a tissue, thus leading to changes in vascular pattern and fused veins rather than to interrupted veins in a similar pattern as wild type. This hypothesis seems also true for root protophloem differentiation. Although the polarity of PDK1 itself does not seem to contribute to direction-dependent promotion of auxin transport, the obvious basal localization of its substrate PAX does. Our results suggest that during cotyledon vein patterning, PDK1 also interacts with the polar localized PAX to achieve its biological function. Recent data indicates that auxin-induced vein-formation occurs independent of all known auxin intercellular transporters, including PINs. The results suggest that the ARF-GEF GNOM acts upstream of auxin signaling and auxin transport in vein formation to determine tissue-cell-polarizing, and that, in line with our hypothesis, PIN proteins act as regulators of this differentiation process to detail the vein pattern by canalization (Verna et al., 2019).

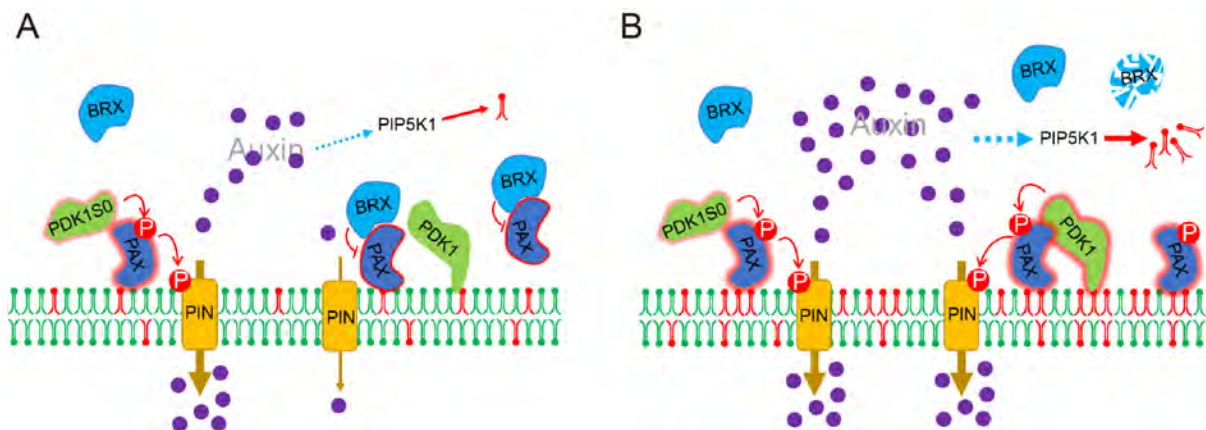


Figure 6. Model showing the role of PDK1 in the auxin sensing rheostat controlling auxin efflux during root protophloem differentiation. Both membrane-localized PDK1 and the cytosolic splice variant PDK1S0 can phosphorylate and activate PAX. When the cellular auxin level is low, PAX is bound by BRX and this prevents its activation by PDK1. Only unbound PAX can be activated by PDK1S0 or PDK1, resulting in limited auxin efflux (A). As cellular auxin levels rise, BRX degradation and subcellular trafficking to the nucleus releases PAX, thereby allowing activation of PAX by PDK1 and the subsequent promotion of auxin efflux by PAX-mediated PIN phosphorylation. In addition, auxin activates PIP5K1 expression and stimulates the PI(4,5)P2 (phospholipid in red) concentration in the PM, resulting in enhanced PDK1 kinase activity (B).

Materials and methods

Plant lines and growth condition

Arabidopsis thaliana (L.) ecotype Columbia 0 (Col-0) was used as wild-type control. *sfc-9* (SALK_069166) (Sieburth et al., 2006), *gnom/emb30-1* (CS6320), *pip5k1 pip5k2* (Tejos et al., 2014), *cvp2 cvl1* (Carland and Nelson, 2009), *d6pk012* (Zourelidou et al., 2009), *pin1* (SALK_047613), *tir1-1 afb1-3 afb2-3 afb3-4* (Parry et al., 2009), *afb5-1* (SALK_023812) have been reported before. *pdk1 pdk2*, *pPDK1::YFP:PDK1 pdk1 pdk2*, *pPDK1::YFP:PDK1S0 pdk1 pdk2*, *pPDK1::turboGFP:GUS* and *pPDK2::turboGFP:GUS* are described in Chapter 2. *pPDK1::YFP:PAX* (PAX) and *pPDK1::YFP:PAX(S596D)* (PAX(SD)) were transformed into the *pdk1-14(-/-) pdk2-4(+/-)* mutant background. *PAX(SD) pdk1-14(-/-) pdk2-4(+/-)* and homozygous segregated from *PAX pdk1-14(-/-) pdk2-4(+/-)* were used for analysis. Plants in

soil were grown at 16h photoperiod and 70% relative humidity at 21°C. For seedling growth, seeds were vernalized in the dark at 4 °C for 2 days after surface-sterilization, subsequently sown on vertical plates with 0.5×Murashige and Skoog (1/2 MS) medium (Duchefa) containing 0.05% MES, 0.8% agar and 1% sucrose. Plates were placed at 22 °C under 16h photoperiod.

Nucleic acid experiments

RNA was isolated from untreated or auxin treated 5-day-old Col-0 seedlings and using NucleoSpin RNA Plant kit (Macherey Nagel, #740949). Subsequent reverse transcription was performed using the RevertAid RT Reverse Transcription Kit (Thermo Scientific™, #K1691). Auxin treatment was performed in 1/2MS liquid media supplemented with 0.01%DMSO (Mock) or with 1µM IAA dissolved in DMSO. The CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) and TB Green Premix Ex Taq II (Tli RNase H Plus) (Takara, #RR820B) were used for qRT-PCR experiment. *PDK1* expression was normalized to the reference gene *PP2A-3* (*AT2G42500*) using the $\Delta\Delta C_t$ method. For *PDK1S0* RT-PCR, a 38-cycle PCR was followed by *BstZ17I* and *NsiI* digestion (Thermo Scientific™).

For *PAX* mutagenesis, *PAX* was amplified from Col-0 cDNA and cloned into *pDONR207*, the entry vector was mutated with QuickChange II XL Site-Directed Mutagenesis Kit (Agilent, #200521). *PAX* and *PAX(S596D)* were then introduced into *pART27-pPDK1::YFP:gateway* by LR reaction (Invitrogen, Gateway BP/LR Clonase II Enzyme Mix #11789020 and #12538120). *pDNOR207-PAX* was also exchanged into *pGEX-gateway* for *in vitro* protein expression. Primers for nucleic acid experiments are listed in Table S1.

Phenotyping and confocal microscopy

For macroscopic phenotyping, plants in the soil and seedlings were photographed with Nikon D5300. Root length was measured based on these images with ImageJ (Fiji) software. Data was analyzed and plotted into graphs with GraphPad Prism 5.

For observation of the cotyledon vein pattern, 5-day-old seedlings were destained overnight in 70% ethanol and placed on slides with chloral hydrate solution (chloral hydrate: glycerol: water = 4:2:1) for 2-day-clearing. A Leica MZ16FA microscope equipped with dark-field optics and a Leica DFC420C camera was used for photographing.

The modified Pseudo-Schiff Propidium Iodide (mPS-PI) method was employed to stain the cell wall of 5-day-old seedling roots for protophloem observation and quantification (Truernit et al., 2008). Samples were observed using a Zeiss LSM5 Exciter/Axiolmager equipped with a 488 nm laser, and the emission signal was collected via a 600nm long pass filter. To quantify gap types, one gap from both strands or more than one gap from both strands were classified into “two strands” or “multiple gaps” group, respectively.

YFP-tagged proteins in the root stele of 4-day-old seedlings were observed using a Zeiss LSM5 Exciter/Axiolmager with a 40× oil immersion objective (NA = 1.2). Samples were excited with a 514 nm laser, and the emission signal was captured using a 530-560nm band pass filter. For YFP-PDK1 subcellular localization analysis, 4-day-old seedlings were treated with 0.1%DMSO (mock), 50 µM brefeldin A (BFA, #B7651, Sigma-Aldrich), 33 µM wortmannin (WM, #W1628, Sigma-Aldrich) 1 µM indole-3-acetic acid (IAA, #I0901, Duchefa Biochemie) or 0.1%

1-butanol (#281549, Sigma-Aldrich) for 1 hour or 30 μ M phenylarsine oxide (PAO, #P3075, Sigma-Aldrich) for 30 min in 1/2MS liquid medium before microscopy observation.

Phylogenetic analysis

Protein sequences of *PDK1* homologous genes from following species were obtained from the National Center for Biotechnology Information (NCBI): *Solanum lycopersicum* (Gene ID: 544184), *Zea mays* (Gene ID: 100384040), *Oryza sativa* (Gene ID: 4324953), *Brachypodium distachyon* (Gene ID: 100835821), *Physcomitrella patens* (Gene ID: 112277681), *Selaginella moellendorffii* (Gene ID: 9631841), *Chlamydomonas reinhardtii* (Gene ID: 5726920), *Arabidopsis thaliana* (Gene ID: 830330). The remaining three PDKs from *Plagiogyria japonica* (onekp:UWOD_scaffold_2139095), *Sceptridium dissectum* (onekp:EEAQ_scaff-old_2087606) and *Prasinococcus capsulatus* (onekp:XMCL_scaffold_2001871) were obtained from data for the 1,000 Plants (1KP) project (<http://www.onekp.com>). 11 sequences were aligned by ClustalW in MEGA-X, and the phylogenetic tree was constructed by maximum-likelihood procedure. At the same time, the secondary protein structures were predicted by InterPro online tool (<https://www.ebi.ac.uk/interpro/>).

Other experiments

Histochemical staining of Promoter-GUS reporter lines for GUS activity and *in vitro* phosphorylation assays were performed as described in Chapter 2 or as published (Huang et al., 2010). A Leica MZ16FA microscope with DIC optics was used to observe the GUS staining photographing.

Acknowledgments

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Supplementary data



Figure S1. Complementation of the *pdk1 pdk2* mutant by *pDPK1* promoter driven *YFP:PDK1*, *YFP:PDK1S0* or *YFP:PAX(SD)* expression. A-O, Phenotype of 13-day-old seedlings of the indicated lines grown on vertical plates. *pdk1 pdk2 pPDK1::YFP-* is omitted in (D-O) for presentation purpose. Note that YFP-PAX only shows partial complementation in the high expressing line #4.7 (see also Figure 2A).



Figure S2. *pPDK1::YFP:PAX* (SD) partially rescues the decreased rosette diameter of *pdk1-14 pdk2-4*, but *pPDK1::YFP:PAX* does not. Rosette phenotype of 26-day-old wild-type (Col-0), *pdk1-14 pdk2-4* mutant, and *pPDK1::YFP:PAX pdk1-14 pdk2-4* (A) and *pPDK1::YFP:PAX pdk1-14 pdk2-4* (B) plants from several independent transgenic lines.

Table S1. Primers used in this research

usage	primer name	sequence
cloning	PAX-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCTGGAAATGGAAAGAGTTG
cloning	PAX-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGAAAACTCAAAGTCTAGAA
mutagenesis	PAX-S596D-FP	AACACACGGTCCATGGACTTTGTTGGAACCCAC
mutagenesis	PAX-S596D-RP	GTGGGTTCCAACAAAGTCCATGGACCGTGTGTT
qRT	qRT-PDK1-FP	CGGTGCTAATGTTTCTAGAAGC
qRT	qRT-PDK1-RP	CTTCTTCTTTGCCCTAACAACC
qRT	PP2A-3-qRT-FP	GATGGATACAACCTGGGCTCACG
qRT	PP3A-3-qRT-RP	TCGGTGCTGGTTCAAACCTGG
RT	PDK1FL-FP-BamHI	CGGGATCCCGATGTTGGCAATGGAGAAAGAATTTG
RT	PDK1S0-RP-EcoRI	CGGAATTCCGTTAGGAGCTTTGAACAAGGTTGCT
RT	ACTIN2-RT-FP	TGAGACCTTTAACTCTCCCGCTA
RT	ACTIN2-RT-RP	TGATTTCTTTGCTCATACGGTCA

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Chapter 4

Two conserved tyrosine residues in the PIN central hydrophilic loop are important for PIN polarity maintenance

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Abstract

Polar transport of the plant hormone auxin is mediated by the PIN-FORMED (PIN) auxin efflux carriers, which determine the direction of this transport through their asymmetric distribution at the plasma membrane. It is well established that PIN polar localization is altered by phosphorylation of these transporters at their central hydrophilic loop by the *Arabidopsis thaliana* AGC3 kinases PINOID (PID), WAG1 and WAG2. However, how the phosphorylation status affects the PIN distribution is yet unknown. Here we identified two functionally important conserved tyrosine residues linked to the AGC3 kinase target serines S1 and S2. PIN1 and PIN2 fluorescent protein fusions in which these tyrosines were substituted by alanines (Y1,2A) lost their ability to complement the corresponding loss-of-function mutants. Moreover, the Y1,2A substitution resulted in reduced polar localization of PIN2, and affected the binding of the PIN2 hydrophilic loop (HL) to the ADAPTOR PROTEIN 1 and 2 μ subunits (AP1M and AP2M). However, the substitutions did not affect phosphorylation of the PIN-HL by PID, nor did they change the *PID* overexpression-induced basal-to-apical shift in PIN2 polarity. Our results indicate that these two tyrosines are involved in AP complex binding and subsequent endocytosis-mediated polarity maintenance, but that the AGC3 kinase-induced shift in PIN polarity requires a different pathway.

