New regulators of PIN polarity and trafficking and auxin-dependent development

Ricardo Tejos

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Front Cover: *Arabidopsis thaliana* seed coat

This work was conducted in the Auxin lab of the Plant Systems Biology department of the Flanders Institute for Biotechnology (VIB)

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New regulators of PIN polarity and trafficking and auxin-dependent development

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ABBREVIATIONS

ABP1	Auxin binding protein 1
AtSFH	*Arabidopsis thaliana* sec fourteen homologues
BFA	Brefeldin A
CME	Clathrin mediated endocytosis
DAG	Diacylglycerol
ER	Endoplasmic reticulum
GFP	Green Fluorescent protein
IP_3	Inositol (1,4,5) trisphosphate
K.O.	Knock out
PA	Phosphatidic acid
PATL	PATELLIN
PI4K	Phosphatydilinositol 4-kinase
PID	PINOID
PIN	PIN-FORMED
PIP5K	Phosphatydilinositol 4-phosphate 5-kinase
PITP	Phosphatydilinositol transfer proteins
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PM	Plasma membrane
PTase	Phosphatase
PtdCho	Phosphatidylcholine
PtdIns 4-P	Phosphatydilinositol 4 phosphate
PtdIns	Phosphatydilinositol
$PtdIns(4,5)P_2$	Phosphatydilinositol 4,5-bi-phosphate
ROS	Reactive oxygen species
TGN	trans Golgi Network
TIR1	Transport inhibitor response 1

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Chapter 1 *Part I:* ***PIN polarity and intracellular trafficking.***

Ricardo Tejos *and Jiří Friml*

RT and **JF** wrote the review.

Part II: ***Phosphoinositide-dependent regulation of vesicle trafficking in Arabidopsis.*** ***Ricardo Tejos*** *and Jiří Friml*

RT and **JF** wrote the review.

Chapter 2 ***Auxin feedback on cell and tissue polarity by phosphoinositide metabolism.***

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RT design and performed most of the experiments (all cell biological, genetic and phenotypic analysis) with the exceptions of the microarray experiment (**MSa, MSc**), expression analysis (**SV**), and total phosphoinositide measurements (**TI, MH**). **RT** and **JF** wrote the chapter.

Chapter 3 ***PI4-P 5-kinases function during reproductive development***

Ricardo Tejos*, José Manuel Ugalde, Jiří Friml and Gabriel León*

RT and **GL** designed the experiments; **RT** and **JMU** performed all experimental part and **RT** wrote the chapter.

Chapter 4 ***PATELLIN family of Phosphatidylinositol transfer proteins regulates embryo patterning***

Ricardo Tejos*, Maciej Adamowski, Michael Sauer, and Jiří Friml*

RT and **JF** designed the experiments. **RT** performed all the experiments with the assistance of **MA** in cloning. **MS** contributed to the selection of PATELLIN gene family and the initial collection of insertional knockout mutants. **RT** and **JF** wrote the chapter.

Chapter 5 ***Fluorescence based screen to identify new components of the BFA dependent PIN2 trafficking pathway***

Ricardo Tejos*, Mugurel I. Feraru and Jiří Friml*

RT and **JF** designed the screen; **RT** performed the screen and the characterization of mutants; **RT** and **MIF** performed the mapping. **RT** wrote the chapter.

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SCOPE

Plants have generated their own specific mechanisms for creating asymmetry. The phytohormone auxin is a mobile signaling molecule essential for many different directional processes. Through the plant, a network of plasma membrane proteins distributes auxin between and within the tissues to create dynamic auxin gradients. These gradients are read-outs to direct processes that are involved in the generation of polar axes or in directional growth responses. Among the many different auxin transporters, the PINFORMED (PIN) family members determine in great part the local auxin gradients. Many internal and environmental cues are integrated into auxin transport by modulating PIN trafficking and polarity.Auxin gradients are maintained or dynamically modified by a positive feedback loop between auxin and its transport machinery. Auxin can modulate PIN transcription, trafficking and polarity. Although the first two interactions are at least partially studied, the auxin effect on PIN polarity is not well defined.

The aim of our study is to characterize new regulators of PIN polarity and trafficking. First, we selected auxin-regulated genes that are potentially involved in auxin-mediated PIN polarity generation, using a microarray approach. We used genetic and cell biology tools to address their contribution to plant development and cell and tissue polarity (**Chapters 2, 3, 4**). Besides, we conducted a fluorescent subcellular genetic screen aiming to find new regulators of PIN trafficking and polarity (**Chapter 5**).

Chapter 1

Part I: PIN polarity and intracellular trafficking

Ricardo Tejos and Jiří Friml

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SUMMARY

Multicellular organisms have to generate asymmetries in cells and tissues to create different organs. Moreover, several responses to environmental factors are directional and hence require an equal directional response. In plants, such a challenge is accomplished by a multitude of polarly localized proteins that are involved in embryonic and post-embryonic development and dynamic polar responses to the environment. The phytohormone auxin and its polar cell-to-cell transport play a key role in several of those events, providing a means to coordinate cell and tissue polarities through regulating the polar localization of plasma membrane localized PIN auxin transporters. In this chapter we discuss the crosstalk between cell trafficking and polarity as a way to integrate external as well as internal signals into asymmetry generation and directional responses in the context of PIN localizations and auxin-dependent processes.

A cell or tissue is considered to be polarized if any of its characteristics is orientated or more pronounced along one preferred direction. In plants, cell polarity is essential for many different developmental processes, including asymmetric cell division, patterning in early embryogenesis, post-embryonic generation of new organs such as flowers, stomata and lateral roots, epidermal cell interdigitation, vascular tissue formation and re-generation and others. In addition, nutrient uptake, gravi- and phototropism, as well as plant's defense to pathogen attack, all involve a polarized response.

One manifestation of cell polarity is the asymmetric distribution of proteins at the plasma membrane (PM). Until now, several examples of polarly localized proteins in plants are documented to be involved in a variety of processes which often require a directional response and/or are essential for the establishment of tissue polarity and patterning (Table 1). We will use the embryo-derived terminology to describe the different polar domains in a cell. During embryogenesis, two axes of polarity are formed: an apical/basal axis following the shoot and root meristems and a radial axis of concentric cell layers. In a similar way, we will refer to the upper and the bottom side of a cell as apical and basal, respectively, to the cell side facing the environment as outer lateral, and to the side oriented to the inner cell layers as inner lateral (Dettmer et al., 2011).

Polarly localized proteins and their roles

Multicellular organisms have to create differences between cells to generate patterns and asymmetry. The orientation of the division plane as well as the (a)symmetry of the somatic divisions play a key role in shaping tissues and organs and this is especially important in plants in which rigid cell walls do not allow cell migration. Division plane determination involves the rearrangement of the cortical microtubule array to form a preprophase band (PPB) which will determine the PM anchoring site of the growing cell plate (Van Damme, 2009). The positioning of the PPB as well as the future guidance of

the cell plate after PPB breaks down is regulated by polarity cues that involve proteins that are localized to PPB site prior and during cytokinesis (Table 1; Camilleri et al., 2002; Buschmann et al., 2006; Walker et al., 2007;Azimzadeh et al., 2008; Xu et al., 2008).

Asymetric cell division is a key process in shaping the tissues and a good example of tissue patterning formation through asymmetric division occurs in the leaf epidermis. The leaf epidermis contains a cell lineage that generates the stomata, a structure that participates in gas exchange and transpiration consisting of two bean-like cells called guard cells (GCs) that surround a pore (Dong and Bergmann 2010). The protein BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) dynamically switches between nuclear and polar PM localization, regulating the asymmetric divisions necessary for stomata formation (Dong et al., 2009). The delivery and maintenance of BASL in a small region of the cell periphery is crucial for the asymmetric division and cell fate determination. Stomatal lineage cells initially all express BASL in the nucleus, after which it also accumulates at the cell periphery, resulting in an asymmetric division, eventually generating the GC and stomatal pore. If BASL is expressed only at the periphery, cells differentiate into pavement cells (PCs). When BASL is expressed both in the nucleus and at the PM, the cell divides asymmetrically again. How BASL exerts its function is unknown, but it is hypothesized that BASL helps to establish a local cell outgrowth upstream or independently from the known small GTPase ROP2, which is involved in the lobe outgrowth during PC formation (Fu et al., 2005). However, due to a rather specific expression pattern of the stomatal cell lineage, we cannot extrapolate BASL activity into a wider developmental context, although when ectopically expressed in roots, hypocotyls and other leaf epidermal cells, BASL is able to retain its polar localization (Dong et al., 2009), suggesting that polar determinants important for its localization are common to other cell types.

Table 1. Polarly localized proteins in plants.
See the text for references and extended description.

Protein Family	Function	Polar localization
PIN	auxin efflux carriers	**PIN1:** basal in vasculature in embryos, leaves and roots; apical in epidermis of shoot apices, embryos and gynoecium; **PIN2:** apical in lateral root cap and epidermis and basal in young cortex cells; **PIN3:** inner lateral in shoot endodermis cells, columella: non-polar, but polarly localized after gravistimulation, lateral in pericycle cells during lateral root formation; **PIN4:** basal localization in embryo and in root meristem; **PIN7:** apical localization until 32-cell stage, then switched to basal localization in the suspensor
AUX/LAX	auxin influx carrier	**AUX1:** preferentially apical in protophloem in roots, axial in epidermis; polarly localized in shoot apical meristem
ATP Binding Cassette (ABC) transporters	pleiotropic plasma membrane transporters. Some of them have been shown to specifically transport auxin.	**PGP1/ABCB1:** above the distal elongation zone in roots, is preferentially basally localized in endodermis and cortex; **PGP4/ABCB4:** basal or apical side in epidermal root cells; **PGP19/ABCB19:** polar in procambial cells in roots; **PIS1/ABCG37:** outer lateral in root epidermis; **DSO/ABCG11:** outer lateral in leaf epidermis; **CER5/ABCG12:** requires DSO/ABCG11 for its plasma membrane localization; **PEN3/PDR8/ABCG36:** outer lateral in root epidermis
AGC kinases	protein phosphorylation, regulate auxin flow in various developmental contexts	**PINOID/WAG1/WAG2:** enriched at apical and basal cell sides; **PHOT1:** enriched at apical and basal cell sides
NHP3-like proteins	functionally interact with PINOID to regulate PIN phosphorylation	**MAB4/ENP** and **MEL1-4:** polarly localized following PIN localization
PIP5K	phosphatidylinositol phosphate kinases	**PIP5K3:** apical in growing root hairs; **PIP5K4/5:** subapical region of pollen tubes; **PIP5K6:** subapical region in growing pollen tubes, and switches to apical when growth stops
Rho-GTPases	molecular switches for multiple cellular mechanisms	various locations: tip of pollen tubes and root hairs, lobes in leaf epidermis cells
BOR	boron efflux	**BOR1:** inner lateral in roots; **BOR4:** outer lateral in root epidermis
CASP	Casparian strip formation	**CASP1:** localized along the Casparian strip in the root endodermis
NIP5;1	boron influx	**NIP5;1:** root epidermis and lateral root cap; outer lateral
LSI	silicon efflux	**LSI1**and **LSI2:** outer lateral exodermis and endodermis in rice roots
NRT1;1	nitrate / auxin influx	epidermis of root primordia possibly axial (anticlinal)
PEN1	SNARE-domain containing syntaxin, pathogen defense	polar PM domain facing pathogen attack site
PEN2	glycosyl hydrolase, pathogen defense	polar PM domain facing pathogen attack site
BASL	regulator for asymmetric cell division	nucleus and polar domains at plasma membrane in stomatal lineage cells
TONNEAU1	regulate microtubule array during cytokinesis	**TON1**: preprophase band and to transversal cell sides in dividing cells.
TONNEAU2/FASS	contains a domain similar to type B subunit of PP2A phosphatase and interacts with type A subunit of PP2A phosphatase.	**TON2**: preprophase band at the late G2 phase, otheriwise is mostly cytosolic
RanGAP1	GTPase activating protein of the small GTPase Ran	localizes to preprophase band in TON2 dependent manner
TANGLED	plays a role in guidance of expanding phragmoplasts to the former PPB	**TAN**: preprophase band, and this localization persist after preprophase band disassembly marking the division plane trough mitosis and cytokinesis
AIR9	microtubule associated protein	decorates microtubules at preprophase band and it marks the former preprophase band site precisely when the cortex is contacted by the growing forming cell plate..