Introduction

Membrane-localized proteins perform various functions in the cell, such as mediating responses to intracellular and extracellular signals, transport of macro-molecules and ions, or the synthesis of biomolecules. The activity and abundance of these proteins are dynamically maintained by continuous vesicle trafficking between source and target membrane compartment. Clathrin-coated vesicles (CCVs) selectively mediate endocytosis from the plasma membrane (PM) to early and late endosomes and the *trans*-Golgi network (TGN), by which they directly regulate transmembrane protein abundance at the PM (Robinson and Pimpl, 2014). In plant cells, clathrin-mediated endocytosis controls many transport and signaling processes at the PM, including auxin transport (Kitakura et al., 2011; Kleine-Vehn et al., 2011), boron transport (Takano et al., 2010), brassinosteroid signaling (Di Rubbo et al., 2013), and defense signaling (Bar and Avni, 2009; Mbengue et al., 2016). The clathrin coat of CCVs consists of clathrin heavy and light chain subunits and heterotetrameric adaptor protein (AP) complexes consisting of four subunits. The medium AP complex subunit (M, μ) mainly functions by direct cargo binding. There are five different putative AP complexes (AP-1 to AP-5) and five μ subunits (AP1M1, AP1M2, AP2M, AP3M, and AP4M) encoded by the *Arabidopsis thaliana* (*Arabidopsis*) genome (Sancho-Andrés et al., 2016).

In the past decades, many transmembrane proteins have been identified that are bound and sorted by the AP1 or AP2 complex in mammalian and plant cells (Traub and Bonifacino, 2013; Sancho-Andrés et al., 2016). Apart from some non-canonical motifs that can be recognized by clathrin-associated sorting proteins, such as β -Arrestin, CALM, Sla1p and Eps15 (Traub and Bonifacino, 2013), these studies have uncovered the tyrosine-based motif (YXX Φ , Φ is a residue with bulky hydrophobic side chain) and the dileucine endocytic motif ([DE]XXXL[L/I]) in the cargo proteins as canonical recognition motifs for AP complex binding in mammalian systems (Bonifacino, 2014). AP complex binding motifs in plant transmembrane proteins have mainly been mapped based on these mammalian motif consensus sequences.

In *Arabidopsis*, the PIN-FORMED 1 (PIN1) protein has been one of the pioneer proteins for studies on the intracellular trafficking in plant cells. It belongs to the family of PIN auxin efflux carriers that has been named after the pin-like inflorescences of the *Arabidopsis pin1* loss-of-function mutant (Okada, 1991). The *Arabidopsis* genome encodes 8 PIN family members that share a similar structure, with two multi-transmembrane domains and a hydrophilic loop (HL) in the middle. Five of the members (PIN1, PIN2, PIN3, PIN4, and PIN7) contain a large central HL, and are predominantly localized at the PM where they show a polar distribution in certain cell types and tissues. The remaining three (PIN5, PIN6, and PIN8) have shorter central HLs and are mainly localized at the endoplasmic reticulum (ER). The subcellular localization variances between different PINs and cell types imply the importance of the hydrophilic loop in providing molecular cues for PIN intracellular trafficking and polarity establishment (Mravec et al., 2009; Ganguly et al., 2012). Besides the strong inflorescence defects observed from the *pin1* mutant, mutations in other *PIN* genes also cause some auxin-related developmental defects or insensitivity to environmental factors, such as the vein patterning defects of the *pin5 pin6 pin8* triple mutant (Sawchuk et al., 2013), root agravitropic growth of the *pin2* mutant (Abas et al., 2006), or the non-phototropic growth of the *pin3* mutant (Ding et al., 2011).

At first, the “NPNTY” sequence at the end of the PIN1 HL was considered as a potential endocytosis motif (Mravec et al., 2009). This hypothesis was supported by substitution of the tyrosine⁵⁰⁵ in this motif to alanine in PIN2, resulting in more lateral localization and reduced internalization of PIN2 in root epidermal cells (Kleine-Vehn et al., 2011). However, the intracellular trafficking and subcellular localization of a chimeric PIN5:PIN2-HL:GFP protein fusion, which still contains the PIN2 tyrosine⁵⁰⁵ motif, was shown to be completely different compared to the PIN2:GFP fusion (Ganguly et al., 2014). This implies that the PIN-HL including the canonical NPNTY motif is not the only determiner for intracellular vesicular transport of PIN cargo proteins. Moreover, recently an atypical phenylalanine (F) residue containing motif in the PIN1HL was demonstrated to be involved in AP complex recognition, suggesting that there is more variety in AP complex binding motifs in plant PM proteins. The PIN1 F165A mutant shows reduced endocytosis and is retained in the ER, similar to a PIN1 mutant version where tyrosine⁴⁸⁰ is replaced by alanine (Y480A) (Sancho-Andrés et al., 2016; Marcote et al., 2016). Sancho-Andrés et al. also identified several tyrosine residues (Y²⁶⁰, Y³²⁸ and Y³⁹⁴) in the PIN1HL involved in μ -adaptin binding *in vitro* (Sancho-Andrés et al., 2016). However, single Y to A substitutions did not alter the functionality nor the subcellular localization of the mutant PIN protein.

Although evidence has been provided for the importance of biosynthetic PIN polar secretion to the PM (Kleine-Vehn et al., 2008; Doyle et al., 2015; Łangowski et al., 2016), PIN polarity is considered to be mainly established and maintained by differential endocytosis and recycling between different sides of the PM and endosomal compartments (Kleine-Vehn et al., 2011). Besides the initiation of endocytosis by AP complex binding and the subsequent assembly of the clathrin coat, the polarity and correlated intracellular trafficking process also depends on factors that direct the recycling, such as the ADP ribosylation factor-guanine exchange factors (ARF-GEFs) GNOM and GNOM-like 1 (GNL), and AGC protein kinases and PP2A phosphatases that antagonistically determine the phosphorylation status of the PIN-HL (Michniewicz et al., 2007; Kleine-Vehn et al., 2009; Dhonukshe et al., 2010; Huang et al., 2010; Zhang et al., 2010). Five phosphorylation sites have been identified to be essential for the correct PIN polarity (Zhang et al., 2010; Huang et al., 2010). Phosphorylation of three of these sites (conserved serine residues S1, S2 and S3) by the PINOID (PID), WAG1 and WAG2 kinases results in sorting of PIN proteins to a GNOM-independent pathway (Kleine-Vehn et al., 2009; Dhonukshe et al., 2010; Ding et al., 2011). However, the mechanism of how the phosphorylation status of the PIN-HL can direct the protein to be sorted into different recycling pathways is still unknown.

In humans, evidence has been obtained that phosphorylation modulates the interaction of the cargo with the AP complex. In the human polymeric immunoglobulin receptor, phosphorylation of a serine near a sorting motif was found to stimulate the recruitment of the protein by the AP-1 complex in the trans-Golgi network (TGN) (Okamoto et al., 1994; Orzech et al., 1999). Phosphorylation of serine (S⁷¹¹) within the Y⁷⁰⁹TSI⁷¹² motif of amyloid precursor protein (APP) by protein kinase C (PKC) was found to disrupt its interaction with the AP-3 complex. As a result, APP intracellular trafficking to the lysosome is reduced (Tam et al., 2016). This led us to hypothesize that phosphorylation of nearby residues potentially interferes with AP complex binding.

In this study, we identified two conserved tyrosine residues in the PIN-HL essential for AP complex binding that are closely linked to the PID/WAG1/WAG2 target serines S1 and S2. Substitution of both tyrosines to alanines in PIN1 and PIN2 resulted in proteins with reduced functionality based on mutant complementation experiments. PIN2 Y-A mutants showed reduced polar localization, but the substitutions did not render PIN2 insensitive to the PID overexpression-induced basal (rootward) to apical (shootward) shift in PIN polarity.

Results

Tyrosine residues closely linked to PID phosphorylation sites S1 and S2 are critical for PIN2 function

Previous research has shown that PIN polarity can switch from the basal to the apical side of the cell after induction of PID expression, and that this polarity switch is not dependent on *de novo* protein synthesis (Friml et al., 2004; Dhonukshe et al., 2010). This implies that the polarity switch is initiated by endocytosis, and the PID-mediated phosphorylation possibly facilitates this process by exposing AP complex binding motifs. In human AP2M1, the amino acid residues F¹⁷⁴, W⁴²¹, and R⁴²³ generate a hydrophobic groove for tyrosine binding, and residues D¹⁷⁶, K²⁰³, and R⁴²³ form hydrogen bonds with the hydroxyl group of tyrosine to enhance the binding (Figure S1). At the same time, a pocket lined with aliphatic residues (L¹⁷³, L¹⁷⁵, L⁴⁰⁴, V⁴⁰¹, K⁴²⁰, and V⁴²²) allows the interaction with a bulky hydrophobic chain of the Φ residue in the YXX Φ motif (Figure S1)(Owen, 1998). In Arabidopsis AP1M2, however, the K⁴²⁰ is substituted by a P, which may alter the specificity for the Φ residue. And in AP2M, W⁴²¹ and R⁴²³ are lost, which most likely completely changes its recognition preference (Figure S1). Moreover, the A and P in respectively the PIN1 Y³²⁸STA and Y³⁹⁴PAP motifs are not typical amino acids with a bulky hydrophobic chain, but still help PIN1 to bind to the AP complex (Boll et al., 1996; Sancho-Andrés et al., 2016). These observations imply that the property of the residue at the Φ position is not an indispensable factor for binding in Arabidopsis.

Based on the prominent role of tyrosine residues in AP complex binding motifs in animal cells, and the most likely weaker role for the Φ residue in plant cells, we searched for conserved tyrosines in the PIN-HLs without considering the canonical endocytosis motifs. This identified five conserved tyrosines, four of which are linked to polarity-related phosphorylation sites. We renamed these five residues to Y1 for Y²⁴⁵ (Y²³⁹ in PIN1), Y2 for Y²⁶⁶ (Y²⁶⁰ in PIN1), Y3 for Y²⁹⁷, Y4 for Y³⁴³ (Y³²⁸ in PIN1) (Figure 1A). Single or multiple tyrosines were substituted with alanines in a functional PIN2-VENUS fusion, and the resulting mutant proteins were tested for complementation of the Arabidopsis *eir1-1* mutant. All single mutants rescued the agravitropic root growth of the *eir1-1* mutant, as demonstrated by the absence of root curling typical for the *eir1-1* mutant (Figure 1B), and quantified by the vertical root growth index (Blancaflor, 2015) (Figure 1C). However, mutant PIN2-VENUS proteins containing the combined Y1,2A substitutions failed to complement *eir1-1* (Figure 1B, C), suggesting that Y1 and Y2 are crucial, but functionally redundant residues. All lines expressing the PIN2-VENUS-Y1,2A mutant protein showed a significantly reduced vertical root growth index (Figure 1C). The inability of PIN2-VENUS-Y1,2A to rescue the *eir1-1* mutant was not due to low expression, as expression levels in these lines were comparable to those in the *eir1-1* PIN2-VENUS control lines (Figure

S2). In fact, *PIN2-VENUS-Y1,2A* was highly expressed in some lines (#9 and #14), but no clear correlation could be observed between the expression levels and the reduced vertical root growth index (Figure 1B, Figure S2). Lines *PIN2-VENUS-Y1,2A* #10 and #17 with a single T-DNA insertion, a similar mRNA abundance and a normal *PIN2-VENUS* expression pattern were used for further analysis.

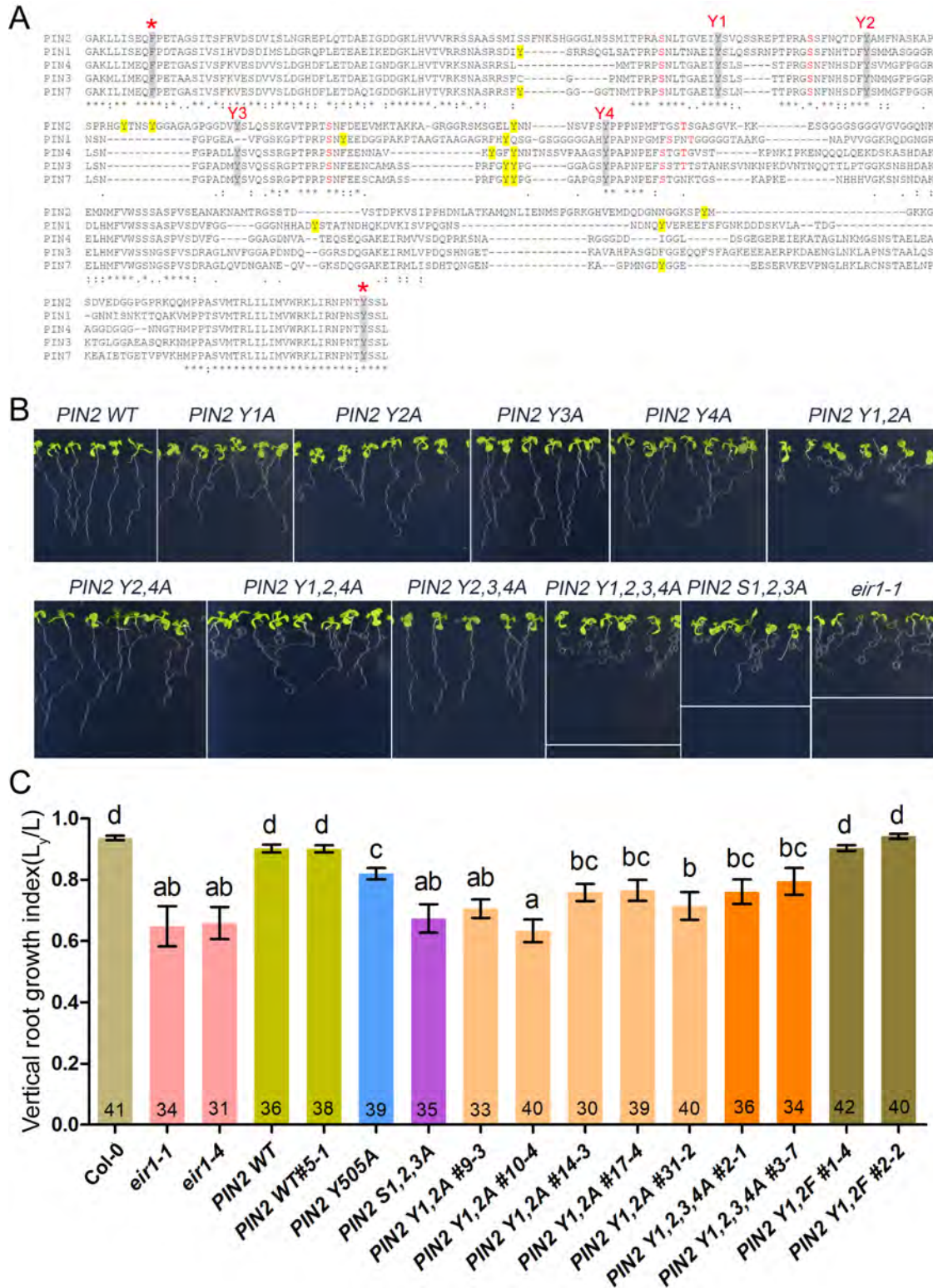


Figure 1. Identification of conserved tyrosine residues essential for proper *PIN2* function. A, Identification of conserved tyrosine residues linked to the conserved TPRXS AGC3 kinase phosphorylation target sites in the *PIN*-

HL. Two reported AP complex binding sites (Kleine-Vehn et al., 2011; Sancho-Andrés et al., 2016) are marked with red asterisks. All tyrosine residues are highlighted in yellow or gray. The conserved tyrosine residues studied in this research are numbered Y1-Y4. Protein sequences are aligned using Clustal Omega <https://www.ebi.ac.uk/Tools/msa/clustalo/>. B, 7 day-old seedlings of transgenic lines containing the indicated PIN2-VENUS wild-type or mutant constructs in the *eir1-1* mutant background to test for rescue of the root curling phenotype. C, The vertical root growth index of wild type (Col-0), *eir1-1*, *eir1-4* and PIN2-VENUS wild-type and mutant constructs in the *eir1-4* mutant background (PIN2-VENUS-Y505A) or in the *eir1-1* background (all other PIN2-VENUS wild-type and mutant constructs). Mean values $\pm$ 95% confidence intervals are shown, the number in each column represents the number of measured roots. Statistically significant groups, as determined by one-way ANOVA and Tukey's test ($p < 0.05$), are indicated with lower case letters. Wild type, mutants and different sets of PIN2-VENUS constructs are marked by different colors.

In parallel, we analyzed whether the two tyrosines could be substituted for the structurally similar, but non-phosphorylatable phenylalanine. Unlike the PIN2-VENUS-Y1,2A version, the PIN2-VENUS-Y1,2F version could completely rescue the *eir1-1* mutant phenotype (Figure 1B). This indicates that the tyrosines are not phosphorylated themselves, and that they are required as hydrophilic structures in the HL for proper PIN2 function.

Mutations leading to the corresponding Y to A substitutions in PIN1 were also introduced in a functional PIN1-GFP fusion construct. As for PIN1, the single Y to A substitution constructs were able to fully rescue the *pin1* mutant phenotypes. However, as shown in Figure S3, three out of four PIN1-GFP-Y1,2A *pin1* and four out of six PIN1-GFP-Y1,2,3A *pin1* F2 plants exhibited *pin*-like inflorescence. These results show that the Y1,2A substitutions weaken PIN1 function, but also that these conserved tyrosine residues are not as critically important for PIN1 as they are for PIN2. We also checked the function of the conserved tyrosine of PIN2 Y505 corresponding site in PIN1 by using PIN1-GFP-Y480A to rescue *pin1* phenotype. Surprisingly, in contrast to reported results (Marcote et al., 2016), four independent PIN1-GFP-Y480A *pin1* lines all showed the same phenotype as wild type (Figure S3B, C). PIN1-GFP-Y480A established the same subcellular localization in root stele cells as wild-type PIN1-GFP, and no accumulation in intracellular compartments was detected (Figure S4).

Y1, 2A causes PIN2 polarity alteration

Roots of 5-day-old seedlings were analyzed with confocal laser scanning microscopy for PIN2 localization defects. In contrast to wild-type PIN2-VENUS (Figure 2A), which showed clear apical localization in the lateral root cap and epidermis cells, PIN2-VENUS-S1,2,3A was localized apolarly in both root cap and epidermal cells close to the root tip (Figure 2B). For both PIN2-VENUS-Y1,2A and PIN2-VENUS-Y505A the difference in polar localization was not as clear as for PIN2-VENUS-S1,2,3A (Figure 2C, D). The polarity index in epidermal cells has often been used to evaluate modest changes in polarity, but the overlap between the lateral signal in epidermal cells and that of either the lateral root cap cell or the cortex cell made us decide not to follow this method (Kleine-Vehn et al., 2011; Łangowski et al., 2016). Instead, we measured the fluorescence intensity at the outer lateral and apical side of lateral root cap cells, respectively (Figure 2I) and calculated the apical to lateral ratio. Compared to wild-type PIN2-VENUS, the fluorescence intensity ratio of PIN2-VENUS-Y1,2A was significantly decreased, implying that the Y1,2A substitutions do cause a reduction in apical PIN2 polarity (Figure 2J). Moreover, longitudinal confocal sections of the epidermis of PIN2-VENUS root tips showed a large number of microdomain structures at the apical side of the cell, whereas these structures were absent in most of the PIN2-VENUS-Y1,2A roots (Figure

2E-H). Additional immunolocalization confirmed that PIN2-VENUS-Y1,2A polarity was still mostly apical, in contrast to what was observed for PIN2-VENUS-S1,2,3A, but that the protein accumulated more at the later, and also the basal side of the epidermis cells (Figure S5).

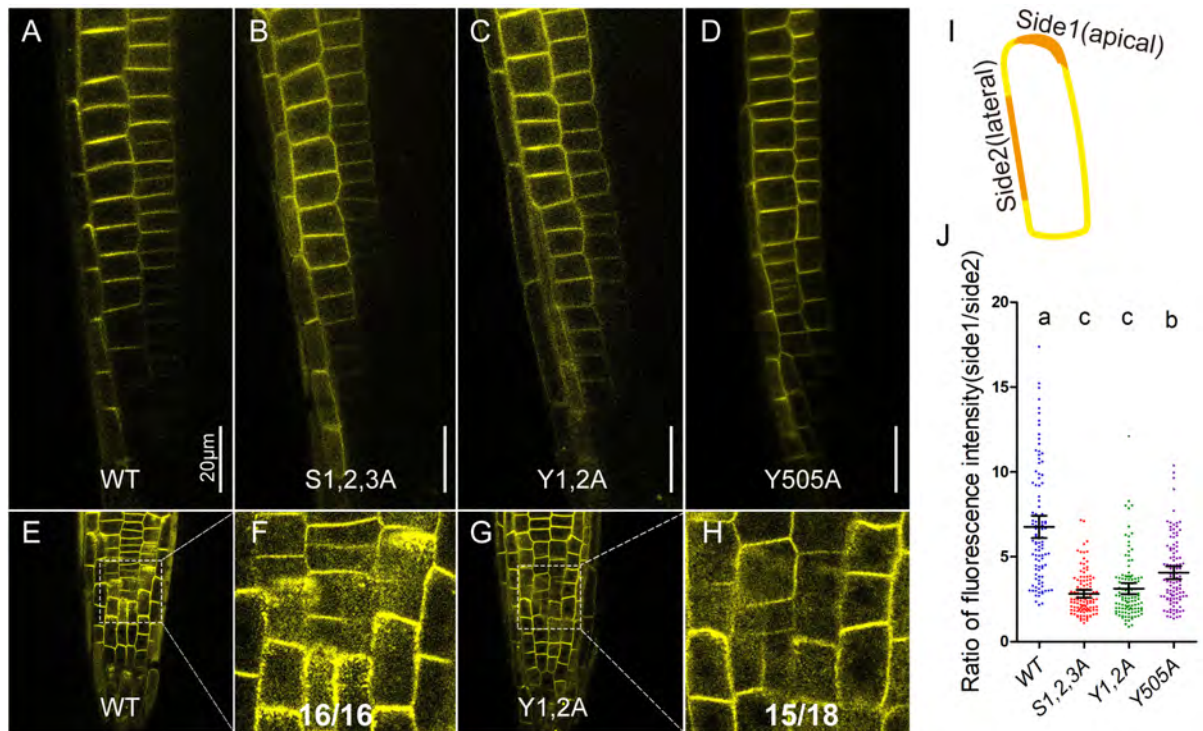


Figure 2. PIN2-VENUS-Y1,2A shows reduced apical polarity in the root cells. A-D, Representative images showing a median confocal section of 7-day-old seedling roots of PIN2-VENUS (WT), PIN2-VENUS-S1,2,3A (S1,2,3A), PIN2-VENUS-Y1,2A (Y1,2A) and PIN2-VENUS-Y505A (Y505A) in the *eir1-1* background. Y1,2A represents #17 line. E-H, Confocal sections through the root epidermis of PIN2-VENUS *eir1-1* (WT; E, F) or PIN2-VENUS-Y1,2A *eir1-1* (Y1,2A; G, H) seedlings. (F, H) are enlargements of the boxed area in (E, G). Numbers in (F) and (H) indicate the number of root tips over the total number observed, for which the image is representative. I, Schematic representation of positions where the fluorescent signal was measured in lateral root cap cells. J, Ratio of fluorescence intensity at side1 and side2 in lateral root cap cells expressing PIN2-VENUS (WT), or the S1,2,3A, Y1,2A or Y505A mutants. More than 103 cells from more than 15 roots were measured for each line. Significantly different means, as determined by one-way ANOVA and Tukey's test ($p < 0.05$), are indicated with lower case letters.

Y1,2 are important for the S1,2-independent PIN2-AP2M interaction

Previous analysis has shown that *hap13-1* (*ap1m2*) mutant roots are agravitropic and that this correlates with irregular epidermis cell shape and PIN2 localization (Wang et al., 2013). Here we examined the root growth of the *ap2m* mutant. Seeds of the *ap2m* mutant are bigger than wild type seeds, as a result, mutant seedlings grow faster (Figure 3A, B). *ap2m* mutant roots were agravitropic as well, as they lacked the typical parallel growth of wild-type roots and instead showed strong waving with a large amplitude (Figure 3A, B). Despite the faster growth, the vertical growth index of *ap2m* roots was significantly reduced compared to wild type (Figure 3C). The agravitropic growth of both *ap1m* and *ap2m* loss-of-function mutants suggests their importance in PIN2 sorting and intracellular trafficking.