A more widely used mechanism for asymmetry generation in plants involves the hormone auxin. Auxin signaling is part of a multitude of processes including *de novo* generation of

cell and tissue asymmetry, re-establishment of axial polarity (e.g. during regeneration of vascular tissues), or directional responses to developmental or environmental cues (Grunewald and Friml, 2010). In most of these events, auxin is distributed differentially within tissues, eliciting cell-specific responses depending on hormone levels and developmental context (Calderón-Villalobos et al., 2012). This differential auxin distribution results mainly from a directional (polar) symplastic transport which depends on PM carriers. These include several ATP binding cassette (ABC) transporters of the subfamily B (ABCB, Geisler et al., 2006), influx carriers constituting the AUXIN RESISTANT1/LIKE AUX1 (AUX/LAX) family, and efflux carriers of the PIN-FORMED (PIN) family (Grunewald and Friml, 2010). Though some of the ABCB and AUX/LAX transporters are polarly localized in certain tissues (Swarup et al., 2001 and 2008; Geisler et al., 2005), the direction of auxin transport seems to be generated by the polarly localized PIN proteins (Wiśniewska et al., 2006). While some of these PIN carriers reside in the endoplasmic reticulum where they contribute to the auxin cellular homeostasis (Mravec et al., 2009; Dal Bosco et al., 2012; Ding et al., 2012), the PM localized PINs can be apically, basally, or laterally distributed depending on the developmental and tissue context (Kleine-Vehn and Friml 2008). Already early in embryogenesis, after division of the zygote, auxin preferentially accumulates in the apical cell, which will form the future proembryo. Later on, the auxin maximum switches from the apical to the basal part of the embryo, where the future root pole will be established. Dynamic PIN relocalization during those early ontogenetic stages redirects the auxin flow, generating distinct maxima at different sites of the developing embryo that result in specification of the main apical-basal plant axis and the embryonic leaves, the cotyledons (Friml et al., 2003). After germination, polar auxin transport and differential auxin distribution coordinate organogenesis (Benková et al., 2003; Reinhardt et al., 2003;

Heisler et al., 2005), vascular tissue formation (Scarpella et al., 2006) and growth responses to light and gravity (Friml et al., 2002; Harrison et al., 2008; Kleine-Vehn et al., 2010; Rakusová et al., 2011; Ding et al., 2011) that involve PIN polarity switches in response to intrinsic or extrinsic cues. On top of these regulations, the feedback loop between auxin signaling and directional auxin transport can coordinate individual cell polarities within tissues, providing a versatile response to the ever changing environmental conditions.

Once the tissue asymmetry is created, cells differentiate into highly specialized tissues with particular functions and characteristics. For instance, polarized epithelia constitute an interface between the environment and the organism, being the first protection barrier against invasion of pathogens. A passive mechanism for pathogen defense is a waxy layer, the cuticle, which covers the entire surface of the plant exposed to air. Two ABC transporters, DSO/ABCG11 and CER5/ABCG12, polarly localize exclusively to the outer PM facing the environment and are crucial in exporting lipids necessary for cuticle synthesis at the epidermal cells surface (Panikashvili et al., 2007; McFarlane et al., 2010). On the other hand, a more active mechanism of plant defense involves the focal secretion at the cell periphery to the actual infection sites. The β-glycosyl hydrolase PENETRATION2 (PEN2), together with the transporter PEN3/PDR8/ABCG36, is necessary for the cytoplasmic synthesis and transport of antimicrobial toxins, while the PM-resident syntaxin PEN1 mediates vesicle fusion processes (Lipka et al., 2005; Stein et al., 2007; Kwon et al., 2008). Each of these proteins relocalizes in response to pathogen attack and becomes concentrated at the invasion point, which is enriched in structural sterols in the form of PM micro domains (Meyer et al., 2009).

On the other hand, the root epidermis works as an uptake surface for nutrients and a layer where detoxification of harmful substances occurs. Here, several proteins have been

reported to reside at the cell's outer lateral side, facing the soil, as well as the inner lateral side oriented toward the internal cell layers and vascular tissues. It is at this layer where uptake of the essential element boron is regulated by the outer laterally localized importers BOR4 and NIP5;1, and the inner laterally localized exporter BOR1 (Takano et al., 2008). A second barrier controlling the diffusion of substances from soil to root inner tissues is the endodermis, a cell layer common to all higher plants. The plant endodermis develops early during its differentiation a belt of specialized cell wall material, the so-called Casparian strip. The CASP1 protein was reported to be polarly localized to the Casparian strip together with other members of the CASP family, defining a physical border at the outer and inner lateral PM domains, where the boron transporters NIP5;1 and BOR1 are polarly localized in the same way as they are in epidermis (Roppolo et al., 2011). In rice and other higher plants, the exodermis is the outer cell layer in the root and constitutes the first diffusion barrier and, as well as the endodermis, it contains a Casparian band (Enstone et al., 2003). Here, similarly as in endodermis, the silicon transporters LSI1 and LSI2, and the boron translocators OsNIP3:1 and OsBOR1 are laterally localized facilitating a controlled uptake of these two important nutrients (Ma et al., 2006 and 2007). Laterally localized proteins in outer root layers are also involved in export of toxic compounds, like the transporter PIS1/PDR9/ABCG37 and the pathogen defense-related protein PEN3/PDR8/ABCG36 (Strader and Bartel 2009; Růzicka et al., 2010).

Other specialized cell types are root hairs and pollen tubes. Both exhibit a highly polarized tip growth necessary for their function (Šamaj et al., 2006;Lee and Yang 2008). This tip growth depends on several polarly localized proteins including ROP GTPases, phospholipases, phosphatidylinositol kinases and phosphatases, which contribute to cytoskeleton orientation, generation of a Ca^{+2} gradient at the tip, and the synthesis of

phospholipids at the PM, which all together are essential for the fast and highly directional secretion of vesicles toward the growing tip, which is tightly coupled with compensatory endocytosis.

Subcellular trafficking in PIN polarity

Despite the profound importance of cell polarity in the sessile life style of plants, the molecular mechanisms for its generation, maintenance and re-specification are not well understood. Nonetheless, some important factors and hypothetical mechanisms have been proposed over the last years, which to some extent resemble those described in animals. Interestingly, plants also developed independent specific polarity determinants. For instance, the phytohormone auxin works in many processes as a cue governing the polar localization of its efflux carriers, the PIN proteins, by which auxin modulates its own transport and polarity of cells and tissues. We focus the following discussion on the interplay between trafficking and polarity of PINs as they are the most studied polar cargos in plants (see Figure 1).

The current notion of PIN polarity establishment highlights the importance of constitutive endocytic recycling (Geldner et al., 2001). PIN endocytosis is clathrin-mediated (Dhonukshe et al., 2007; Chen et al., 2011); internalization also depends on the ARF GEFs GNOM and GNL1 (Teh and Moore 2007; Naramoto et al., 2010) and the activity of the RabGTPaseARA7 (Dhonukshe et al., 2008a). The recycling back to the PM also requires the activity of GNOM (Geldner et al., 2001) and the early endosomal protein BEN1/MIN7 (Tanaka et al., 2009). Such constitutive PIN recycling might provide a mechanism for rapid changes in polarity and concomitant redirectioning of the auxin flow at certain developmental events (Dettmer and Friml 2011). This polarity switch does not involve *de novo* PIN protein synthesis but follows a transcytosis-like mechanism from one cell side to another, as described for PIN3 repolarization during gravitropic

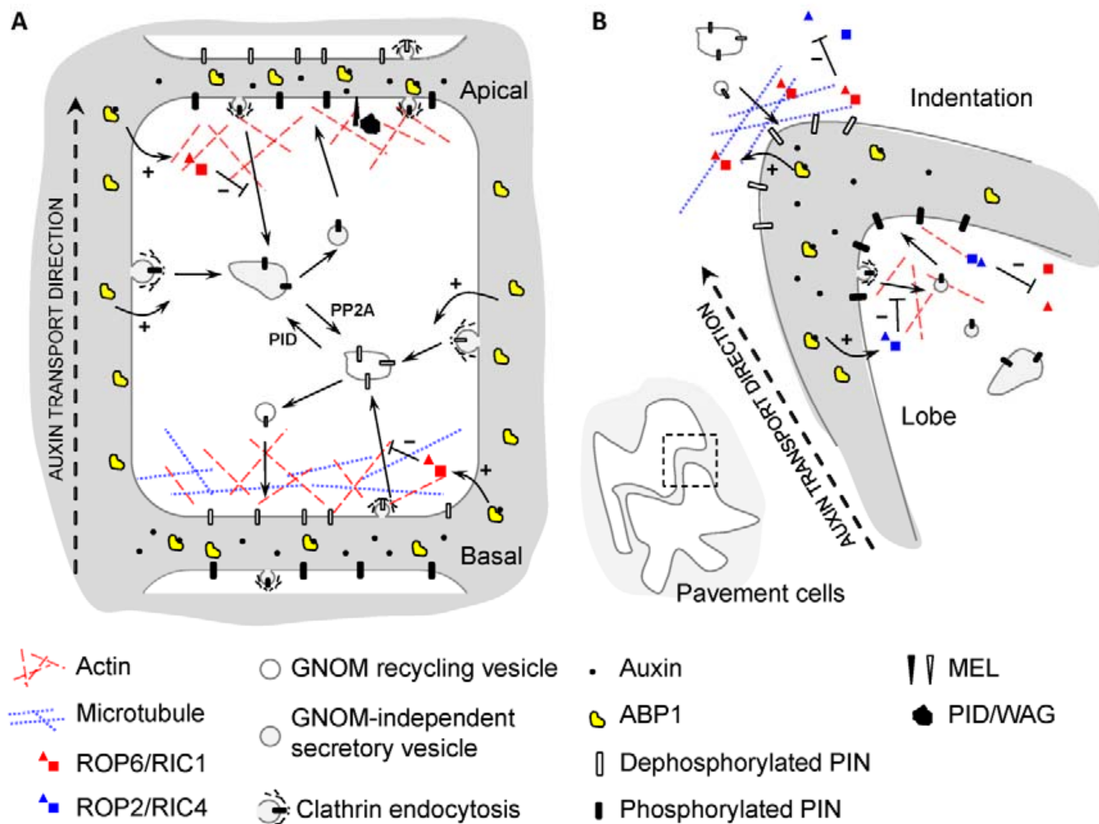

Figure 1 Trafficking-regulated polar delivery of PIN proteins

A. In roots, the PID/WAG-phosphorylated PINs are recruited into a GNOM-independent recycling pathway, which is thought to depend preferentially on the actin cytoskeleton. The PID/WAG trafficking pathway is enhanced by the action of the MEL proteins possibly at the place of endocytosis. On the other hand, dephosphorylated PINs are transported by the GNOM pathway, which depends on both microtubules and the actin cytoskeleton. Putative extracellular ABP1 signaling, which positively regulates PIN endocytosis, is inhibited by auxin, thereby enhancing PIN polar localization at the auxin efflux sites.

B. In the pavement leaf cells, lobe tip PIN localization depends on the PID activity and is regulated by remodeling of the actin cytoskeleton and by PIN trafficking by the ROP6/RIC1 module, which acts antagonistically to the microtubule remodeling ROP2/RIC1 pathway. Here, ABP1 auxin perception maintains ROP/RIC signaling by enhancing their activity.

stimulation (Kleine-Vehn et al., 2010) and for PIN basal to apical translocat ion following inhibition of the basal GNOM-mediated recycling by brefeldin A (BFA) (Kleine-Vehn et al., 2008a and 2008b). Endocytosis coupled with ongoing recycling is clearly required also for the maintenance of polar PIN distribution (Dhonukshe et al., 2008a; Kitakura et al., 2011; Kleine-Vehn et al., 2012), however, it still remains unclear how PIN polarity is originally established. Indirect observations suggest that newly synthesized PINs are initially secreted in a non-polar manner and subsequently polarized by a recycling

mechanism (Dhonukshe et al., 2008a), though a default polar secretion cannot be excluded based on the available data.

Genetic analysis of the components encoding the clathrin machinery in Arabidopsis clearly established a role of clathrin-mediated endocytosis (CME) in PIN polarity establishment, impacting plant development (Kitakura et al., 2011). CME is essential for the previously mentioned PIN constitutive recycling (Dhonukshe et al., 2007) and, together with dynamin-related proteins, for the re-establishment of PIN polarity after cytokinesis (Mravec et al., 2011). Moreover, the feedback loop between auxin signaling and transport seems to involve the putative auxin receptor AUXIN BINDING PROTEIN 1 (ABP1)-mediated regulation of CME (Robert et al., 2010). Auxin itself is able to promote its own transport by inhibiting CME endocytosis (Paciorek et al., 2005) and putative extracellular ABP1 signaling has a positive effect on PIN endocytosis. This enhancing effect of ABP1 on PIN endocytosis is inhibited by auxin, resulting in elevated levels of those efflux carriers in the PM of cells with higher auxin content, subsequently leading to intensified hormone efflux rates. Although there is no experimental demonstration of the effect of ABP1 on PIN polarity, a mathematical model that takes into account this hypothetical extracellular auxin signaling can reproduce PIN polarity and auxin gradients seen *in planta* (Wabnik et al., 2010).

The phosphorylation status of PIN proteins is an important cue for their polar localization. The phosphorylation of serine residues in the PIN hydrophilic loop (Zhang et al., 2010; Huang et al., 2010) by the AGC3 kinases, PINOID (PID) and its homologs WAG1 and WAG2, directs PINs for apical targeting in root and embryonic protodermal cells (Friml et al., 2004; Dhonukshe et al., 2010). The opposite effect is exerted by protein phosphatase 2A (PP2A), which preferentially leads to the basal delivery of dephosphorylated PINs (Michniewicz et al., 2007). The gene *ENHANCER OF PINOID*

(ENP)/MACCHI-BOU4 (MAB4) is suggested to control PIN1 polarity in concert with PID, as the reversal from apical to basal localization seen sporadically in *pid* embryos is much more pronounced in the double *pid enp* mutant (Treml et al., 2005). The gene *ENP/MAB4* encodes a protein similar to NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3), an interacting partner of the light-activated Ser/Thr kinase PHOTOTROPIN1 (PHOT1), which functions as a scaffold protein involved in signal transduction (Motchoulski et al., 1999) and has been implicated in modulating PIN2 trafficking in response to blue light (Wan et al., 2012).The Arabidopsis genome encodes four other ENP/MAB4-like (MEL) proteins whose asymmetric localization resembles PIN polarity in all tissues and cell types examined. These molecular players are regarded to modulate PIN trafficking subsequently regulating the polarity of those carriers (Furutani et al., 2011).

The process of polar sorting in plants is not known in detail and thus mechanistic understanding of how phosphorylation influences PIN targeting is lacking. It has been suggested that PINs are phosphorylated at the PM by the AGC3 kinases and from there internalized and recruited into the GNOM-independent apical recycling pathway (Kleine-Vehn et al., 2009; Dhonukshe et al., 2010). Consequently, the GNOM recycling pathway preferentially recruits dephosphorylated basal cargos. Besides influencing apical and basal PIN targeting, PID-mediated phosphorylation also provides entry points for various external (Ding et al., 2010; Rakusová et al., 2011) and internal (Sorefan et al., 2009) signals that influence PIN localization and auxin response.

PINOID phosphorylation and ROP/RIC interactions with the cytoskeleton: common mechanisms for PIN polar localization

In animal and plant cells, the actin and microtubule cytoskeletons cooperatively interact to regulate cell polarity and vesicle trafficking (Goode et al., 2000; Petrásek and

Schwarzerová 2009). Actin filaments are considered to provide guidance for vesicle trafficking and polar growth in plants (Voigt et al., 2005) and have been shown to play important roles in auxin transport, and PIN recycling (Dhonukshe et al., 2008b; Geldner et al., 2001) and polarity (Baluška et al. 2001; Kleine-Vehn et al., 2008a). Although an intact actin cytoskeleton is necessary for endocytic uptake (Baluška et al. 2002 and 2004) and PIN recycling (Geldner et al., 2001) and thus for both apical and basal PIN localization in root cells, it is the apical delivery route that is more sensitive to actin filament disruption by the actin depolymerizing agent latrunculin B. On the other hand, the basally localized PIN1 in stele and PIN2 in young cortex cells are sensitive to microtubule disruption by oryzalin, while apical PIN2 in the epidermis is largely insensitive to the disruption of the microtubule arrays (Kleine-Vehn et al., 2008a). These data suggest that the delivery and maintenance of polar cargos to apical or basal cell domains are dependent on different arrays of cytoskeletal components.

Similarly, in leaf PCs, the coordinated communication between adjacent cells permits a local growth inhibition concomitant to the activation of the outgrowth of the neighbor cell by the regulation of cytoskeletal components. Two mutually exclusive ROP pathways modulate this process (Fu et al., 2005 and 2009). While cortical microtubules inhibit the growth at the indentation, the F-actin array at the lobe promotes the local elongation (Figure 1B). Active ROP2 locally stimulates RIC4 at the lobe tips to allow the formation of fine cortical actin filaments and lobe development, while ROP6 activates RIC1 leading to organized microtubule formation at the indentation, which constrains growth. RIC1-dependent microtubule organization not only locally inhibits the outgrowth, but in turn also suppresses the activity of ROP2, while lobe-activated ROP2 inhibits RIC1 activity and microtubule organization. Thus, both pathways antagonize each other for cell shape formation using different cytoskeletal components.

Auxin promotes and is required for PC interdigitation. Auxin can increase the average number of cell lobes, and this response is dependent on ROP2/ROP6 action and ABP1 auxin perception. Indeed, auxin rapidly activates ROP2-RIC4 and ROP6-RIC1 pathways through ABP1 activity (Xu et al., 2010). The enrichment of PIN1 at the expanding lobe tip is necessary for the generation of a local gradient of auxin and for auxin-promoted PC interdigitation. At the lobe tip, ROP2 attenuates the mechanism of auxin-inhibited, clathrin-mediated PIN1 endocytosis (Nagawa et al., 2012). ROP2-activated, RIC4-dependent cortical F-actin formation at the lobe mediates the inhibition of PIN1 endocytosis in a similar way shown for chemical stabilization of actin filaments in root tips (Dhonukshe et al., 2008b), suggesting a conserved function of the actin cytoskeleton in the regulation of PIN internalization. This is further supported by observations that ROP/RIC signaling is required for clathrin-mediated PIN internalization in roots (Chen et al., 2012; Lin et al., 2012). PIN1 polar localization in PCs also depends on its phosphorylation status. The degree of PC interdigitation was greatly reduced when the PP2A phosphatase FYPP1 was knocked out or when PID was overexpressed. PIN1 localization in those genetic backgrounds changed from lobe to indentation (Li et al., 2011), pointing out the importance of PIN phosphorylation for the polar localization in this developmental context as it is for the apical basal axis in root cells.

The regulation of PIN polarity in root cells and PCs shares some common components (see Figure 1). In roots, phosphorylated PIN2 is recruited to apical cell surface in epidermis and PIN1 to basal cell surfaces, in both cases pointing toward the direction of the auxin flow; while in PCs the phosphorylated PIN1 is located at the lobe tip, also here being polarized with the auxin flow. In both cases, the genetic interference of PIN phosphorylation recruits dephosphorylated PINs to the ‘opposite pole’, being in this case the cell side facing the auxin efflux from the neighboring cell. Actin filaments and

microtubules have a different contribution in opposite cell sites. While actin filaments contribute to a polar localization at the cell side where phosphorylated PINs are targeted, the microtubule array contributes to the localization at the ‘opposite pole’. The ABP1-auxin effect on clathrin-mediated PIN endocytosis through modulation of ROP/RIC activity is also a common module for trafficking regulation in both cell types. To which extent the ABP1/ROP-RIC and the PID/GNOM pathways overlap to regulate PIN trafficking and polarity is still unclear, but it may account for a general mechanism for polarity establishment.

PIN polarity regulation in response to environmental signals

Plants are able to respond to environmental signals such as light and gravity by modulating their growth. These growth changes result from an asymmetric auxin distribution facilitated by PIN-dependent auxin transport. One of the typical adaptation responses to light is the so-called shade avoidance response. Wavelength ratio changes from red to far red (R:FR) occur typically in areas with dense vegetation where light availability is limited. The change in R:FR is perceived by the plant and translated into a switch in polar PIN3 localization to the outer lateral side in the endodermis, resulting in a transient increase in auxin levels at the outer layers, which in turn induces cell elongation and permits shade avoidance (Keuskamp et al., 2010). Another example is phototropism, a directional growth by which plants respond to a light stimulus. When exposed to unidirectional light, auxin accumulates on the shaded side, leading to a differential elongation and bending towards the light. Arabidopsis PHOT1 and its homolog PHOT2 are PM localized Ser/Thr kinases that function as blue light receptors (Christie 2007). The auxin transporter ABCB19/PGP19 is phosphorylated by PHOT1 in response to light, thus inhibiting its export activity, thereby increasing the auxin content in and above the hypocotyl (Christie et al., 2011), priming phototropic bending. In a subsequent process, a

blue light-dependent signaling polarizes the cellular localization of PIN3 in hypocotyl endodermis cells away from light coinciding with the establishment of an asymmetric auxin distribution with a maximum at the shaded side (Ding et al., 2011). This PIN3 polarization is dependent on GNOM-mediated trafficking and PID activity that phosphorylates PIN3. Roots are also able to respond to unidirectional light but in this case, they show a negative phototropism. Here, PIN2 mediates a PHOT1/NPH3 transduction pathway to generate an asymmetric auxin distribution and root bending away from the light source (Wan et al., 2012). Since PHOT1 does not phosphorylate PIN3 directly (Ding et al., 2011), and probably neither PIN2, possibly a downstream PHOT1-dependent signaling cascade is able to modulate GNOM-dependent trafficking or PIN phosphorylation by PID, or both, by a mechanism that is not fully understood but might involve the activity of NPH3-like proteins (Furutani et al., 2011; Wan et al., 2012).

Similar to phototropism, also during gravitropic response, PIN proteins are central players in auxin-mediated gravitropic bending. PIN3 and PIN7 are symmetrically localized in the first two rows of the root columella, the gravity sensing tissue. Following the gravity stimulation, PIN3 and PIN7 become polarized toward the bottom side of the cells. This gravity-induced PIN3 polarization requires the activity of GNOM and might involve endosome-based PIN3 transcytosis from one cell side to another (Kleine-Vehn 2010). A similar situation occurs in the hypocotyl gravitropic response. Here, PIN3, together with PIN7, polarizes to the bottom side of gravity-sensing endodermis cells and mediates the differential auxin accumulation at the lower side of the hypocotyl required for asymmetric growth and bending (Rakusová et al., 2011). Similar to light, gravity-mediated PIN relocalization also depends on GNOM and PID activity but how the sedimentation of amyloplasts triggers changes in subcellular PIN trafficking is unclear. Overall, these examples show that external signals, such as light and gravity, possess a potential to

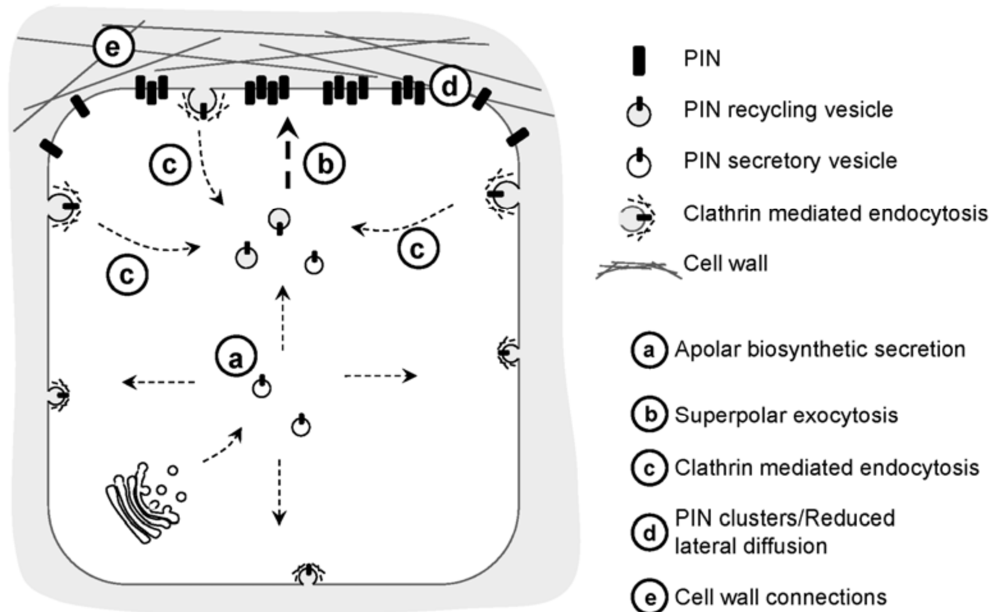

Figure 2 Mechanism of PIN2 polar localization and maintenance of its polarity
After initial post-synthetic secretion that can be apolar or polar (a), the PIN proteins get recruited into an apical recycling pathway by clathrin-mediated endocytosis (CME). The apical domain is generated by the contribution of the super-polar exocytosis (b) and the contribution of CME (c). The PIN clustering (d) presumably reduces the lateral diffusion within the plasma membrane while the connections with the cell wall (e) contribute to maintain this polar domain.

regulate an auxin-mediated response via the modulation of polar PIN trafficking representing a plant-specific mechanism for adaptive development.