In line with the mutant analysis, AP1M1 and AP2M have been shown to bind the PIN2HL *in vitro* in a Y2-dependent manner (Sancho-Andrés et al., 2016). Also in our hands, AP1M2 and AP2M pulled down the PIN2HL, but not the mutant PIN2HL Y1,2A *in vitro*, confirming that both AP subunits interact with the PIN2HL in a tyrosine-dependent manner (Figure 3D).

To explain our observation that *PIN2Y1,2F-VENUS* can rescue the *eir1-1* mutant phenotype, we checked the interaction between the mutant PIN2HL-Y1,2F and AP1M2 or AP2M as well. The Y1,2F substitution completely disturbed the interaction between PIN2HL and AP1M2, but not between the PIN2HL and AP2M (Figure 3D). This suggests that phenylalanine can structurally replace tyrosine to mediate the interaction with some, but not all Arabidopsis AP complexes.

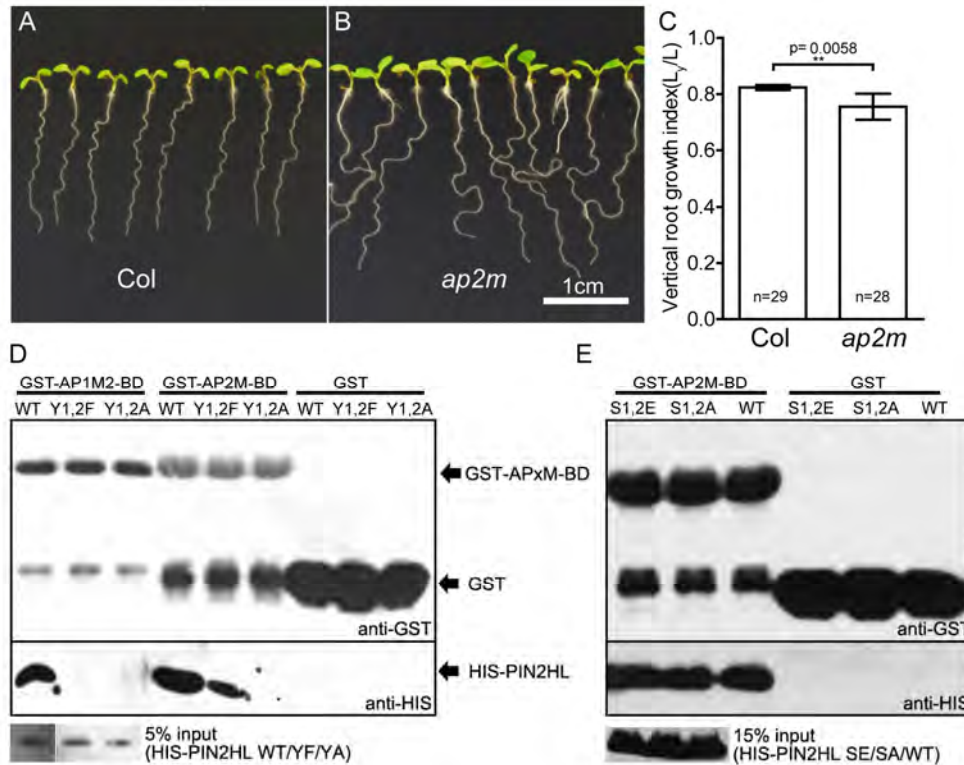


Figure 3. Agravitropic root growth of the *ap2m* mutant correlates with the Y1,2-dependent binding of the PIN2HL fragment to AP2M. A-C, 7-day-old seedlings of the *ap2m* loss-of-function mutant show strongly increased root waving (A, B) and decreased vertical root growth index (C) compared to wild type (Col-0). C shows means with 95% confidence intervals, the *t*-test was used for significance analysis. The number of seedlings used for the measurements is indicated in each column. D and E, Pull-down assays using the GST-tagged binding domain (BD) of the indicated AP μ subunits and HIS-tagged PIN2HL (WT) or the Y1,2F or Y1,2A mutant versions in (D) or the S1,2E or S1,2A mutant versions in (E). The upper panels in (D, E) are Western blots hybridized with anti-GST, the middle and the lower panels are blots hybridized with anti-HIS antibody. The upper and middle panels show the proteins after pull-down, the lower panels show 5% (D) or 15% (E) of the HIS-PIN2HL input before pull-down.

The PID-induced PIN polarity shift does not require Y1,2-dependent AP complex binding to the PIN-HL

Our initial hypothesis was that PIN phosphorylation by the AGC kinases PID, WAG1 or WAG2 alters its subcellular localization by enhancing AP complex binding to conserved tyrosine motifs in the PIN-HL, thereby promoting PIN endocytosis and intracellular trafficking. Results above demonstrated that Y1 and Y2 are functional APxM binding sites, but whether this binding is regulated by phosphorylation of the closely linked S1 and S2 was still not clear. *In vitro* pull-down experiments showed that AP2M binds to wild-type PIN2HL or the loss-of-phosphorylation (PIN2HL-S1,2A) or phosphomimic versions (PIN2HL-S1,2E) with the same efficiency (Figure 3E). Moreover, immunolocalization showed that PIN2-*VENUS*-Y1,2A, like PIN2-*VENUS*, predominantly localized to the apical side of cortex cells in the *35S::PID#21*

background (Figure 4A-D). These results indicated that the binding of the tyrosine residues in the PIN2HL by APxM subunits is not dependent on the phosphorylation state of the PIN-HL, and that the *PID* overexpression-induced basal-to-apical switch in PIN polarity does not require Y1 and Y2.

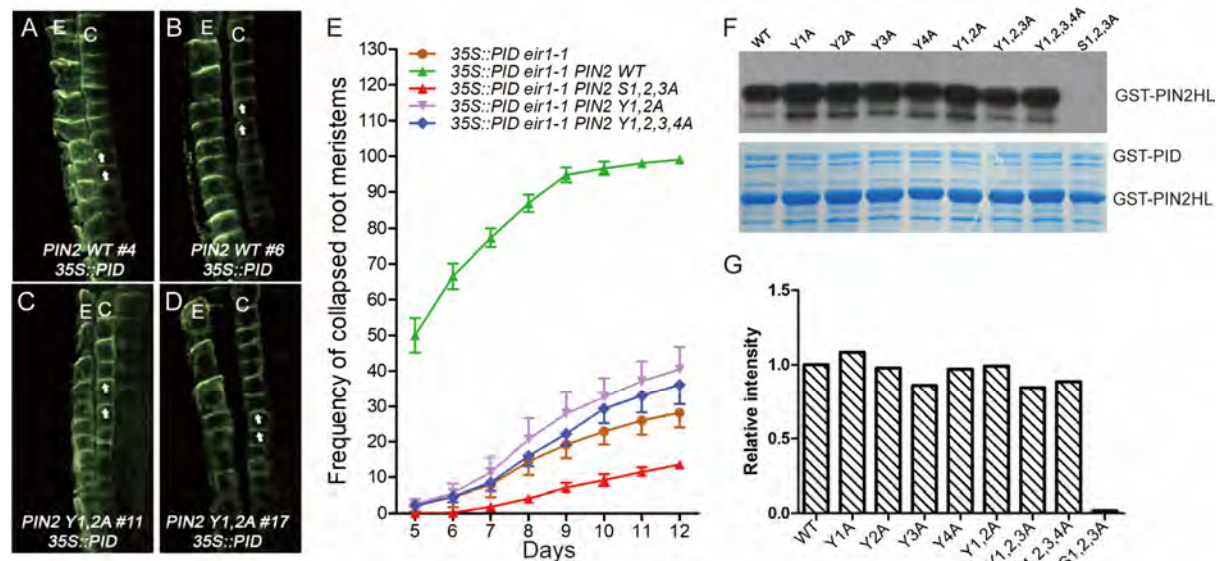


Figure 4. Tyrosine 1,2 to alanine substitution does not interfere with PID phosphorylation and the resulting basal-to-apical polarity shift. A-D, Confocal median section of a 4-day-old seedling root, showing immunolocalized PIN2-VENUS (PIN2 WT) (A, B) or PIN2-VENUS-Y1,2A (PIN2 Y1,2A) (C, D) in the 35S::PID background. “E” and “C” indicate the epidermis and cortex layer, respectively. Polarity is indicated with white arrows. E, Monitoring of the *PID* overexpression-induced root collapse in seedlings of the indicated lines, starting at 5 days after germination. Means from four independent experiments (> 79 seedlings per experiment) are shown with 95% confidence intervals. For presentation purposes, only a one-sided error bar is shown in some cases (S1,2,3A; Y1,2A and Y1,2,3,4). F, Phosphorylation assay of PID on the wildtype (WT) or the indicated Y to A or S to A substitution versions of the PIN2HL. Upper panel: autoradiograph; lower panel: PageBlue stained protein gel. The positions of GST-PID and GST-PIN2HL are indicated. G, Relative autoradiograph band intensity corrected for protein loading using the PageBlue staining image from F. The value for GST-PIN2HL (WT) was put at 1.

The changes in PIN polarity by *PID* overexpression result in disappearance of the auxin maximum from the main root meristem, eventually leading to meristem collapse (Benjamins et al., 2001; Friml et al., 2004). These root meristems can be partially rescued by bringing the 35S::PID construct into the *eir1-1* background (Friml et al., 2004)(Figure 4E), indicating that repolarized PIN2 in the cortex provides an important contribution to the root meristem collapse. Introducing the wild-type *PIN2-VENUS* or the loss-of-phosphorylation *PIN2-VENUS* S1,2,3A into the 35S::PID *eir1-1* background significantly enhanced or decreased the root meristem collapse, respectively (Figure 4E). *PIN2-VENUS*-Y1,2A or *PIN2-VENUS*-Y1,2,3,4A did not trigger a significant increase like *PIN2-VENUS*-WT or a decrease like *PIN2-VENUS*-S1,2,3A in root meristem collapse of the 35S::PID *eir1-1* background, suggesting that the Y to A mutant PIN2-VENUS versions only undergo partial *PID* overexpression-induced basal-to-apical relocation in the cortex (Figure 4A-D), more than PIN2-VENUS-S1,2,3A but not as strong as the wild-type PID-VENUS. This result is in line with the reduced apical polarity of PIN2-VENUS-Y1,2A in wild-type lateral root cap cells (Figure 2). This reduced apical polarity was not caused by a negative effect of the Y to A substitutions on the phosphorylation of S1, S2, and S3, since the different PIN2HL tyrosine mutant versions were phosphorylated at a similar level by PID as the wild-type version (Figure 4F, G). Overall, PID S1,2,3 phosphorylation and Y1,2-dependent AP complex binding act independent of each other, with Y1,2 playing a more

general role in intracellular trafficking, like F165 in PIN1 and Y505 in PIN2 (Kleine-Vehn et al., 2011; Sancho-Andrés et al., 2016).

Discussion

The polarity establishment and the post-transcriptional regulation of PIN proteins have been studied for many years now. Phosphorylation and ubiquitination of the PIN-HL have been identified as the two crucial molecular cues that trigger and alter trafficking of PIN cargo loaded membranes, and thereby regulate the abundance and polarity of PIN proteins at the PM of plant cells (Zhang et al., 2010; Huang et al., 2010; Leitner et al., 2012). In this study, we discovered that two conserved tyrosine residues close to PID phosphorylation sites in PIN HL are responsible for AP complex binding and sorting. However, we did not find a strong correlation between tyrosine binding and PID-directed changes in PIN polarity.

How does PID induce PIN polarity shifting?

PID phosphorylation may be not the only reason causing PIN polarity shifting, but seems sufficient to direct PIN protein sorting into the apical recycling pathway. In line with this, PIN1 S1,2,3A and PIN1 S1,2,3E do not follow wild-type PIN intracellular trafficking behavior and react differently to dark- and BFA treatment (Kleine-Vehn et al., 2009; Huang et al., 2010; Huang, unpublished data). Similarly, the phosphorylation status of S³³⁷/T³⁴⁰ in the PIN1HL has been reported to redirect PIN1 polarity as well (Zhang et al., 2010). This indicates that the phosphorylation state of the PIN-HL does change PIN trafficking. The goal of our studies was to uncover the possible link between serine phosphorylation and AP-mediated intracellular trafficking. In our present study, the conserved Y1 and Y2 were proven to be involved in AP2M binding, but this event seemed independent of S1,2 phosphorylation. On the one hand, a broader binding motif screening could be carried out to investigate how phosphorylation controls differential PIN protein sorting; on the other hand, quantitative mass spectrometry-based proteomics could be used to detect differential protein binding to PIN-HLs with different phosphorylation patterns.

NPNTY, F¹⁶⁵ and Y1,2, which one is the real binding motif?