A mechanism for PIN polar maintenance

The examples mentioned above demonstrated how flexible PIN polarity can be. However, any polarly localized protein requires a mechanism to maintain its asymmetric disposition within a fluid membrane. With the only known exception of the Casparian band in the endodermis, in plant cells it is not possible to detect a diffusion barrier separating the PM domains. In addition, the canonical polarity modules and regulators highly conserved among animals (Assémat et al., 2008) are absent in plant genomes, though some functional homologs to those proteins are present and partially characterized in terms of cell trafficking or polarity (e.g., the Ser/Thr kinases). This strongly suggests the existence of a plant-specific mechanism for maintaining different polar PM domains. Recently, a

model, which involves the combination of directional exocytosis to the middle of the polar domain, reduced lateral diffusion at the PM and constitutive endocytosis at margins of the polar domain has been proposed to maintain PIN localization (Figure 2, Kleine-Vehn et al., 2011). PIN2 is apically localized in epidermal root cells with some degree of enrichment at the central part of the apical surface of the cell, the so called ‘super-polar domain’. This focused localization is gradually becoming more pronounced during cell differentiation and has not been observed in other PM proteins. Apparently, this super-polar localization is generated by preferential polar exocytosis to the center of the polar domain and limited lateral diffusion within this domain. This poor lateral diffusion is presumably related to the enrichment of PIN proteins into immobile ‘clusters’ within the PM. Super resolution microscopy confirmed that these clusters are of 100−200 nm in size and pharmacological treatment suggested that they are enriched in structural sterols. It is unclear how those clusters are immobilized but this might be due to connections with the cell wall that have been shown to contribute to maintain the polar domain and/or slowdown the diffusion rate of the PINs and other proteins at the PM (Feraru et al., 2011; Martinière et al., 2012). In addition to super-polar exocytosis and limited lateral diffusion, endocytosis has been shown to be absolutely essential for PIN polar localization (Dhonukshe et al., 2008a; Kitakura et al 2011), probably by retrieving PINs that escaped from the polar domain back into the super-polar exocytosis trafficking route.

CONCLUSIONS

Plants rely on polarly localized proteins to mediate several directional responses and *de novo* establishment of cellular and tissue asymmetries. Environmental as well as developmental cues are sensed and integrated into trafficking processes, thereby influencing the localization and abundance of polar proteins. It is important to note that despite increasing the wealth of information about the mechanisms for generation and

maintenance of PIN polarity and the interplay between cell trafficking and polarity, it is currently unclear to which extent this information can be translated to other polar proteins or if the outer and inner lateral domains share some cellular mechanisms and molecular components with the apical-basal polar targeting machinery.

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Chapter 1

Part II: Phosphoinositide-dependent regulation of vesicle trafficking in Arabidopsis

Ricardo Tejos and Jiří Friml

SUMMARY

Phosphatidylinositols (PtdIns) can be phosphorylated, dephosphorylated or cleaved to generate a variety of signaling molecules that participate in several processes, including polar secretion, stress responses, organelle morphology and intracellular trafficking. Phosphorylated forms of PtdIns, collectively called phosphoinositides, are differentially distributed in membranes within the cell by equally differentially distributed kinases, phosphatases and phospholipases, which regulate where and when a particular phosphoinositide is produced. As such, phosphoinositides work as scaffold signals to recruit effector proteins or they can be used as substrates to generate other second messengers. In the present review we attempt to give a glimpse of what is known about PtdIns signaling in plants, their roles in cell trafficking and in developmental processes and emphasize its crosstalk with auxin signaling.

Phospholipids are major structural components of eukaryotic membranes that undergo constant metabolic changes. Among the phosphorylated membrane lipids, the phosphatidylinositols (PtdIns) can be phosphorylated at three different positions at the cytosol facing inositol ring potentially generating seven phosphorylated PtdIns forms, so called Phosphoinositides (Figure 1A). Since almost all structural lipids or their precursors are synthesized at the endoplasmic reticulum (ER), the final steady state composition at the destination membrane is reached by a complex network of phospholipid kinases and phosphatases, and phospholipases, and with the assistance of phosphatidylinositol transfer proteins (Figure 1B and Table 1). Phosphoinositides form gradients and clusters at membranes as a direct consequence of the compartmentalization of the enzymes that metabolize these lipids. Phosphoinositides can work as scaffold molecules, namely working as direct binding signals for cytosolic proteins or cytosolic domains of integral membrane proteins, or as precursors for secondary signaling molecules like other PtdIns, other lipid compounds (e.g. phosphatidic acid (PA) and diacylglycerol (DAG)) or soluble molecules like inositol-1,4,5-trisphosphate (IP_3). This dual function, as well as their intracellular differential distribution, makes phosphoinositides important mediators of a wide variety of cellular processes like membrane trafficking, membrane homeostasis, nuclear signaling and stress responses.

PtdIns3-P and PtdIns(3,5)P_2 regulation of vacuolar trafficking and morphology

Using the endosome binding domain (EBD) of the animal EEA1 protein as an *in vivo* probe for PtdIns3-P in plants, it was possible to demonstrate that different pools of PtdIns3-P are present in plant cells and that this phosphoinositide is transported from endosomes to the vacuole for degradation (Kim et al., 2001). When the PtdIns3-P levels where modulated by the overexpression of the PtdIns3-P binding domain FYVE, which also sequesters the phosphoinositide, or by the treatment with a specific PtdIns3-kinase

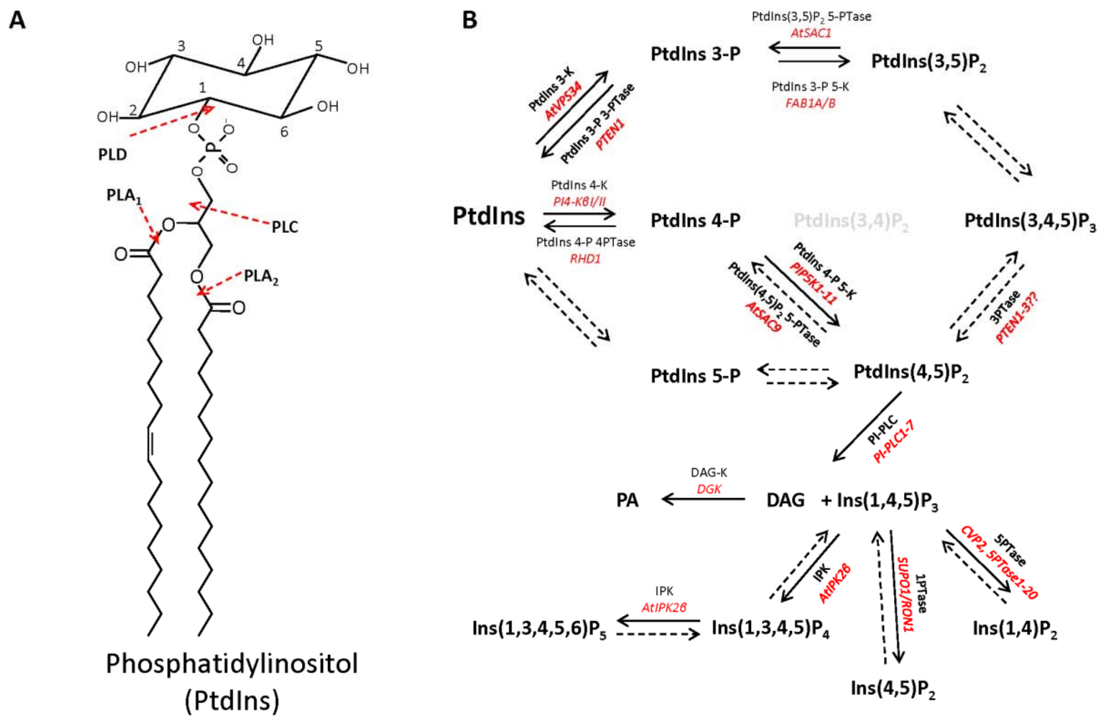

Figure 1. Phosphatidylinositol metabolism.
A. Cartoon of the structure of phosphatidylinositol (PtdIns). The cytosol facing inositol ring and the places where it can be phosphorylated are indicated, together with the places where different phospholipases cleave phospholipids. PLC is the only one cleaving specifically phosphatidylinositol.
B. PtdIns phosphorylated forms, the phosphoinositides, are produced by the action of phosphatases and kinases. Here, we depict the biosynthetic route of all phosphoinositides and soluble inositol polyphosphates described in plants (in bold) and the described enzymes (in red).

inhibitor LY294002, root hair elongation was impaired presumably by the inhibition of endocytosis, by a change in the vacuole morphology at the tip or by a decrease in the level of intracellular reactive oxygen species (ROS) (Lee et al., 2008b). The only Arabidopsis homolog of the yeast PtdIns 3-kinase VPS34, AtVPS34, has been shown to be essential for plant development (Welters et al., 1994). A heterozygous knockout *atvps34* mutant displays early male gametophyte formation defects probably as a consequence of aberrant vacuole morphology and cell division during microgametogenesis (Lee et al., 2008a). Both pieces of evidence support a crucial role of PtdIns3-P in vacuole morphogenesis and trafficking toward the vacuole for degradation, which is essential for proper plant development. In addition to its own roles in membrane trafficking, PtdIns3-P serves as a precursor of other two phosphoinositides: PtdIns(3,4)P_2

Table 1. Enzymes modulating phospholipid homeostasis and signaling and their involvement in vesicular trafficking and auxin dependent responses

Enzyme	Characteristics	References
Phosphatidylinositol kinases:		
PI4Kβ1/2	PtdIns 4-kinase, RABA4b effector. Mutants result in abnormally large secretory vesicles produced at trans Golgi Network (TGN) and defects in delivery of cell wall molecules to cell plate and cell wall. As a consequence the cell patterning in roots is lost.	Preuss et al., 2006, Kang et al., 2011
AtVPS34	PtdIns 3-kinase localized at endosomal vesicles showing specificity toward PtdIns. The knockout mutant shows male gametophyte lethality and vacuole morphology and elongation defects in root hairs, while knockdown deregulation of *AtVPS34* generates strong growth defects in adult plants.	Welters et al., 1994; Lee et al., 2008a
Phosphatidylinositol monophosphate kinases:		
AtIPK2α	Inositol polyphosphate kinase, produces IP_4 and IP_5 phosphorylating Ins(1,4,5)P_3 at positions 3 and 6. It negatively regulates plant growth possibly trough modulating Ca^{2+} signaling.	Xu et al., 2005
AtIPK2β	Inositol polyphosphate 6-/3- kinase, is involved in axillary shoot branching via regulating auxin transcriptional signaling.	Zhang et al., 2007
FAB1A/B	PtdIns 3-P 5-kinases, localize at endosomes in roots. They are involved in vacuolar acidification, endocytosis and endomembrane homeostasis. Conditional knockdown mutants as well as overexpressing plants show various common phenotypes related to auxin signaling.	Whitley et al., 2009; Hirano et al., 2010
PIP5K1	Is able to synthesize PtdIns(3,5)P_2 and PtdIns(4,5)P_2*in vitro* and PtdIns(3,4,5)P_3 in insect cells. Its transcription is rapidly induced upon water stress.	Mikami et al., 1998; Elge et al., 2001; Westergren et al., 2001
PIP5K2	Balances the rate between exocytosis and endocytosis in root cells. Regulates lateral root formation and root gravitropic response, presumably affecting PIN cycling.	Mei et al., 2012
PIP5K3	Type B PtdIns 4-P 5-kinase expressed in trichoblast and root hairs. PIP5K3 localizes to the bulging places in trichoblast and at the apical PM domain in elongating root hairs.	Kusano et al. 2008; Stenzel et al., 2008
PIP5K4/5	Pollen expressed type B PtdIns 4-P 5-kinases. Both localize to the apical PM in elongating pollen tubes. A double *pip5k4pip5k5*mutant shows reduced pollen germination and pollen tube abnormalities while overexpression of both genes generates multiple branching events, due to defects in apical pectin secretion, and/or vesicle trafficking events but not callose deposition neither cytoskeleton abnormalities.	Ischebeck et al., 2008, Ischebeck et al., 2010b; Souza et al., 2008
PIP5K6	Type B PtdIns 4-P 5-kinase. It is localized to the subapical membrane in pollen tubes. Knockdown inhibits clathrin-mediated endocytosis while overexpression induces massive aggregation of membrane at the pollen tube tip. The later can be overcome by inhibiting endocytosis using the dominant negative clathrin version or by modulating the PtdIns4P/PtdIns(4,5)P_2 balance.	Zhao et al., 2010
PIP5K9	Type B PtdIns 4-P 5-kinase. Interacts with a cytosolic invertase to regulate sugar metabolism and root growth	Lou et al., 2007
PIP5K10/11	Pollen-specific type A PtdIns4P 5-K, localized to the subapical PM domain. Overexpression in tabaco pollen generates severe tube swelling and defects in actin cytoskeleton partially by regulating the Rac5/RhoGDI2 interaction.	Ischebeck et al., 2011
Phospholipid phosphatases:		
PTEN1	Dual lipid-protein phosphatase specifically expressed in pollen. Regulates autophagy in pollen tubes by modulating PtdIns 3-P content in endosomal compartments.	Gupta et al., 2002; Zhang et al., 2011b
RHD4	Sac1p-like PtdIns 4-P PTase is localized to the tip of root hairs together with RabA4b. *rhd4* mutants show several bulging places in growing root hairs due to higher levels of PtdIns 4-P in internal vesicles and mislocalization of RabA4b at the root hair tip.	Thole et al., 2008
FRA7/AtSAC1	Phosphoinositide 5-PTase showing high degree of specificity towardPtdIns(3,5)P_2. It is required for normal cell morphogenesis, cell wall biosynthesis and actin cytoskeleton organization.	Zhong et al., 2005
FRA3	Type II 5-PTase hydrolyzing PtdIns(4,5)P_2, PtdIns(3,4,5)P_3 and Ins(1,4,5)P_3, with the highest substrate affinity toward PtdIns(4,5)P_2.A *fra3* mutant accumulates elevated levels of PtdIns(4,5)P_2 and IP_3 and displays defects in cell wall biosynthesis and actin organization.	Zhong et al., 2004
AtSAC9	Phosphoinositide 5-PTase, mutant *sac9* over accumulates PtdIns(4,5)P_2 and develops a short root and a constitutively expressed stress response.	Williams et al., 2005
5PTase7	Inositol polyphosphate 5-PTase 7, it localizes to thePM and nucleus. It positively regulates ROS production and stress response.	Kaye et al., 2007
5PTase13	WD-containing inositol polyphosphate 5-PTase 13, regulates PIN2 trafficking and mediates root gravitropism and vein formation. Functionally interacts with PHOT1 to regulate blue light response probably trough interacting with Ca^{+2} signaling.	Chen et al., 2008, Wang et al., 2009, Lin et al., 2005
CVP2	Inositol polyphosphate 5-PTase 6, negatively regulates IP_3 levels. A knockout mutant displays vasculature defects due to increased IP_3 levels and reduced recruitment of cells for vein formation.	Carland et al., 2004
SUPO1/FRY1/ RON1	Encodes a protein with inositol polyphosphate 1-PTase and 3'(2'),5'-bisphosphate nucleotidase activities. *Supo1* mutants show higher levels of Ins(1,4,5)P_3 and display auxin related phenotypes. Its function was linked to PIN polarity trough the modulation of Ca^{+2} signaling.	Zhang et al., 2011, Robles et al., 2010
Phospholipases:		
PLC	By using the PLC-specific inhibitor U73122, it was possible to establish a role of PLC-derived PA in gravitropism and actin organization.	Andreeva et al., 2010
PLDζ1	Regulates tip growth by modulating vesicular trafficking and exocytosis.	Ohashi et al., 2003
PLDζ2	PLDζ2 expression is auxin-regulated and a GFP fusion localizes to the tonoplast membrane. PLDζ2 deficiency inhibited primary root growth and lateral root formation. It is involved in the regulation of PIN2 cycling.	Li et al., 2007; Yamaryo et al., 2008
$PLA_2α$	It is involved in the regulation of PIN protein trafficking to the PM, most likely acting in Golgi-related compartments. It is able to translocate to the apoplast and to the nucleus in response to developmental and biotic cues.	Lee et al., 2010; Jung et al., 2012; Froidure et al., 2010
$PLA_2β$	Transcriptionally induced by auxin. It regulates the process of cell elongation during shoot gravitropism. It is secreted into the extracellular space where signaling for cell wall acidification is thought to occur.	Lee et al., 2003
$PLA_2β/δ/γ$	RNAi lines down regulating all three result in defects during male gametophyte development. They localize either to Golgi or ER.	Kim et al., 2011
Lipid transfer proteins:		
AtSFH1/ COW1	Phosphatidylinositol transfer protein complements the yeast SEC14p mutant. It is essential for root hair growth and a GFP tagged version localized at the tip of growing root hairs.	Böhme et al., 2004; Vincent et al., 2005
PATL1	Localized to the forming cell plate in late cytokinesis, and in Golgi and PM in interphase cells.	Peterman et al., 2010