Up to now, three kinds of AP complex binding motifs in PIN proteins have been identified. They all disable PIN protein functionality by affecting proper PIN subcellular localization. “NPNTY” is the first described sorting motif of PIN that can modify PIN polarity (Mravec et al., 2009). The Y⁵⁰⁵ (in PIN2) and Y⁴⁸⁰ (in PIN1) in this motif have also been reported to be essential for normal intracellular trafficking and polarity establishment (Kleine-Vehn et al., 2011; Marcote et al., 2016). However, when we checked the *PIN2-VENUS-Y505A eir1-4* seedling phenotype, we did not observe a striking difference from wild type seedlings. Some of the older plants showed dwarf stature and some exhibited strong auxin-related phenotypes at the flowering stage, such as pin-like inflorescence. This suggests that the *PIN2-VENUS-Y505A eir1-4* line holds at least two T-DNA insertions of *PIN2-VENUS-Y505A*, and one of the insertion sites is auxin-related. As for PIN1 Y⁴⁸⁰, in contrast to the reported result (Marcote et al., 2016), PIN1-GFP-Y480A is functioning and localizing the same as wild-type PIN1-GFP.

Moreover, an “NPNTY” to “NSLSL” substitution in PIN1 has been reported to result in its endoplasmic reticulum (ER) localization (Mravec et al., 2009), which also conflicts with reported PIN1-GFP-Y480A localization (Marcote et al., 2016). These results made us hesitant to conclude the importance of Y⁵⁰⁵ or Y⁴⁸⁰ for PIN2 or PIN1 endocytosis and trafficking. PIN1-GFP-F165A similarly shows obvious ER localization but with special dot-like endomembrane structure, it accumulates in these intracellular punctate firstly after photobleaching (Figure S4)(Sancho-Andrés et al., 2016). This reminds us the ER-retention of BRI1 in an Endoplasmic Reticulum Quality Control (ERQC) deficient mutant (Liu and Li, 2014; Liu et al., 2015). Since PIN1 Y⁴⁸⁰ and PIN1 F¹⁶⁵ are close to the junction between the transmembrane domain and cytosolic loop, it's highly risky to state that the protein mislocalization is mainly caused by decreased AP complex binding affinity without checking mistakes in protein modification or folding. Instead of the canonical endocytosis motif, we identified two novel tyrosine residues involved in PIN polarity maintenance, most likely through endocytosis by binding to the AP complex. Partial rescue in *eir1-1* and *pin1* suggests that Y1,2 has a significant but limited effect on PIN intracellular trafficking. This is in line with the observation that apical localization is still dominant in epidermal cells. Like the predominantly apical PIN2-VENUS-Y1,2A, also the predominantly basal PIN1-GFP-Y1,2A showed more lateral localization, which indicates that Y1,2-mediated intracellular protein trafficking is a general process for all PIN proteins in the cell, and these tyrosine residues do not differentiate between apical or basal localization.

Different from studies in animal cells, all the current plant tyrosine motifs have been discovered by prediction and mutation. The animal YXXΦ motif has been widely used in *in silico* screens for endocytosis motifs in plant proteins. However, the conserved tyrosines we revealed in this study are part of YXXQ and YXXM/F motifs, which do not follow the Φ rule. *In vitro* pull-down also demonstrated that AP2M recognizes both phenylalanine and tyrosine. As the key residues for tyrosine binding are missing in Arabidopsis AP2M, its binding preference may be completely reformed. Recently, the polar localization of BOR1, another well-known model for membrane protein trafficking, was reported to be dependent on AP2 function but independent of YXXΦ motifs (Yoshinari et al., 2019). Reevaluating our way of searching protein sorting motifs in plant proteins is urgently required.

Materials and methods

Plant lines and growth condition

All plant lines are in the Columbia (Col-0) background. *eir1-4* (SALK_091142) and *ap2m* (SAIL_165_A05) were obtained from the Nottingham Arabidopsis Stock Centre. *p35S::PID#21* (Benjamins et al., 2001), *eir1-1* (Roman et al., 1995), *pin1* (SALK_047613) (Huang et al., 2010), *PIN2-VENUS* (Dhonukshe et al., 2010), *PIN2-VENUS-Y505A eir1-4* (Kleine-Vehn et al., 2011), and *PIN2-VENUS-S1,2,3A eir1-1* (Dhonukshe et al., 2010) have been described previously. Different versions of *pPIN2::PIN2-VENUS* and *pPIN1::PIN1-GFP* were transformed into *eir1-1* and Col-0 respectively using the Agrobacterium floral dip method (Clough and Bent, 1998). Root growth was checked at least three independent lines for each mutated *PIN2-VENUS* version. Eight homozygous lines of *PIN2-VENUS-Y1,2A* were quantified. For each *PIN1-GFP* mutant version, at least four independent lines were crossed

with *pin1*. For presentation purposes, GFP or VENUS tag is not shown in some images. Primers for genotyping are summarized in Table S1. Plants were grown in soil at 21 °C, 16-hour photoperiod, and 70% relative humidity. For seedlings grown on the plates, seeds were surface-sterilized and vernalized at 4 °C in darkness for 2 days, and then placed on plates with 0.5 × Murashige and Skoog (1/2 MS) medium (Duchefa) containing 0.05% MES, 0.8% agar and 1% sucrose. Plates were incubated vertically at 22 °C and 16-hour photoperiod.

Phenotypic analysis

7-day-old plate grown seedlings were used to quantify the vertical root growth index (Blancaflor, 2015). During the genetic screening of PIN2 tyrosine sites and *ap2m* phenotyping, plates were set at a 15° slope from vertical to enhance the agravitropic phenotype. Images were photographed with Nikon D5300, and measurements were done in Image J. GraphPad Prism 5 was used for data analysis and graph plotting. F2 generation seedlings *pin1* × *PIN1-GFP* crosses were germinated and grown on soil for 10 days, then sprayed with 15ug/mL Phosphinothricin (PPT) for three alternate days to select for the presence of the PIN1-GFP construct. Surviving plants were counted and classified into two groups (pin-like and wild-type-like) according to the inflorescence phenotype. Theoretically, *PIN1-GFP* restoring *pin1* phenotype would not result in plants with pin-like inflorescences after PPT selection. Representing images were taken with Leica MZ16FA equipped with DFC 420 C digital camera.

Cloning procedures

To obtain the *pGREEN0229-pPIN1::PIN1-GFP* tyrosine substitution mutant constructs, *pDONR207-PIN1-GFP* was mutated using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent, #200521) and the corresponding primers (Table S1). Subsequently, the unique *NcoI* and *XhoI* sites were used to transfer the mutated PIN1 HL coding region from *pDONR207-PIN1-GFP* to *pGREEN0229-pPIN1::PIN1-GFP* (Huang et al., 2010).

For mutagenesis of the *pPZP221-pPIN2::PIN2-VENUS* construct (Dhonukshe et al., 2010), a *BamHI-PIN2-AvrII* fragment was amplified from *pPZP221-pPIN2::PIN2-VENUS* with PCR with primers described in Table S1, and cloned into *pDONR207* using gateway technology (Invitrogen, Gateway BP Clonase II Enzyme Mix #11789020). *pDONR207-BamHI-PIN2-AvrII* was used for site directed mutagenesis as described above, and the mutant *BamHI-PIN2-AvrII* fragments were used to replace the wild-type fragment in *pPZP221-pPIN2::PIN2-VENUS*. Vectors for *in vitro* protein expression were created as follows. Short versions (residues 220-280) of the wild type and mutant *PIN2HLs* were amplified from *pPZP221-pPIN2::PIN2-VENUS* or *pDONR207-PIN2-VENUS* vectors and inserted as *EcoRI* and *XhoI* fragments into pET16H. *AP1M2-BD (-mHD)* and *AP2M-BD (-mHD)* were PCR amplified using cDNA of 5-day-old seedlings as template, and exchanged into *pGEX-gateway* vector. *pGEX-PID* and *pGEX-PIN2HL* (WT and S1,2,3A) have been described before (Huang et al., 2010; Dhonukshe et al., 2010). *EcoRI-(PIN2Y→A)-AvrII* fragments were PCR amplified from the corresponding mutated *pDONR207-BamHI-(PIN2Y→A)-AvrII* and used to substitute the wild-type coding region in *pGEX-PIN2HL*. All primers used for cloning are listed in Table S1.

***In vitro* phosphorylation and pull-down**

For *in vitro* phosphorylation assays, we followed the previously described protocol (Huang et al., 2010). Pull-down assays were performed by combining several published protocols with some modifications (Einarson et al., 2007; Huang et al., 2010; Sancho-Andrés et al., 2016), as described below.

pGEX-KG, pGEX-AP1M2/AP2M-BD and pET16H-PIN2HL were transformed into Rosetta™ strain (DE3) pLysS. 3mL overnight cultures were subcultured to 50mL fresh LC supplemented with antibiotics and grown at 37 °C until OD₆₀₀=0.6. Protein expression was induced for 3 h with 1 mM IPTG at 30 °C, after which the cultures were centrifuged at 4000 RPM for 10min. The pellets were frozen in liquid nitrogen and stored at -80 °C. Purification of GST-AP1M2/AP2M-BD and His-PIN2HL was performed following Molecular Cloning: a laboratory manual (Green and Sambrook, 2012) using Pierce™ Glutathione Agarose (#16100, Thermo Scientific™) and Ni-NTA Agarose (#30210, QIAGEN). GST fusion protein purification excluded the elution step, instead, the protein bound to GSH beads was directly used after washing twice with binding buffer (100 mM Tris-HCl, 5 mM EDTA, 0.05% Triton X-100, pH=7.5). Purified His-PIN2HL was 100-fold diluted into binding buffer supplemented with 1% BSA (w/v), and concentrated to 50 µL with Vivaspin® 500, 3 kDa MWCO Polyethersulfone (GE28-9322-18, Sigma). His-PIN2HL was added to GST and GST-AP1M2/AP2M-BD beads, and filled up to 100 µL. The mixture was incubated at 4°C for 2 hours on a rotator, after which the beads were washed three times with 500µL binding buffer. The sample was suspended in 30 µL 2 × SDS loading buffer and boiled at 98 °C for three minutes. 5 µL sample was separated by 12% SDS-PAGE gel electrophoresis, and proteins were detected by anti-GST immunoblotting (GST Antibody (B-14) HRP, sc-138 HRP, Santa Cruz Biotechnology). For detection by anti-His (His-probe Antibody (H-3) HRP, sc-8036 HRP, Santa Cruz Biotechnology) immunoblotting, 25 µL sample was loaded on the 12% SDS-PAGE gel.

Immunolocalization, confocal microscopy and fluorescent analysis

Whole-mount immunolocalization on 4-day-old seedlings was performed as described (Pasternak et al., 2015), using rabbit anti-GFP (dilution 1:750, #A-11122, Invitrogen) as primary antibody, and Alexa Fluor 488 conjugated goat anti-rabbit (dilution 1:500, # A-11034, Invitrogen) as secondary antibody. Immunofluorescence, PIN2-VENUS or PIN1-GFP in roots were visualized and captured with Zeiss LSM5 Exciter. Alexa Fluor 488 and GFP were excited with a 488 nm laser. The emission signal was captured with 505–530 nm band pass filter. VENUS was excited with a 514 nm laser, and the emission signal was captured with 530-560nm band pass filter. To determine PIN2-VENUS polarity alteration, fluorescent intensity was measured at the outer later (side1) and apical (side2) PM of lateral root cap cells by quantifying the mean fluorescence intensity on a free-hand line drawn along the PM using ImageJ. The ratio was calculated by dividing the intensity of side1 by the intensity of side2. Data were analyzed and plotted into graphs using GraphPad Prism 5.

Quantitative RT-PCR

Total RNA was extracted from 5-day-old seedlings using the NucleoSpin RNA Plant kit (Macherey Nagel, #740949) and reverse transcribed using the RevertAid RT Reverse Transcription Kit (Thermo Scientific™, #K1691). The CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) and TB Green Premix Ex Taq II (Tli RNase H Plus) (Takara, #RR820B) were used to perform qRT-PCR. Gene expression was normalized to the reference gene *PP2A-3* (*AT2G42500*) using the $\Delta\Delta C_t$ method.

Acknowledgments

We would like to sincerely thank Christian Luschnig (BOKU) and María Jesús Marcote (Universidad de Valencia) for sharing *PIN2-VENUS-Y505A eir1-4* and *PIN1-GFP-F165A* seeds, respectively. Gerda Lamers and Joost Willemse kindly helped with confocal microscopy work. Nick Surtel, Ward de Winter and Jan Vink provided daily technical assistance. This project was supported by the China Scholarship Council.