and PtdIns(3,5)P_2.While the first molecule has not been characterized in plant cells, PtdIns(3,5)P_2 synthesis has been shown to be rapidly induced upon hyperosmotic stress (Meijer et al., 1999). The Arabidopsis genome contains four putative kinases that could produce such phosphoinositide by phosphorylating PtdIns3-P in the position five (Mueller-Roeber and Pical 2002). From those kinases, two close genes, *FAB1A* and *FAB1B*, codify for proteins that localize to endosomes and they were shown to redundantly regulate pollen development through modulating membrane homeostasis, vacuole acidification and vacuole morphogenesis, as well as endocytosis in roots (Whitley et al., 2009; Hirano et al., 2010). Most probably a portion of the phenotypes related to auxin signaling observed in the knockdown and overexpression mutants can be explained by their impact on PIN trafficking (Hirano et al., 2011). Although the impact of the abundance of these phosphoinositides on PIN trafficking is not totally established, the fact that the PtdIns 3-kinase inhibitor Wortmanin induces the accumulation of PIN2 on its way to the vacuole gives additional insights into a role of PtdIns3-P in PIN trafficking (Jaillais et al., 2006; Kleine-Vehn et al., 2008). All these observations strengthen the hypothesis that a fine-tuned balance between PtdIns3-P and PtdIns(3,5)P_2 is necessary for a proper vacuolar trafficking pathway and hence for vacuolar biogenesis and probably also for proper PIN-mediated auxin responses.

In *Saccharomices cereviseae*, the *FAB1* gene encodes the only protein with a PtdIns3P 5-kinase activity. A knockout *fab1* mutant has almost undetectable PtdIns(3,5)P_2 levels and develops a single, extremely large vacuole (Gary et al., 1998). Interestingly, the overexpression of *FAB1* does not increase the PtdIns(3,5)P_2 levels, but for doing so, it needs the function of Vac7, an integral vacuolar membrane protein whose mutant mimics all *fab1* phenotypes (Gary et al., 2002). These observations suggests that Vac7 acts upstream of FAB1 and has a positive effect on FAB1 kinase activity. Moreover, the

$PtdIns(3,5)P_2$ 5-phosphatase (PTase) FIG4 when mutated is able to overcome the vacuole morphology defects and to restore normal $PtdIns(3,5)P_2$ levels observed in the *vac7* mutant. FIG4 forms a signaling complex together with Vac7 and FAB1which has beem implicated in the phosphoinositide turnover at the vacuolar membrane crucial for vacuole morphology and homeostasis (Botelho et al., 2008). In Arabidopsis, the characterized FAB1A/B proteins (two out of four homologous genes) and the only one SAC1-containing phosphatase (out of nine present in the Arabidopsis genome) are localized to endosomes and not to vacuole (Hirano et al., 2010; Zhong et al., 2005), and besides not a single Vac7-like protein has been characterized so far. Apparently the downstream processes controlled by these phosphoinositides (e.g. vacuolar trafficking and vacuolar morphology) seem to be conserved in Arabidopsis compared to yeast, the mechanisms necessary to generate and maintain the balance between PtdIns3-P/ $PtdIns(3,5)P_2$ and to control the trafficking to the vacuole and the vacuole homeostasis are not clearly established yet in Arabidopsis.

PtdIns 4-P mediated modulation of trafficking at the Golgi-plasma membrane interphase

PtdIns 4-P is synthesized by PtdIns 4-kinases (PI4K) phosphorylating position 4 of the inositol ring. It is the major source of the signaling lipid $PtdIns(4,5)P_2$, but by itself PtdIns 4-P has an important role in membrane trafficking as well as in hormonal responses (Krinke et al., 2007), stomatal movements (Jung et al., 2002) and fertility (Chapman et al., 2011). In Arabidopsis, PtdIns 4-P is present in different cell compartments. By using an YFP fused to the Pleckstrin Homology (PH) domain of the animal four-phosphate-adaptor protein 1 (FAPP1) protein that specifically recognizes PtdInd 4-P, it was possible to visualize this phosphoinositide in Golgi vesicles as well as at the PM in root tip cells

(PH-FAPP1-YFP; Vermeer et al., 2009). Moreover, this PtdIns 4-P marker is enriched at the tip of the PM in growing root hairs (Thole et al., 2008).

PI4Ks regulate vesicle export from the trans Golgi Network (TGN) by recruiting effector proteins that regulate vesicle budding and membrane dynamics (Santiago-Tirado and Bretscher 2011; Graham and Burd 2011). In animals, two major classes of PI4K can be distinguished, classes II and III (Mueller-Roeber et al., 2002). The Arabidopsis genome contains eight putative PI4Ks that belong to group II (PI4Kγ1-8), but so far none of them has been characterized. In group III, there are four PI4Ks divided in the subgroups α and β, however most of the information regarding their localization, activity and function comes from subgroup β.

PI4Kβ1 colocalizes at the tip of growing root hairs with its interacting partner, the small GTPase RabA4b (Preuss et al., 2006) most probably in Golgi compartments (Kang et al., 2011). The double mutant *pi4kβ1/β2* has an altered TGN morphology and as a consequence, this double knockout mutant produced growth and fertilization defects as well as aberrant root hair and pollen morphology (Chapman et al., 2011; Preuss et al., 2006). On the other hand, the PtdIns4-P PTase ROOT HAIR DEFECTIVE1 (RHD1) was shown to dephosphorylate preferentially PtdIns4-P *in vitro*, and as expected the knockout mutant *rhd1* accumulates elevated amounts of PtdIns 4-P (Thole et al., 2008). RHD1 has a similar tip-focused localization as PI4Kβ1, and the *rhd1* mutant showed increased numbers of branched, bulged and wavy root hairs. Moreover, *rhd1*accumulated the PtdIns 4-P marker and RabA4b in internal structures, it had defects in the actin cytoskeleton affecting the tip-focused exocytosis of membranes and cell wall material. Like that, PtdIns 4-PPTase RHD1 and PtdIns 4-kinase PI4Kβ1 functionally interact to regulate the overall PtdIns 4-P levels at the TGN, which is important for regulated tip focused exocytosis in root hairs. All together, the current data place PtdIns 4-P as an essential

phosphoinositide for actin cytoskeleton-mediated trafficking from TGN compartments to the PM.

Endocytic trafficking regulation by PtdIns(4,5)P_2

The phosphatidylinositol 4-phosphate 5-kinase (PIP5K) phosphorylates PtdIns 4-P in position 5 in the inositol ring. The product of this reaction, PtdIns(4,5)P_2, has been shown to participate in multiple processes as a precursor of other signaling molecules as well as a signaling molecule itself in animal systems: PtdIns(4,5)P_2 is crucial for several steps during clathrin-mediated endocytosis, participates in exocytosis and actin arrangement and moreover it is a substrate for the canonical phospholipase C (PLC) signaling pathway (see below, van den Bout and Divecha 2009).

In Arabidopsis, there are eleven genes encoding PIP5Ks and they can be classified in two distinct subfamilies designated A and B (Mueller-Roeber and Pical 2002). Both subfamilies share a high homology in the phosphatidylinositol phosphate kinase domain, but differ in their overall protein organization. While the subfamily A is composed by two members, nine proteins belong to the subfamily B. These two subgroups differ in the presence of an additional conserved domain at the amino terminus in the subfamily A which seems to be unique among the PIP5Ks. This domain is preceding the dimerization domain and consists of eight repeats of a conserved 23-amino acid long motif, which was subsequently identified as a membrane occupation and recognition nexus (MORN) domain. This MORN domain, together with a linker domain located between the dimerization domain and the MORN motif (Stenzel et al., 2011), has been shown to participate in the targeting of the protein to the PM and moreover, it participates in the specific interaction with a number of Rab GTPases of the subclass E, causing its rapid relocation from Golgi to PM (Camacho et al., 2009).

In Arabidopsis and tobacco BY-2 cells, it was possible to indirectly visualize PtdIns(4,5)P_2 using a fluorescent marker similar to the one described for PtdIns 4-P (PH-PLCδ1-YFP, van Leeuwen et al., 2007). The PtdIns(4,5)P_2 marker is predominantly cytosolic but also accumulates at the PM. An unknown mechanism, involving clathrin-mediated endocytosis, dynamically changes PH-PLCδ1-YFP localization upon salt and heat stress (van Leeuwen et al., 2007;Konig et al., 2008) involving CME and PtdIns(4,5)P_2 in a mechanism sensing stress responses.

Most of our knowledge regarding the function of the PIP5Ks in plant trafficking has come from studies in single cell types. They are proposed to regulate polar tip growth through the modulation of different trafficking and secretion processes including the cytoskeleton architecture, the Ca^{+2} gradient and signaling, and the clathrin-mediated endocytosis (Ischebeck et al., 2010a). For instance, PIP5K3 is expressed in root trichoblast cells where it marks the places where the new root hair will emerge, and later it localizes to the tip of growing root hairs (Kusano et al., 2008). Its overexpression leads to an increased accumulation of the PtdIns(4,5)P_2 marker at the tip of root hairs (Stenzel et al., 2008), pointing out the importance of the polarized synthesis of PtdIns(4,5)P_2 for proper apical growth. In pollen tubes, PIP5K4, 5, 6, 10 and 11 are localized in a subapicalPM microdomain in growing tubes, where they regulate at different levels processes important for endocytic and exocytic events at the root hair tip (Ischebeck et al., 2008, 2010b; Sousa et al., 2008; Zhao et al., 2010). Manipulation of PIP5K levels in pollen leads to apparently opposite effects in the tube growth, despite the fact that they are located at the same PM domain. This might indicate that different PtdIns(4,5)P_2 pools might exist in pollen tubes, which would require a strict regulation of the localization of the PtdIns(4,5)P_2 synthesizing enzymes as well as their interacting partners.