Supplementary data

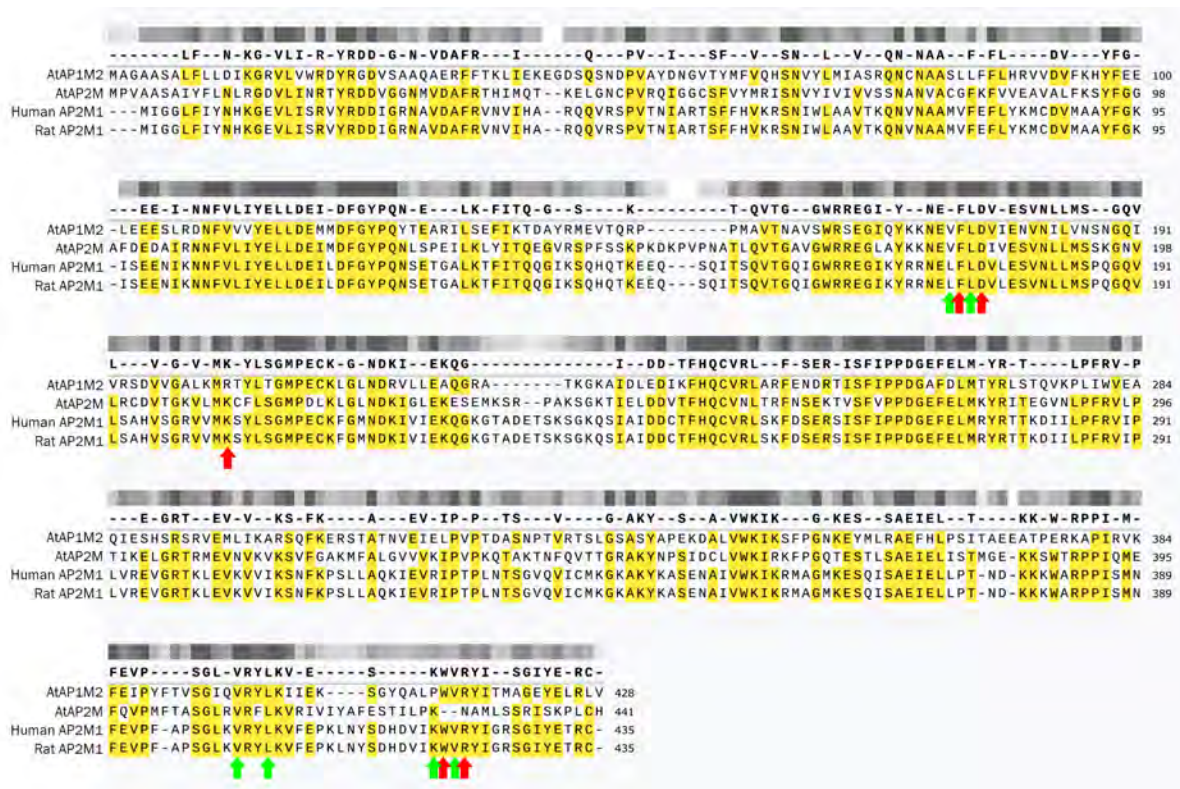


Figure S1. Alignment of Arabidopsis AP1M2 and AP2M with Human AP2M1 and Rat AP2M1 using Clustal Omega <https://www.ebi.ac.uk/Tools/msa/clustalo/>. Red arrows indicate residues critical for tyrosine binding, green arrows indicate residues creating the hydrophobic groove for Φ .

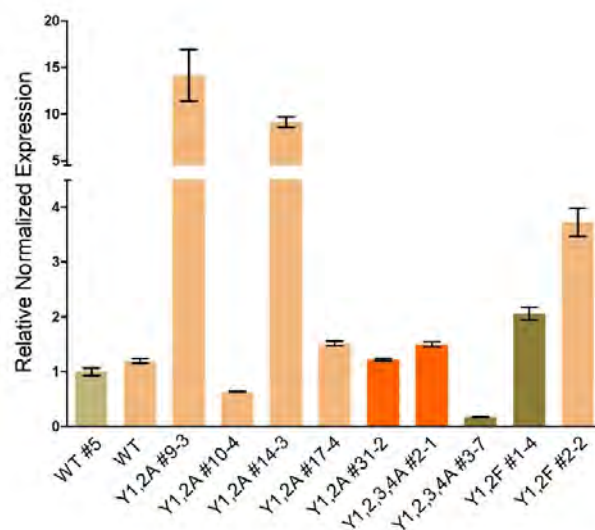


Figure S2. PIN2-VENUS expression levels in different lines. The expression level in our own PIN2-VENUS (WT) #5 line was put at 1. WT represents the previously described PIN2-VENUS line (Dhonukshe et al., 2010). All lines are in *eir1-1* background.

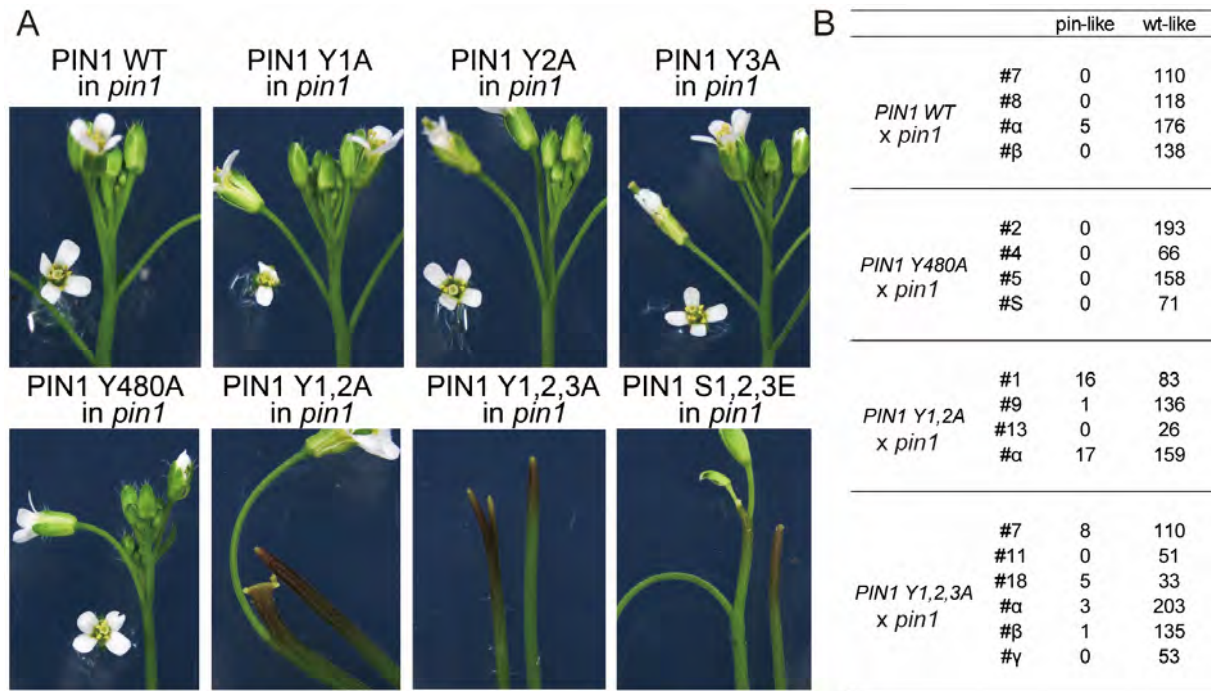


Figure S3. Conserved tyrosines in the PIN1HL are necessary for full PIN1 functionality. A, Inflorescence phenotypes of flowering plants containing *PIN1-GFP* (PIN1 WT) or different Y to A or S to E *PIN1-GFP* substitution versions in the *pin1* mutant background. For Y1A, Y2A and Y3A, four independent lines were checked, all showed wild-type inflorescences. B, Table indicating the number F2 plants showing pin-like or wild-type inflorescences. F2 plants were from crosses between the *pin1* mutant and different lines containing *PIN1-GFP* wild type (*PIN1* WT) or tyrosine substitution constructs (*PIN* Y-A).

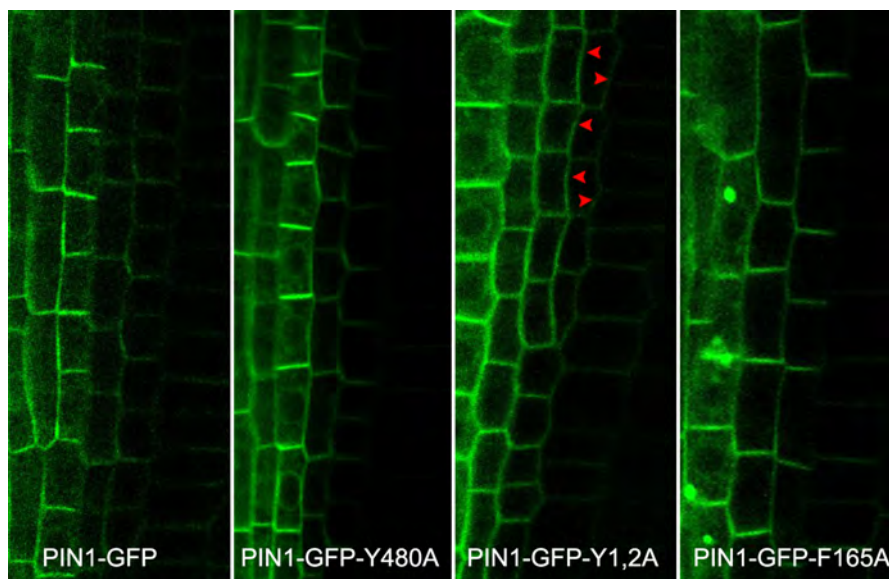


Figure S4. Median confocal sections of roots of 5-day-old *PIN1-GFP*, *PIN1-GFP-Y480A*, *PIN1-GFP-Y1,2A* or *PIN1-GFP-F165A* seedlings. Arrowheads indicate the enhanced lateral localization of PIN1-GFP-Y1,2A.

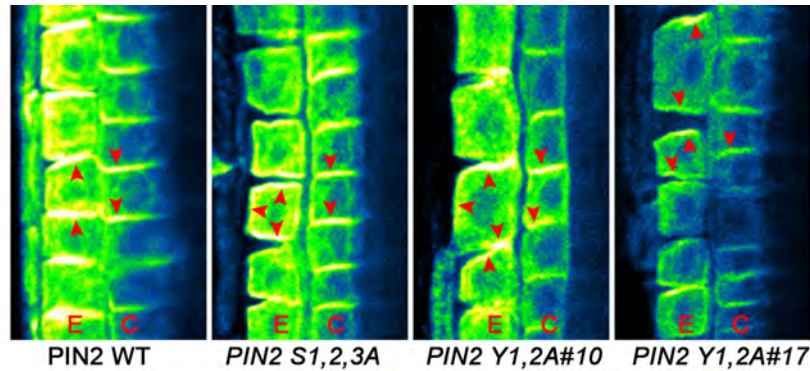


Figure S5. Immunolocalization of the indicated PIN2-VENUS versions in the root epidermis (E) or cortex (C). Red arrowheads point out PIN2-VENUS polarity .

Table S1. Primers used in this study.