PIP5K2 is the only example of PIP5K studied at tissue level in terms of membrane trafficking. *PIP5K2* expression is auxin inducible and a knockout *pip5k2* mutant displays phenotypes related to an improper auxin response and distribution. The uptake of the membrane dye FM4-64 is slightly reduced, and PIN protein cycling is also affected (Mei et al., 2012), pointing toward a key role of PIP5K2-produced PtdIns(4,5)P_2 for general membrane trafficking rather than for PIN-specific regulation as the general endocytosis seems to be affected. A couple of phosphatase mutants with elevated amounts of PtdIns(4,5)P_2 show defects in cell wall biosynthesis, actin organization and root growth (Zhong et al., 2004; Williams et al., 2005), although a link with the above mentioned functions of PIP5K-produced PtdIns(4,5)P_2 is not illustrated clearly.

Regulation of vesicle trafficking by phospholipase-derived second messengers

In animal systems, the classic lipid signaling pathway involves stress-triggered PLC activity which uses PtdIns(4,5)P_2 to produce the soluble IP_3 and the membrane lipid DAG. IP_3 is able to open intracellular calcium stores and DAG stays in the membrane where it is thought to be rapidly phosphorylated by diacylglycerol kinases to produce PA, another important secondary messenger. In plants, PLC signaling has been implicated in osmotic stress responses, plant defense, seed germination and stomatal opening (Meijer et al., 2003). Moreover, using the PLC-specific inhibitor U73122, it became possible to establish a role of PLC-based signaling during gravitropic response and in actin organization (Andreeva et al., 2010). If the PLC-mediated classic signaling described in animal systems is present as such in plants is still not clear. For instance, although IP_3 is able to release intracellular Ca^{+2} in plant cells, the IP_3-gated calcium channel is so far not described, and most probably another soluble inositol phosphate, IP_6, is the responsible for the increase in cytosolic calcium concentrations. Similarly, while DAG is an important component of animal PLC signaling, its function in plants is not characterized;

instead PA could work as the membrane bound function of the signaling event following PLC action (Testerink et al., 2005).

Two other types of phospholipases are present in plants which vary in the phospholipid site they hydrolyze (Figure 1A). Together with the previously described PtdIns-specific PLC, there are phospholipases A (PLA) and phospholipases D (PLD), which use other phospholipid substrates than phosphoinositides, hence generating different secondary messengers. PLA2 uses phosphatidylcholine (PtdCho) or phosphatidylethanolamine (PtdE) to produce LysoPtdCho and LysoPtdE, respectively, and free fatty acids. On the other hand, PLD uses PtdCho as a substrate to generate PA.

Plant PLA2s can be classified as low molecular weight PLA2s and patatin-like PLA2s. They are involved in different processes like gravitropism, cell elongation, auxin response and guard cell movement. PLA2α is localized to the Golgi. Impairing its function or using a PLA2-specific inhibitor dramatically reduces the PIN protein amount at the PM accumulating them in intracellular structures. Moreover, PIN PM localization can be rescued by the external application of lysoPE on PLA2α knockdown lines (Lee et al., 2010). This suggests a role of PLA2 signaling, in particular lysoPE, in PIN trafficking and this effect might also help to explain the previously observed effects of PLA2 on auxin-dependent transcription (Scherer et al., 2007), cell elongation and gravitropism (Lee et al., 2003).

Phospholipases D are a common hub for a variety of external and internal signals, and their activity and localization make them versatile effectors for multiple plant processes. Arabidopsis PLDs and PLD-derived PA are involved in plant growth, development and response to biotic and abiotic stress (Li et al., 2009). A PLD isoform, PLDζ2, was shown to participate in auxin response in roots by modulating PIN2 trafficking (Li and Xue 2007). External application of PA enhances gravitropic and auxin responses monitored by

DR5-GUS and other auxin-regulated genes. By using the PLD-specific inhibitor 1-butanol it is possible to distinguish the PLD-derived PA from other sources, linking PLD activity directly to auxin-regulated responses. Moreover, general endocytosis and in particular PIN2 trafficking are affected in *pldζ2* mutants and PLDζ2 overexpressing plants. However, considering the PLDζ2 localization to tonoplast membranes (Yamaryo et al., 2008) it appears unlikely a direct local-effect of PLDζ2 over PIN trafficking, and most probably a bunch of PA-binding proteins is affected in *pldζ2* or by external PA treatments.

Phosphatidylinositol transfer proteins

In the early 1980s, the pioneering work of Novick and colleagues established one of the first genetic approaches aiming the identification of components of the secretory pathway in yeast (Novick et al., 1980). They were able to identify 23 complementation groups of *sec* mutants. Those mutants have defects in the secretion of a couple of marker proteins and displayed a variety of morphological defects in intracellular membranous compartments, indicating that they were affected somewhere in the secretory pathway (Novick et al., 1980; Schekman and Novick 2004). Currently we know in great detail the function of these *SEC* genes and their counterparts in other model systems. One of these mutant groups, *sec14*, was characterized as accumulating vesicles and forming some cup- or ring-shaped organelles called Berkeley bodies (Novick et al., 1980). *Sec14* encodes a phosphatidylinositol/phosphatidylcholine transfer protein (PITP) primarily named PIT1 (Aitken et al., 1990). Initially characterized as essential for yeast growth (Aitken et al., 1990), PIT1/Sec14p was later described as an important ubiquitous cytosolic membrane-associated protein involved in trafficking from the Golgi complex (Bankaitis et al., 1989, Bankaitis et al., 1990). Since the early 1990s we know the existence of PITPs, but till now they form an enigmatic class of proteins.

Phosphatidylinositol transfer proteins are able to transfer PtdIns or PtdCho between membranes *in vitro* in a mutually exclusive manner, in other words, they are seen as lipid transfer proteins as they can move PtdIns from membranes rich in these phospholipids, like the primary place of synthesis at the ER, to poorer membranes, such as the PM, in exchange of PtdCho (Figure 2A). In other *in vitro* assays in mammals and yeast, PITPs are able to stimulate Ca^{+2}-triggered exocytosis (Hay and Martin 1993), regulate budding and vesicle formation at TGN (Simon et al., 1998) and assist PLC-mediated PtdIns(4,5)P_2 hydrolysis (Cunningham et al., 1995), pointing to a key central role played by PITP in vesicle trafficking trough modulating phosphoinositide-dependent processes and membrane homeostasis. The current understanding places PITPs as active mediators of the synthesis of phosphoinositides like PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 and hence also of second messengers such as IP_3 and DAG, and instead of being seen as lipid transfer proteins they may rather work as lipid biosensors or PtdIns-presenting molecules, making PtdIns a better substrate for lipid kinases (Figures 2B and 2C). In this sense, it is suggested that PITP might work regulating the interphase between phosphoinositide metabolisms and signaling (Cockcroft 2001).

In spite of being present in all eukaryotes and in relatively high abundance (over 500 genes among all species sequenced; Mousley et al., 2007) there is little information about the physiological role of Sec14-like proteins and PITPs in general. This point is even more extreme in plants, where there have been only few PITP functional analyses. The first description in plants came from a study in soybean where two proteins named Ssh1 and Ssh2, were recovered based on their ability to complement the growth of the yeast *sec14* mutants (Kearns et al., 1998).

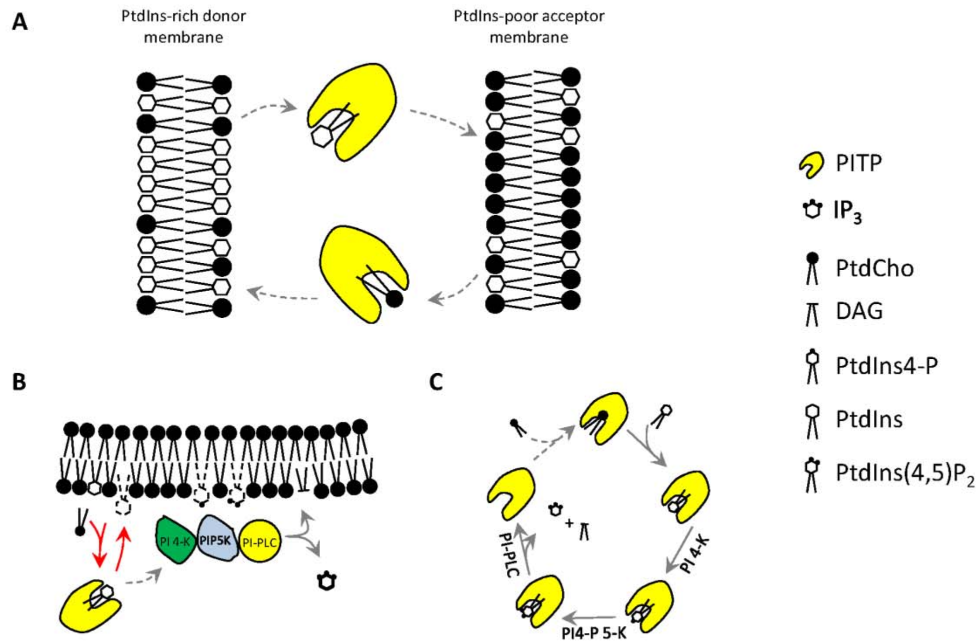

Figure 2. Phospholipid transfer proteins: mode of action.
A. Phospholipid transfer model. Based on *in vitro* activities, it is hypothesized that PITPs are able to transfer PtdIns from their place of synthesis to membranes where this lipid is not abundant in exchange of phosphatidylcholine (PtdCho).
B-C. Nanoreactor model. B. PITPs might be part of the phospholipid signaling pathway providing the precursor for the formation of the secondary messengers IP_3 and DAG to the complex of phospholipid kinases (PI4K, PIP5K) and phosphoinositol-specific PLC (PI-PLC) either transferring PtdIns(4,5)P_2 in the membrane where this lipid is present in low levels (red arrows) or directly presenting PtdIns(4,5)P_2 to the enzymatic complex (discontinuedline arrow). C. Possibly PITPs cycle between a PtdCho-bound and a PtdIns(4,5)P_2-bound state and due to their interaction with phosphoinositide kinases and phospholipases, they generate every cycle IP_3 and DAG.

Ssh1/2 proteins share a rather low sequence similarity compared to yeast PITP and differ in their lipid binding affinities to what was previously described for other PITPs. While Sec14p binds PtdIns and PtdCho with a high affinity, and transfers both of them in *in vitro* assays, Ssh1 and Ssh2 are able to bind and transfer only PtdIns(4,5)P_2 and not PtdCho. Besides, Ssh1 is phosphorylated upon hyperosmotic stress and is rapidly translocated from its membrane bound form to the cytoplasm (Kearns et al., 1998; Monks et al., 2001), where it is supposed to trigger a nuclear protective response. Other examples are the nodule-specific regulation of the *Lotus japonicus* PITP-like protein (LjPLP; Kapranov et al., 2001) and a *Nicothiana bentamiana* Sec14p-like (NbSEC14) protein,

which is upregulated upon pathogen attack (Kiba et al., 2012). All these examples point to the further functional diversification that PITPs reached in the plant kingdom.

Arabidopsis thaliana contains 32 Sec14p-like proteins and recent evidence has suggested they are involved in membrane morphogenesis and trafficking processes. Several Arabidopsis thaliana Sec14 homologs (AtSFHs) are able to rescue the growth deficiency of the yeast *sec14p* mutant as well as its secretory activity (Vincent et al., 2005). One of them, AtSFH1, is highly enriched at the tip of root hairs and a null *atsfh1* mutant displays defects in the actin and microtubule cytoskeleton, mislocalizes the PtdIns(4,5)P_2 marker and displays abnormal Ca^{+2} gradients at the tip, thereby generating defects in root hair patterning and polar growth (Böhme et al., 2004; Vincent et al., 2005). The authors propose a model where AtSFH1 stimulates the apical PtdIns(4,5)P_2 synthesis by a direct interaction with enzymes of the phosphoinositol metabolic pathway. As several secretion processes in polar tip growth rely on a focused synthesis of PtdIns(4,5)P_2, the logic consequence of its disruption is the loss of regulated polar exocytosis and endocytosis.

PATELLINs (PATLs) form a small subgroup of six PITP proteins which also contain in tandem with the Sec14-like domain a Golgi dynamic (GOLD) domain. PATL1 is a cytosolic protein that peripherally associates to the forming cell plate in cells with ongoing cytokinesis and to Golgi-like vesicles (Peterman et al., 2004). Similarly to other plant PITPs, PATL1 mainly associates to the phosphoinositides PtdIns 5-P and PtdIns(4,5)P_2 but not to PtdCho, and most probably PATL1 regulates exclusively phosphoinositide homeostasis. About PATLs there is only some suggestive data that could give them a very essential role due to their participation in several signaling pathways (Deng et al., 2007; Tang et al., 2008; Černý et al., 2011; Benschop et al., 2007; Elmore et al., 2012; Kiba et al., 2012). However, so far there is no functional characterization for any PATL protein.

PDK1-PINOID as a hub for lipid action and auxin signaling

Lipid-derived signals are essential for a multitude of processes as described in the previous sections, but the downstream effectors are so far not totally elucidated. Members of the Arabidopsis AGC kinases family (named after the cAMP-dependent protein kinase A/cGMP-dependent protein kinase G/phospholipid dependent protein kinase C) are common downstream effectors of key intracellular second messengers such as phospholipids or Ca^{+2} in animals and yeast. The Arabidopsis homolog of the mammalian 3-phosphoinositide-dependent protein kinase-1 (PDK1) could couple lipid signals to the downstream effect of several kinases of the AGC family (Bögre et al., 2003). PDK1 phosphorylates the activation loop present in some AGC proteins, including PINOID (PID) and enhances its kinase activity. PDK1 binds and phosphorylates PID, which in turn activates PID autophosphorylation activity, enhancing its own kinase activity (Zegzouti et al., 2006). PDK1 is able to bind PA, PtdIns 3P, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 and to a lesser extent PtdIns(4,5)P_2 and PtdIns 4-P through its pleckstrin homology domain. PDK1 is also able to activate a mammalian protein in the presence of PtdIns(3,4,5)P_3 (Deak et al., 1999), indicating a possible regulatory role of those phospholipids on PDK1 activity (Anthony et al., 2006).

Hydrolysis of PtdIns(4,5)P_2 by PLCs leads to the synthesis of IP_3 and DAG, and a subsequent phosphorylating reaction produces PA from DAG (Wang 2004). IP_3 is a major second messenger in all eukaryotic cells and its more prominent action is the modulation of the cytosolic Ca^{+2} levels and hence the calcium response (van Leeuwen et al 2004; Krinke et al 2007). Genetic evidence suggested a crosstalk of IP_3 signaling with the auxin response and transport.

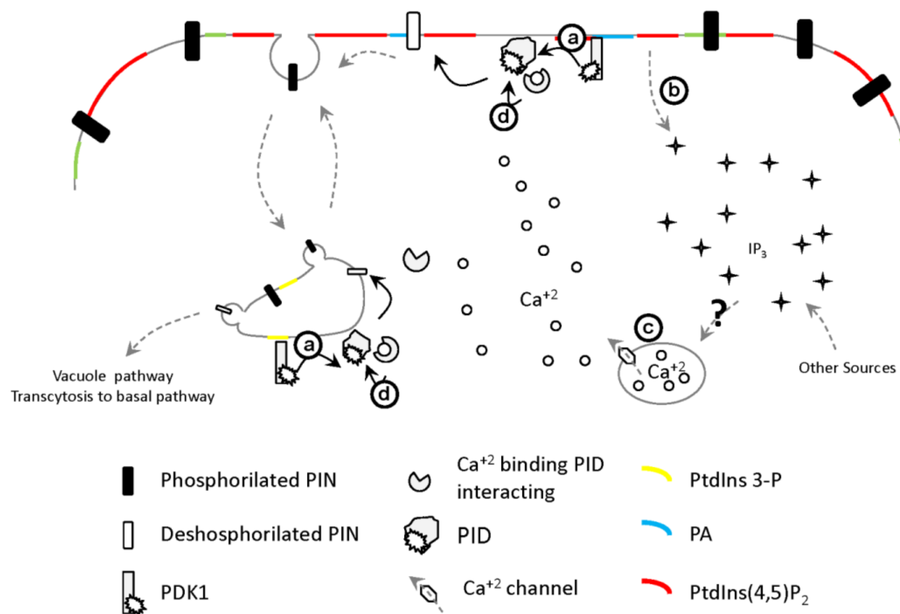

Figure 3. PINOID pathway interaction with phosphoinositide signaling.
PINOID (PID) kinase activity is enhanced by phosphorylation of PDK1, a phosphatidylinositol-dependent kinase which binds phosphoinositides (a). IP3 production by PLC (b) or coming from other sources mediates Ca^{+2} release from intracellular stores (c). Ca^{+2}-binding proteins transphosphorylate PID, enhancing its kinase activity (d). By modulating PID activity, phosphoinositide signaling might influence the PIN phosphorylation status, hence the polar localization or recruitment to different trafficking pathways.

Several inositol PTase mutants showing an altered IP_3 content have altered auxin levels and responses (Table 1; Xue et al., 2007). Moreover, the VAN3 protein, which encodes a TGN-localized ARF-GAP, is antagonistically regulated by IP_3 and PtdIns 4-P and genetically interacts with PIN1 in regulating the auxin-mediated vascular development (Koizumi et al., 2005; Naramoto et al., 2009). More recently it was demonstrated that IP_3-induced calcium signaling modulates auxin transport through an effect on PIN polarity, where presumably PID plays an important role (Zhang et al., 2011a). Ca^{+2} inhibits PID autophosphorylation activity and its kinase activity *in vitro* (Zegzouti et al., 2006). Moreover, two PID interacting proteins, a calmodulin-related protein TOUCH3 (TCH3) and an EF hand-containing protein PINOID BINDING PROTEIN1 (PBP1) were shown to bind PID in a Ca^{+2}-dependent manner and modulate its activity (Benjamins et al.,

2003). These pieces of evidence point to the kinase PINOID as a good candidate for the crosstalk between auxin and lipid signaling (Figure 3).

CONLUDING REMARKS

Phosphoinositides are versatile lipid messengers which modulate a big variety of processes in plants. Its versatility arises from the rapid metabolic changes that phosphoinositide-modifying enzymes exert over all phospholipid species, which also compartmentalizes their synthesis and turnover, generating gradients of membrane bound signals and mobile PtdIns-derived messengers. The huge variety of processes under PtdIns control is therefore not surprising, as is the fact that PtdIns signaling overlaps with or is part of stress responses, developmental processes, directional growth and hormonal control. Despite the increasing information regarding PtdIns functions and the characterization of their metabolizing enzymes in plants, many links are missing, especially the ones concerning the crosstalk with other pathways like auxin signaling. Although several evidences indicate that at least a part of phosphoinositide input on auxin signaling is at the level of PIN-mediated auxin transport, the PtdIns effectors mediating this modulation are not fully established. Moreover, the fact that several PtdIns species including the PtdIns-derived second messengers have an impact on PIN trafficking just adds more pieces to the puzzle and suggests a high degree of diversification and complexity in auxin-phosphoinositide crosstalk.

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Chapter 2

Auxin feedback on cell and tissue polarity by modulation of phosphoinositide metabolism

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SUMMARY

Coordinated cell polarization in developing tissues is a recurrent problem during development of multicellular organisms. In plants, a dynamic distribution of the plant hormone auxin is at the core of many developmental programs. Via feedback regulation by auxin on the subcellular localization of PIN auxin transporters, a self-organizing mechanism for coordinated cell polarization is proposed for plant development. Although there is ample evidence for such a feedback regulation in different developmental processes, the mechanisms which link auxin signaling to polar auxin transport are not well understood. We found that auxin has the capacity to regulate overal PtdIns(4,5)P_2 levels and in particular the plasma membrane levels of PtdIns(4,5)P_2, a phosphoinositide that is tightly coupled to polarization of tip growth in root hairs and pollen tubes. Moreover, we have identified phosphatidylinositol 4-phosphate 5-kinase1 (PIP5K1), a PtdIns(4,5)P_2-forming enzyme, to be transcriptionally induced by auxin and polarly localized in root cells. PIP5K1 and its close homolog PIP5K2 were redundantly required for PIN polarity, differential auxin distribution and auxin-related developmental processes. Our results provide a novel link between auxin signaling and plasma membrane phosphoinositides in the regulation of PIN polarity during plant development.

The life strategy of plants is characterized by an exceptional developmental flexibility often involving (re)specification of tissue and cell polarity. A fundamental problem concerns how individual cells sense their own cellular context, i.e.their position within a tissue or organ, and integrate this information to coordinate their own polarity (Berleth and Sachs 2001). It has been established that the plant hormone auxin can act as both a coordinative signal and a polarizing cue (Boutté et al., 2007; Leyser 2011; Reinhardt et al., 2003; Vanneste and Friml 2009). Auxin has the unique property to be directionally transported between cells and the directionality of its polar movement combined with the capacity for feedback regulation has been proposed to enable flexible and self-organizing patterning events ranging from embryogenesis, organogenesis, leaf venation or tissue regeneration following wounding (Berleth and Sachs 2001).

The polar cell-to-cell transport is generated by the concerted action of auxin transporters. Among them, the polarly localized PIN-FORMED (PIN) auxin efflux carriers (Petrásek et al., 2006) determine the directionality of auxin transport (Wisniewska et al., 2006). Auxin has been shown to regulate the capacity of its own transport through inhibiting clathrin-mediated endocytosis (Paciorek et al., 2005; Robert et al., 2010)and dynamically changing PIN polarity in different developmental contexts (Heisler et al., 2005; Benková et al., 2003; Ikeda et al., 2009; Scarpella et al., 2006; Sauer et al., 2006; Balla et al., 2011; Leyer 2003). It is, however, entirely unclear how auxin modulates PIN polarity: while the underlying mechanism requires canonical SCF^{TIR1}-AUX/IAA-ARF auxin signaling for the regulation of gene expression (Chapman and Estelle 2009), it does not involve any known regulator of PIN trafficking and polarity (Sauer et al., 2006).

Plasma membrane lipid composition had been previously demonstrated to be required for proper cell trafficking and polarity (Fischer et al., 2004). Sterols and PIN2 follow a similar trafficking pathway (Grebe et al., 2003) and a chemically or genetically altered

PM sterol composition disrupts PIN polarity (Men et al., 2008; Willemsen et al., 2003; Markham et al., 2011) and PIN2 clustering at the PM (Kleine-Vehn et al., 2011). Phosphoinositides are minor but functionally important lipid constituents of all cellular membranes. They can function as precursors for other signaling molecules or as signaling molecules themselves. Previous reports have highlighted the importance of phosphoinositides in membrane trafficking and auxin-dependent development (Xue et al., 2007). Mutants in the key enzyme involved in *de novo* synthesis of myo-inositol, a common precursor of various inositol-derived molecules including PtdIns and phosphoinositides, showed several auxin-related mutant phenotypes and PIN polarity and trafficking defects, which can be explained by an improper balance in membrane phosphoinositides (Luo et al., 2011; Chen et al., 2010). Moreover, the phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2) as well as the PtdIns(4,5)P_2–derived second messengers phosphatidic acid (PA) and inositol (1,4,5) triphosphate (IP_3) were shown to modulate PIN trafficking or polarity (Li et al., 2007; Mei et al., 2012; Zhang et al., 2011), pointing to a pivotal role of PtdIns(4,5)P_2 in regulating trafficking processes during auxin-dependent development.

Here, we report the characterization of phosphatidylinositol 4-phosphate 5-kinase 1 (PIP5K1) as a key regulator of PIN polarity downstream of auxin signaling. Double mutants of PIP5K1 and the close homologous PIP5K2 show several auxin-related phenotypes that are correlated with disrupted auxin content and response and aberrant PIN polarity. Moreover, PtdIns 4-P, PtdIns(4,5)P_2 and PIP5K1 are enriched to apical-basal membranes where they colocalize with polar PIN1 and PIN2. Auxin was able to modulate global PtdIns(4,5)P_2 levels and in particular phosphoinositide contents at the PM. Our results suggest that an auxin-PIP5K-dependent phosphoinositide pool is

necessary for the correct PIN polarity establishment and hence for auxin-dependent developmental processes.

RESULTS

Microarray analysis of the auxin effect on PIN polarity identifies PIP5K1

Auxin induces a change of PIN polarity in *Arabidopsis thaliana* roots manifested by basaltoinner lateral repolarization of PIN1 in endodermis and pericycle cells and a basaltoouter lateral repolarization of PIN2 in cortex cells (Figure 1A). This effect depends on the SCF^{TIR1} -Aux/IAA-ARF signaling pathway since ectopic heat shockinducible expression of a dominant negative mutant of the auxin signaling repressor IAA17/AXR3 (*HS::axr3-1*; Knox et al., 2003) leads to a loss of PIN lateralization after auxin treatment (Sauer et al., 2006).