usage	name	sequence
genotyping	LBb1.3	ATTTTGCCGATTTTCGGAAC
genotyping	Salk_083693C-LP	GCACAAAGAAAAGTCAGTGCC
genotyping	Salk_083693C-RP	GCAATGCTAATGTTGCTTGTG
genotyping	SALK_047613-LP	CAAAAACACCCCCAAAATTTC
genotyping	SALK_047613-RP	AATCATCACAGCCACTGATCC
mutagenesis	PIN2-Y245A-FP	TCACCGGCGTAGAGATTGCCTCCGTTCAATCGTCAC
mutagenesis	PIN2-Y245A-RP	GTGACGATTGAACGGAGGCAATCTCTACGCCGGTGA
mutagenesis	PIN2-Y266A-FP	CTTTAATCAGACAGATTTTCGCCGCAATGTTTAACGCAAG
mutagenesis	PIN2-Y266A-RP	CTTGCGTTAAACATTGCGGCGAAATCTGTCTGATTAAAG
mutagenesis	PIN2-Y297A-FP	GGTCCAGGTGGAGATGTTGCCTCACTTCAGTCTTCTAAAG
mutagenesis	PIN2-Y297A-RP	CTTTAGAAGACTGAAGTGAGGCAACATCTCCACCTGGACC
mutagenesis	PIN2-Y343A-FP	CAATAATAGTGTTCCGTCGGCCCCACCGCCGAACCC
mutagenesis	PIN2-Y343A-RP	GGGTTTCGGCGGTGGGGCCGACGGAACACTATTATTG
mutagenesis	PIN2-Y245F-FP	TCACCGGCGTAGAGATTTTCTCCGTTCAATCGTCAC
mutagenesis	PIN2-Y245F-RP	GTGACGATTGAACGGAGAAAATCTCTACGCCGGTGA
mutagenesis	PIN2-Y266F-FP	CTTTAATCAGACAGATTTCTTCGCAATGTTTAACGCAAG
mutagenesis	PIN2-Y266F-RP	CTTGCGTTAAACATTGCGAAGAAATCTGTCTGATTAAAG
mutagenesis	PIN1-Y239A-FP	CTAACCAACGCTGAGATAGCTTCGCTTCAGAGTTCAAG
mutagenesis	PIN1-Y239A-RP	CTTGAACCTCTGAAGCGAAGCTATCTCAGCGTTGGTTAG
mutagenesis	PIN1-Y260A-FP	GTTTTAATCATACTGATTTTGCCCTCGATGATGGCTTCTGG
mutagenesis	PIN1-Y260A-RP	CCAGAAGCCATCATCGAGGCAAAATCAGTATGATTAAAAAC
mutagenesis	PIN1-Y328A-FP	GGTGGAGGAGCGCATGCTCCGGCGCCGAACCC
mutagenesis	PIN1-Y328A-RP	GGGTTTCGGCGCCGGAGCATGCGCTCCTCCACC
mutagenesis	PIN1-Y480A-FP	TCGTAATCCCAACTCTGCCTCCAGTTTATTCGGGCATC
mutagenesis	PIN1-Y480A-RP	GATGCCGAATAAACTGGAGGCAGAGTTGGGATTACGA
cloning	PIN2-BamHI-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGTATTACACGTACAAGTGATCC
cloning	PIN2-AvrII-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTAACCTGTGGCCGTTTACGTC
cloning/pET16H	PIN2HL-N220-XhoI	CCGCTCGAGAACAAATCTCACGCGGAGGAC
cloning/pET16H	PIN2HL-G280-EcoRI	GGGAATTCCTAACCGTGACGAGGGCTTGAGCT
cloning/pGEX	PIN2CL-FP-EcoRI	CCCCAGGAATTCGTGAACCCCT
cloning/pGEX	PIN2CL-RP	CTGTTTCTAGGACCGGGACC
cloning	AP1M2-mHD-attB1	AAAAAGCAGGCTTCgtctctctggaggagtggaggaa
cloning	AP1M2-mHD-attB2	AGAAAGCTGGGTCTtacacgagtccttagtctgtac
cloning	AP2M-mHD-attB1	AAAAAGCAGGCTTCgttggttgagagaagagagggcc
cloning	AP2M-mHD-attB2	AGAAAGCTGGGTCTtaatagacacaatggcttagagatt
cloning	Full length attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT
cloning	Full length attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT
qRT	PIN2-venus qRT-FP	AAGGTAGCGACGTGGAAGAC
qRT	PIN2-venus qRT-RP	CTGAACCTGTGGCCGTTTAC
qRT	PP2A-3-qRT-FP	GATGGATACAACCTGGGCTCACG
qRT	PP3A-3-qRT-RP	TCGGTGCTGGTTCAAACCTGG

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Summary

By taking advantage of advanced technologies, especially new molecular genetic, biochemical and cell biology tools, research on auxin biosynthesis, transport and signaling have made significant progress in the past decades. In **chapter 1** of this thesis we review this progress. An important player in the regulation of polar auxin transport is the *Arabidopsis thaliana* (Arabidopsis) AGC3 kinase PINOID (PID). PID has been shown to control the direction of polar auxin flow by phosphorylating the PIN FORMED (PIN) auxin efflux carrier proteins in their large central hydrophilic loop, and thereby to regulate their polar distribution at the plasma membrane (PM) (Friml et al., 2004; Michniewicz et al., 2007; Huang et al., 2010). Several PID post-translational regulators have been discovered by comparative studies with animal AGC kinases, yeast two-hybrid screens and co-immunoprecipitation-mass spectrometry. Unfortunately, however, genetic evidence for the *in planta* function of all these putative PID regulatory proteins is still lacking.

The prime goal of our research was to discover the genetic link between PID and its *in vitro* activator 3-PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE 1 (PDK1). This study is described in **chapter 2**. In Arabidopsis, PDK1 is encoded by two genes, *PDK1* and *PDK2*. Since reported *pdk1 pdk2* double mutants did not display striking developmental defects like the *pid* loss-of-function mutant, we first overexpressed *PDK1* in Arabidopsis to examine if this would phenocopy *PID* overexpression. However, *PDK1* overexpression did not lead to any noticeable phenotypic alteration. Because tissue-specific constraints and protein activation requirements could not be excluded in the 35S promoter-driven overexpression lines, we decided to study the available *pdk1 pdk2* loss-of-function mutants in more detail. Careful analysis of the T-DNA insertion sites of the existed *pdk1* mutant alleles showed that these T-DNA alleles do not lead to *pdk1* loss-of-function. RT-PCR experiments and *in vitro* phosphorylation assays confirmed this analysis, as a partial PDK1 protein with a higher kinase activity is still expressed in these mutant alleles. Subsequently, we created multiple *pdk1 pdk2* loss-of-function mutants using CRISPR-Cas9 technology. Even though the phenotype of these mutants still completely differed from *pid*, which indicated that PDK1 and PID are involved in different signaling pathways, various organ defects suggested that PDK1 function is also tightly correlated to auxin transport.

To better understand the function of PDK1 *in planta*, we analyzed the global expression pattern of *PDK1* and *PDK2*, and described the alternative splicing of *PDK1* and the subcellular localization using YFP-PDK1 fluorescent reporter fusions. Detailed phenotypic observations of *pdk1 pdk2* during all stages of plant development were also recorded. Until now, we can conclude that PDK1 is involved in various developmental processes including reproductive organ and embryo development, hypocotyl phototropism and root gravitropism, vascular patterning etc. (Figure 1). Most of the *pdk1 pdk2* phenotypes are caused by reduced auxin transport and altered auxin distribution, but this does not rely on direct phosphorylation of co-localized PIN proteins by PDK1. Surprisingly, the *PDK1* gene produced a splice variant encoding a shorter but functional PDK1 version (PDK1S0) lacking the phospholipid interacting pleckstrin homology (PH) domain. As expected, this shorter version is cytosolic, but the fact

that it can still rescue most developmental defects of the *pdk1 pdk2* mutant indicates that (polar) PM localization of full length PDK1 is not essential for most of its *in planta* functions. In view of the previously suggested role of PDK1 as upstream regulator of several members of the AGC1 kinase subgroup (Zegzouti et al., 2006), we compared the *pdk1 pdk2* phenotypes with those of loss-of-function mutants in *AGC1* kinase genes. Strikingly, the reduced lateral root numbers, fused cotyledons and agravitropic hypocotyl growth were also observed for the *d6pk012 (agc1.1 agc1.2 pk5)* triple mutant, the short root for loss-of-function mutants in the *PROTEIN KINASE ASSOCIATED WITH BREVIS RADIX (PAX/ACG1.3)* gene, and the defective pollen tube growth for the *agc1.5 agc1.7* double mutant (Zhang et al., 2009; Zourelidou et al., 2009; Li et al., 2018; Marhava et al., 2018). Together our data corroborates the existing model that PDK1 is an upstream regulator of AGC kinases, but surprisingly it does not act through the AGC3 kinase PID, but rather by activating several AGC1 kinases to promote polar auxin transport.

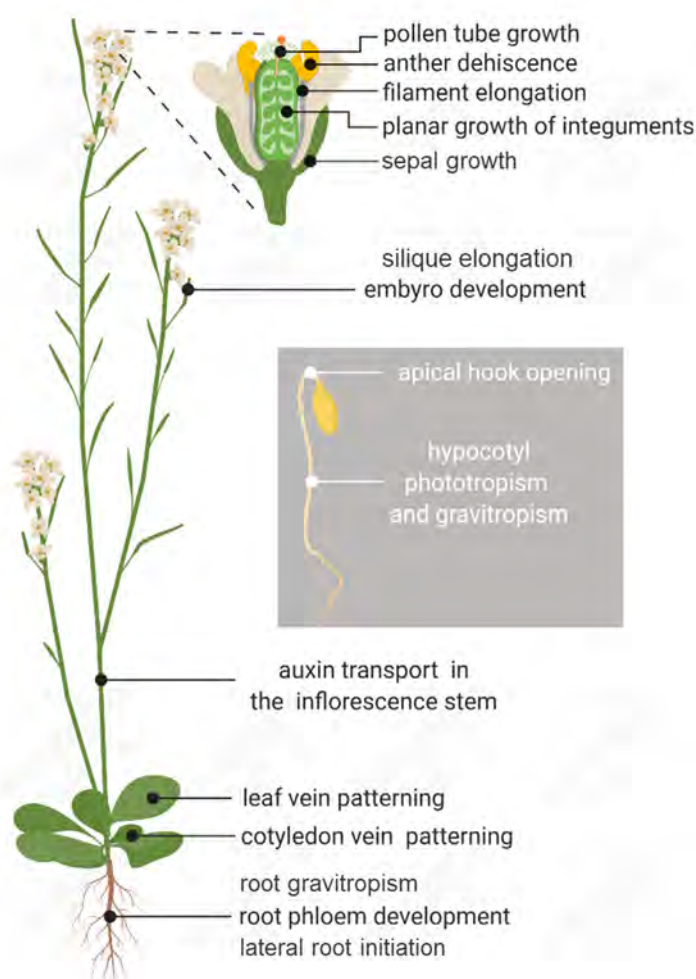


Figure 1. PDK1 is a positive regulator of polar auxin transport upstream of the AGC1 kinases, and is involved in many aspects of development in *Arabidopsis thaliana*. The model is created with the BioRender online tool. The detailed schematic representation of the flower is modified based on the image from TaylorCustom.

In **chapter 3** we investigated one of the PDK1-AGC1 kinase interactions more in detail. Close observations on the cause of the short root phenotype in *pdk1 pdk2* double mutants showed fragmented phloem strands in the root stele, indicating defects in protophloem differentiation

similar to that in the *pax* mutant. Expression of a phosphomimic version of PAX mutant (PAX(SD)) under the *PDK1* promoter in the *pdk1 pdk2* background resulted in rescue of the root protophloem differentiation and the primary root length. This showed that PDK1 is part of the auxin sensing rheostat controlling protophloem differentiation in the root, as upstream regulator of PAX. In root protophloem cells PDK1 co-localized with PAX and the PIN auxin efflux carriers at the basal PM, suggesting that its phospholipid-mediated PM polar PM localization is important for its function in vascular development. Interestingly, however, the cytosolic PDK1S0 lacking the PH domain could still rescue the root phloem and -growth defects of the *pdk1 pdk2* mutant, indicating that polar PM association of PDK1 is not essential in this developmental process. This conclusion is corroborated by evolutionary analysis of PDK1, which shows that the ancestral plant PDK1 did have a PH-domain, and that this domain is present in flowering plants, but has been lost in some early vascular plant (ferns).

Except for investigating signal transduction upstream of PID, we also attempted to explain the mechanism of PID phosphorylation-directed PIN polarity shift. In **chapter 4**, we identified two conserved tyrosine residues in the PIN HL (Y1 and Y2), closely linked to the AGC3 kinase PID/WAG1/WAG2 phosphorylation targets S1 and S2, which appeared to be crucial for AP complex binding and PIN polarity maintenance. Further research suggested that the Y1,2-mediated intracellular PIN trafficking and the PIN phosphorylation-directed change in subcellular localization act independent of each other. Since Y1,2 are not part of a canonical endocytosis motif known from animal cells, we propose that the use of these canonical motifs protein database searches should be carefully applied, and that a systematic screening for plant AP complex binding motifs is urgently required.

In conclusion, we discovered two novel factors regulating polar auxin transport. Due to time limitations, many of the *pdk1 pdk2* phenotypes and their corresponding PDK1-AGC1 kinase interactions have not been further studied. PDK1 mediated crosstalk between light and auxin is also not presented in this thesis. The role of PDK1 in phosphorylation and activation of D6PK/D6PKLs and AGC1.5/AGC1.7 can be easily demonstrated by expressing the corresponding wild-type and phosphomimic kinases into the *pdk1 pdk2* background in the future. This may contribute to uncovering the mechanism of the phototropism, photomorphogenesis and phospholipid-dependent pollen tube growth. The importance of the PDK1 PH domain in these processes still needs to be defined as well. Since *pdk1 pdk2* T-DNA insertion mutants display insensitivity to fungus infection (Camehl et al., 2011), we suspect that the PH domain may be only be essential for stress adaption, and for reproduction, as hinted by the reduced silique length and seed production of *pdk1 pdk2* T-DNA insertion mutants. PDK1 alternative splicing of *PDK1* transcripts may provide a novel and unique regulation mechanism for balancing growth, reproduction and defense in Arabidopsis. The study on *PDK1* gene function in this thesis benefits from CRISPR-Cas9 gene-editing technology. In fact, because of gene redundancy and lacking multiple loss-of-function mutants, the function of AGC kinase family is not well-defined in plants. For example, it is almost impossible to get a double mutant of S6K1 (AT3G08730) and S6K2 (AT3G08720) with the classical cross, thus the genetic study about S6K is largely absent. CRISPR-Cas9 will be a powerful tool for a better understanding the function of genes that are part of highly redundant families.

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Samenvatting

Door gebruik te maken van geavanceerde technologieën, waaronder nieuwe moleculair genetische, biochemische en celbiologische methoden, heeft het onderzoek naar auxinebiosynthese, -transport en -signalering de afgelopen decennia aanzienlijke vooruitgang geboekt. In **Hoofdstuk 1** van dit proefschrift wordt deze vooruitgang gepresenteerd en bediscussieerd. Een belangrijke speler in de regulering van het polaire auxine transport is het *Arabidopsis thaliana* (Arabidopsis) AGC3 proteïne kinase PINOID (PID). Dit kinase bepaalt de richting van de polaire auxinestroom door de PIN FORMED (PIN) auxine-transporteiwitten in hun grote centrale hydrofiele lus te fosforyleren, en daarmee hun polaire verdeling over het plasmamembraan (PM) te reguleren (Friml et al., 2004; Michniewicz et al., 2007; Huang et al., 2010). Door de jaren heen zijn er meerdere mogelijke post-translationale regulatoren van PID geïdentificeerd, onder andere door vergelijkende studies met dierlijke AGC kinases, door gist twee-hybride screens en door co-immunoprecipitatie gevolgd door massaspectrometrie. Helaas ontbreekt nog steeds het genetische bewijs voor de *in planta* functie van deze vermeende PID-regulerende eiwitten.

Het hoofddoel van het in dit proefschrift beschreven onderzoek was om de genetische link tussen PID en zijn mogelijke activator, het 3-FOSFOINOSITIDE-AFHANKELIJKE EIWIT KINASE 1 (PDK1), te achterhalen. Deze studie wordt beschreven in **Hoofdstuk 2**. PDK1 wordt in Arabidopsis gecodeerd door twee genen, *PDK1* en *PDK2*. Omdat gepubliceerde *pdk1 pdk2* dubbel mutanten geen opvallende ontwikkelingsdefecten - zoals die van de *pid* verlies-van-functie mutant - vertonen, hebben we eerst het *PDK1* gen in Arabidopsis tot overexpressie gebracht om te onderzoeken of dit vergelijkbare fenotypes zou opleveren als overexpressie van *PID*. Overexpressie van *PDK1* leidde echter ook niet tot een duidelijke fenotypische verandering. Omdat weefsel-specifieke beperkingen en de noodzaak voor eiwit-activatie niet konden worden uitgesloten in de 35S promotor-gestuurde overexpressielijnen, hebben we besloten om de beschikbare *pdk1 pdk2* verlies-van-functie mutanten in meer detail te bestuderen. Zorgvuldige analyse van de T-DNA-insertieplaatsen van de bestaande *pdk1* mutante allelen toonde aan dat deze T-DNA-allelen niet leiden tot *pdk1* verlies-van-functie. RT-PCR-experimenten en *in vitro* fosforylatiereacties bevestigden dat een gedeeltelijk PDK1 eiwit met een hogere kinase-activiteit nog steeds tot expressie komt in deze mutante allelen. Vervolgens hebben we met behulp van de CRISPR-Cas9-technologie meerdere nieuwe *pdk1 pdk2* verlies-van-functie mutante combinaties gecreëerd. Het fenotype van deze mutanten kwam niet overeen met dat van de *pid* mutant, wat aangaf dat PDK1 en PID bij verschillende signaaltransductieroutes betrokken zijn. Maar de sterke ontwikkelingsdefecten van de *pdk1 pdk2* dubbelmutant suggereerden wel dat PDK1 een centrale rol heeft in de regulatie van auxinetransport.

Om de *in planta* functie van PDK1 beter te begrijpen, hebben we het globale expressiepatroon van *PDK1* en *PDK2* geanalyseerd en de alternatieve splicing van het *PDK1* transcript en de subcellulaire lokalisatie met behulp van een complementerende YFP-PDK1 reporterfusie beschreven. Tevens zijn de *pdk1 pdk2* mutante fenotypes tijdens alle ontwikkelingsstadia van de plant bestudeerd. Uit deze analyses kunnen we opmaken dat PDK1 bij verschillende ontwikkelingsprocessen betrokken is, waaronder de ontwikkeling van voortplantingsorganen

en embryo's, de hypocotylfototropie en wortelgeotropie, en de vasculaire patroonvorming. De meeste fenotypes van de *pdk1 pdk2* dubbelmutant worden veroorzaakt door verminderd auxinetransport en de daarmee samenhangende veranderingen in auxinedistributie. Dit bleek echter niet afhankelijk van directe fosforylering van gecolocaliseerde PIN-eiwitten door PDK1. Verrassend genoeg produceert het *PDK1* gen een splitsingsvariant die codeert voor een kortere maar functionele PDK1 versie (PDK1S0) die het pleckstrine homologie (PH) fosfolipide-interactiedomein mist. Zoals verwacht, lokaliseert deze kortere versie in het cytosol, maar het feit dat het in een complementatie-experiment nog steeds de meeste ontwikkelingsdefecten van de *pdk1 pdk2* mutant kan opheffen, geeft aan dat (polaire) PM-lokalisatie van het volledige PDK1 eiwit niet essentieel is voor de meeste *in planta* functies. Met het oog op de eerder voorgestelde rol van PDK1 als regulator van verschillende leden van de AGC1-kinase-subgroep (Zegzouti et al., 2006), hebben we de *pdk1 pdk2* mutante fenotypes met die van verlies-van-functie mutanten in *AGC1-kinase* genen vergeleken. Het verminderde aantal zijwortels, de gefuseerde zaadlobben en de ageotrope hypocotylgroei werden ook waargenomen bij de drievoudige *d6pk012 (agc1.1 agc1.2 pk5)* mutant, de korte wortel kwam overeen met die van verlies-van-functie mutanten in het *PROTEIN KINASE GEASSOCIEERD MET BREVIS RADIX (PAX / ACG1.3)* gen en de defecte pollenbuisgroei was eerder geobserveerd bij de *agc1.5 agc1.7* dubbelmutant (Zhang et al., 2009; Zourelidou et al., 2009; Li et al., 2018; Marhava et al., 2018). Samen bevestigen onze data het model dat PDK1 een regulator van AGC-kinases is. Verrassend genoeg reguleert PDK1 niet het AGC3-kinase PID, maar bevordert het polair auxinetransport door verschillende AGC1 kinases te activeren, die op hun beurt de PIN eiwitten fosforyleren.

In **Hoofdstuk 3** hebben we één van de PDK1-AGC1-kinase-interacties nader onderzocht. Een detailanalyse van de oorzaak van het korte wortelfenotype van de *pdk1 pdk2* dubbelmutant liet zien dat floëemstrengen in de wortel gefragmenteerd zijn, wat vergelijkbaar is met defecten in protofloëem-differentiatie in de *pax* mutant. *PDK1* promotor gedreven expressie van een fosfomimic versie van PAX (PAX (SD)) in de *pdk1 pdk2* mutante achtergrond kon het defect in protophloëemdifferentiatie en primaire wortellengte opheffen. Dit toonde aan dat PDK1 als regulator van PAX deel uitmaakt van de auxine-responsieve reostaat die protofloëemdifferentiatie in de wortel controleert. In protofloëemcellen in de wortel lokaliseert PDK1 samen met PAX en de PIN-auxine-transporters op het basale PM. Dit suggereert dat de fosfolipide-afhankelijke polaire PM-lokalisatie belangrijk is voor PDK1 functie in vasculaire ontwikkeling. Interessant genoeg kan het cytosol gelokaliseerde PDK1S0 zonder het PH-domein nog steeds de wortelfloëem- en groeidefecten van de *pdk1 pdk2* mutant teniet doen, wat aangeeft dat polaire PM-associatie van PDK1 niet essentieel is in dit ontwikkelingsproces. Deze conclusie wordt ondersteund door de evolutionaire analyse van PDK1, waaruit blijkt dat het voorouder PDK1 eiwit in planten wel een PH-domein had en dat dit domein aanwezig is in bloeiende planten, maar verloren is gegaan in enkele vroege vasculaire planten (varens). Behalve het onderzoek naar de signaaltransductie stroomopwaarts van PID, hebben we ook geprobeerd het mechanisme van PIN-fosforylatie-geïnduceerde polariteitsverschuiving te verklaren. In **Hoofdstuk 4** hebben we twee geconserveerde tyrosine residuen in de PIN HL (Y1 en Y2) geïdentificeerd, vlak bij de AGC3-kinase PID/WAG1/WAG2-fosforylatietargets S1 en S2 die cruciaal bleken voor AP-complexe binding en het behoud van PIN-polariteit. Verder onderzoek suggereerde dat de Y1,2-gemedieerde intracellulaire PIN-trafficking en de PIN-

fosforylatie-afhankelijke verandering in subcellulaire lokalisatie onafhankelijk van elkaar werken. Aangezien Y1 en Y2 geen deel uitmaken van een geconserveerd endocytose-motief uit dierlijke cellen, stellen wij voor dat dergelijke geconserveerde motieven voorzichtig gebruikt moeten worden in eiwitanalyseonderzoek, en dat een systematische screening voor plant-AP-complex bindende motieven dringend vereist is.

Concluderend hebben we twee nieuwe factoren ontdekt die het polaire auxinetransport reguleren. Vanwege tijdgebrek zijn veel van de *pdk1 pdk2* mutante fenotypes en hun overeenkomstige PDK1-AGC1 kinase interacties, waaronder de PDK1-gemedieerde interactie tussen licht- en auxinesignalering, niet verder onderzocht. De rol van PDK1 in fosforylering en activering van D6PK/D6PKL's en AGC1.5/AGC1.7 kan eenvoudig worden aangetoond door de overeenkomstige wildtype en fosfomimic versies van de kinases in de *pdk1 pdk2* achtergrond tot expressie te brengen. Dit kan bijdragen aan het ophelderen van het mechanisme van fototropisme, fotomorfogenese en fosfolipide-afhankelijke pollenbuisgroei. Het belang van het PDK1 PH-domein in deze processen moet ook nog worden bepaald. Omdat *pdk1 pdk2* T-DNA insertiemutanten ongevoelig zijn voor schimmelinfectie (Camehl et al., 2011), vermoeden we dat het PH-domein alleen essentieel kan zijn voor stressadaptatie en voor reproductie, zoals gebleken is uit de verminderde zaadproductie en lengte van de hawtjes van de *pdk1 pdk2* T-DNA-insertiemutanten. Alternatieve splicing van *PDK1* transcripten vormt mogelijk een nieuw en uniek regulatiemechanisme in het balanceren van groei, reproductie en verdediging in Arabidopsis. De in dit proefschrift beschreven studie naar de functie van het *PDK1* gen heeft veel baat gehad bij de CRISPR-Cas9-technologie. Genredundantie en het ontbreken van meervoudige verlies-van-functie mutanten hebben het onderzoek naar de functie van veel leden van de AGC-kinase familie in planten vertraagd. Zo is het bijvoorbeeld bijna onmogelijk om een dubbelmutant van de twee naast elkaar gelegen *S6K1* (AT3G08730) en *S6K2* (AT3G08720) genen te verkrijgen via klassieke mutagenese en kruising, en een genetische studie naar de functie van S6K ontbreekt om deze reden nog. De CRISPR-Cas9 technologie is een krachtig hulpmiddel om de functie van genen die deel uitmaken van dergelijke functioneel redundante genfamilies te achterhalen.

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Curriculum Vitae

Yao Xiao was born in 1989, in Jingshan, Hubei province, China. In 2011, he obtained his bachelor's degree in Life Science and Biotechnology at Lanzhou University, China. The thesis was about "Polyclonal antibody production of three important proteins in brassinosteroid signaling pathway, BRI1, BAK1 and BES1", under the supervision of Prof. dr. Jia Li. After that, he identified several novel brassinosteroid-related genes by combining genetic methods with BES1 detection. During his master's study in Plant Biology and Molecular Genetics, he worked in the same lab focusing on three genes that are involved in the cross-talk between brassinosteroid and other signaling pathways, including auxin, cell death and flowering time control. In 2014, the China Scholarship Council supported his doctoral research in the laboratory of Prof. dr. Remko Offringa at Leiden University, the Netherlands. From 2014 to 2017, his research focussed on the interaction between PIN phosphorylation and intracellular trafficking. In 2017, he also started to work on signal transduction upstream of PINOID, and discovered novel roles of PDK1 in plant development. From January 2020 on, Yao Xiao will work as a postdoc in the laboratory of Prof. dr. Claus Schwechheimer at the Technical University of Munich to continue his studies on the Arabidopsis AGC kinase family.