To identify downstream factors required for this auxin effect on PIN polarity, we designed a microarray experiment to search for genes that respond differentially to auxin between wild type (WT; Col-0) and *HS::axr3-1* in roots and thus are potential mediators of this effect. We applied a 40-minute heat shock to seedlings 5 days after germination (DAG) of Col-0 and *HS::axr3-1*, allowed them to recover 1.5 hours and treated them subsequently with 10 µM 1-naphthaleneacetic acid (NAA) or solvent control for 4 hours. RNA of the lower third of the root was extracted and hybridized to Affymetrix ATH1 microarrays (Figure 1A). In a first step, we identified 523 (logit-t $p = 0.05$) auxin-regulated genes in Col-0 and 667 (logit-t $p = 0.05$) genes differentially regulated between *HS::axr3-1* and Col-0 in the presence of auxin. Overlap of these genes yielded a list of 245 candidate genes that are auxin-regulated in an AXR3-dependent manner and thus are potential regulators of PIN polarity (Figure 1B, Annex 1). This list was manually examined for genes with a possible direct role in protein trafficking, thus excluding genes coding for transcription factors, enzymes and metabolic processes. As one of the

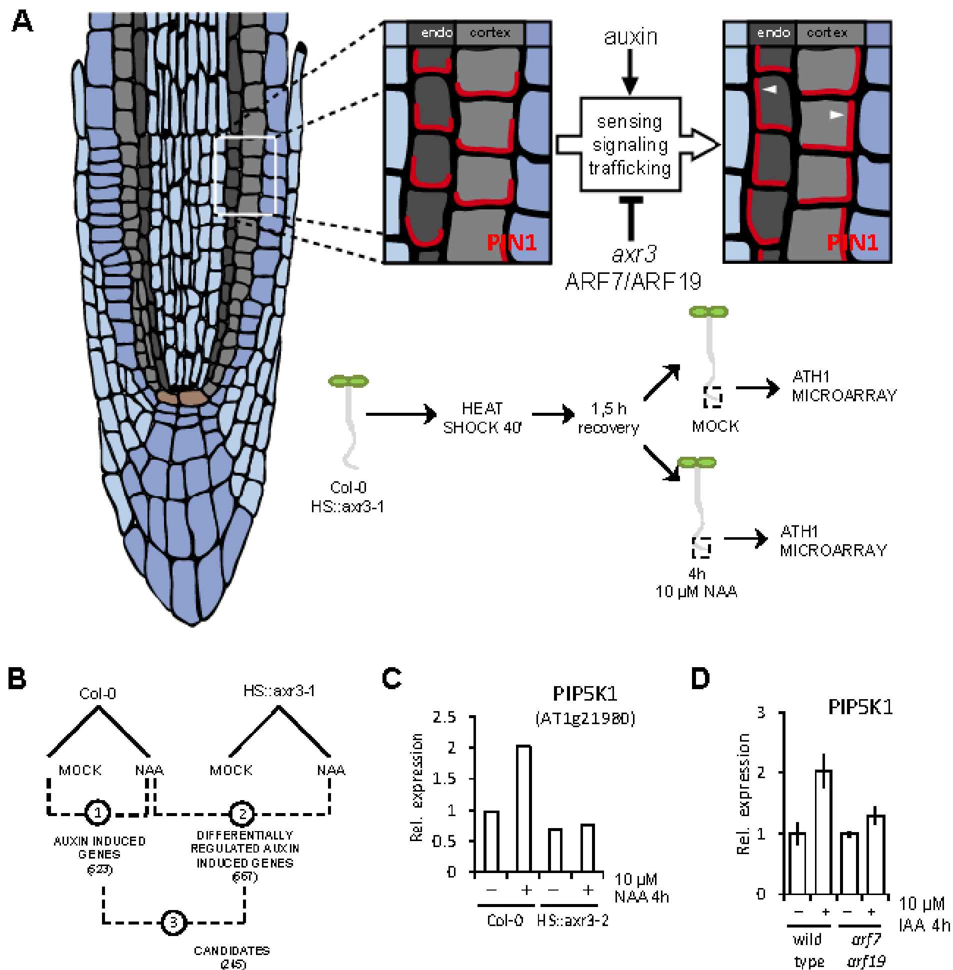


Figure 1. Identification and validation of PIP5K1 as a candidate gene mediating auxin-dependent PIN repolarization.

A. Summary of microarray set-up. In untreated roots, PIN1 in the endodermis and PIN2 in young cortex cells localize to the lower (basal) cell sides. After auxin induction, PINs repolarize to inner and outer lateral cell sides in endodermis and cortex, respectively. This process can be inhibited by heatshock induction of dominant negative *axr3*. After heatshock and recovery, both WT and *HS::axr3-1* were treated with 10µM NAA for 4 h. RNA from the lower third of the roots was used for microarray analysis.

B. Diagram showing a summary of the selection criteria on the resulting microarray data: 1) differential expression between treated and untreated Col-0 (523 genes) and 2) differential expression between auxin-treated WT and *HS::axr3-1*. 3) For 245 genes, both criteria were met (candidate genes).

C. PIP5K1 met both criteria, being auxin-inducible in an *axr3*-dependent manner (microarray data).

D. Auxin-inducible expression of PIP5K1 was abolished in roots of *arf7 arf19* double mutants as shown by Q-RT-PCR.

prominent hits, we identified *PIP5K1* (At1G21980) as a likely player involved in protein trafficking events at the PM (Figure 1C). PIP5K phosphorylates PtdIns4-P. The product of this reaction, PtdIns(4,5)P_2 participates in multiple processes as a precursor of other signaling molecules as well as a signaling molecule itself, and it has been implicated in

the control of membrane trafficking and polarity generation (Di Paolo and De Camilli 2006; Shewan et al., 2011). In plants, PtdIns(4,5)P_2 and PIP5K function are required for various trafficking processes, among others for polar tip growth (Ischebeck et al., 2010) and PIN-mediated root gravitropism (Mei et al., 2012). This makes PIP5K1 a promising candidate for regulating trafficking processes governing PIN polarity *in planta*.

To corroborate the microarray data, we first addressed the kinetics of *PIP5K1* induction by auxin using quantitative RT-PCR. At 4 DAG, seedlings were transferred to medium supplemented with auxin (10 µM IAA). During a 6-h time course, root material was harvested at specific intervals for RNA extraction and subsequent cDNA synthesis. As a positive control, we analyzed the relative expression levels of two known early auxin inducible genes (*SHY2/IAA3*, *BDL*/*IAA12*). Within half an hour these genes were strongly induced and stayed induced for several hours, after which the induction became attenuated. Interestingly, *PIP5K1* followed a similar pattern, confirming the auxin induction observed in the microarray analysis (Figure S1). Because auxin-induced PIN lateralization depends on the activity of ARF7 and ARF19 transcription factors (Sauer et al., 2006), we analyzed *PIP5K1* auxin inducibility in *arf7arf19* double mutants. While in WT background, *PIP5K1* was induced, in *arf7 arf19*, this induction was largely lost (Figure 1D). These data confirm that *PIP5K1* is an early auxin inducible gene, downstream of the canonical auxin signaling pathway, as predicted from the microarray analysis. In summary, our microarray experiment identified auxin inducible PIP5K1, a phosphatidylinositol metabolic enzyme, as a potential mediator of auxin effects on PIN polarity.

***pip5k* mutants show defects in multiple developmental processes**

Among the subfamily B of the Arabidopsis PIP5K genes, the closest homolog of PIP5K1 is PIP5K2, and both are thought to originate from a gene duplication event (Mueller-

Roeber and Pical 2002). Besides, both genes transcription are regulated by auxin (Figure 1D; Mei et al., 2011). We first checked the expression pattern using 2000 bp of PIP5K1 and PIP5K2 promoters fused to nuclear targeted GFP-GUS. Both kinases were expressed in the vascular system of cotyledons and leaves (Figure 2A and 2B). In the root apical meristem PIP5K1 was expressed in the preprocambial cells (Figure 2C) and PIP5K2 was expressed in lateral root cap and epidermis (Figure 2D). During embryo development, both genes had partially overlapping expression patterns, in early stages in the basal embryo and switching to the apical part in later stages (Figures 2E-2J). Additionally, PIP5K2 was also expressed in the suspensor (Figure 2H and 2I , arrowhead).

To gain insights into the function of PIP5K1 and its closest homolog, we isolated representative full knockout SALK insertional mutants in both genes (Figure S2A) and because of their close homology we also generated *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$ double mutants. Each single mutant displayed significantly shorter primary roots and reduced lateral root densities as compared to WT (Figure S2B). In the double mutant, root length was even shorter than in the single mutants, and fewer lateral roots were formed (Figure S2B). Beside stronger effects found already in the single mutants, the double mutants also showed dramatically smaller root apical meristems which contained less starchcontaining root cap cells (Figure S2C). *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$ cotyledons showed severe defects in vascular development, all having disconnected vascular strands and 90% of them showing completely disconnected vascular fragments – so calledvascular islands (n = 159, Figure 2K, arrowhead indicates a vascular island). Similar vascular defects were also observed in primary leaves of 7 days old *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$plants (Table 1) as well as in adult plants (Figures S2D-S2G). At a low frequency (0.5%, n = 854) seedlings from the segregating *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$ population displayed single or fused cotyledons (Figures 2N and 2O), and in other extreme cases some root-like structures (Figure 2P, arrowhead)

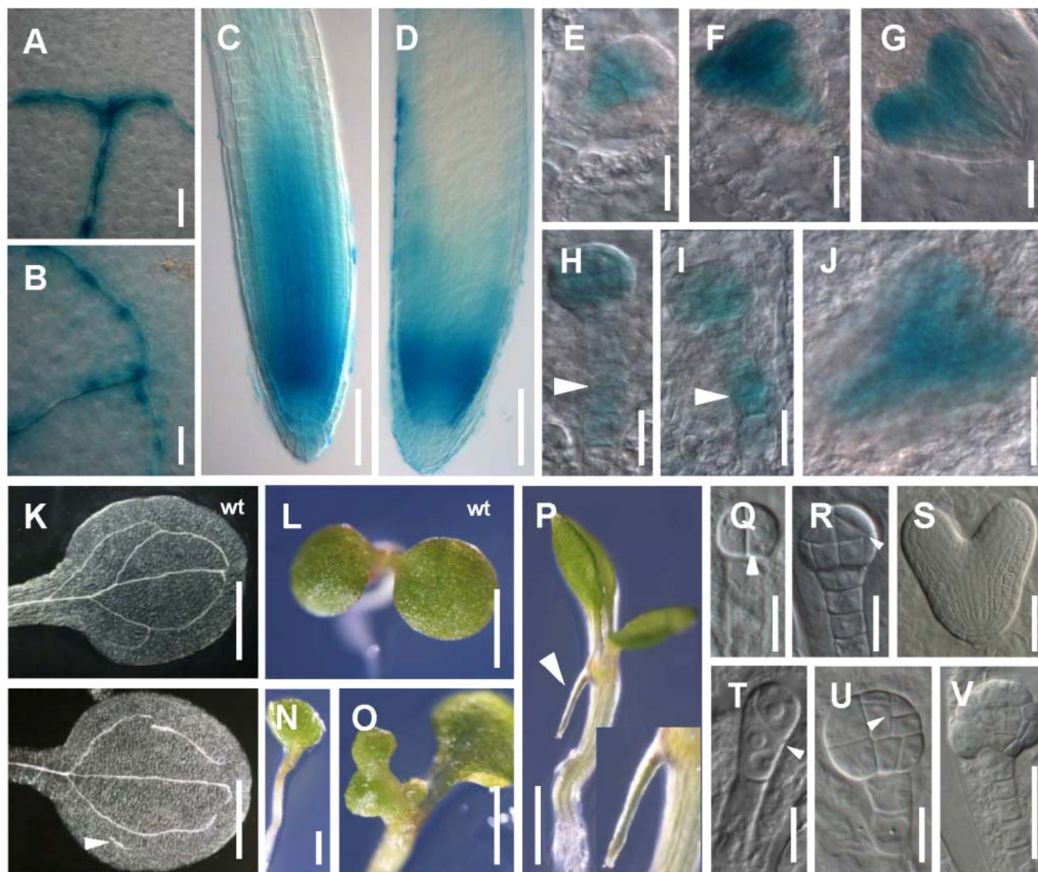


Figure 2. ***PIP5K1*** **and** ***PIP5K2*** **are expressed and required for multiple developmental processes.**
A-J. Expression pattern of *PIP5K1* (A, C, E-G) and *PIP5K2* (B, D, H-J) in cotyledon vascular tissues (A, B), primary roots (C, D) and embryo (E-J). The arrowhead indicates *PIP5K2* suspensor expression (H, I).
K-P. Morphological phenotypes of seedlings 7 DAG. *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$ shows open vascular loops and vascular islands (K) (Arrowhead indicates the vascular island). Instead of oval-shaped cotyledons (L), some double mutants show a misshapen and single cotyledon (N) or fused cotyledon phenotypes (O). In few extreme cases we found some root like structures emerging from the hypocotyl (P).
Q-S. Different stages of embryo development in WT: two-celled stage (Q), dermatogen stage (R) and torpedo stage (S); and in segregating *pip5k1*$^{-/-}$ *pip5k2*$^{+/-}$: two-celled stage (T), dermatogen stage (U) and arrested mutant embryo (V). Arrowheads indicate aberrant cell divisions. Bar sizes: A-D, J, V, S 50 µm; E-I 20 µm, K 500 µm; L-P 0.1 cm.

originate close to the shoot apical meristem,resembling mutants globally affected in auxin transport or signaling (Aida et al., 2004; Dhonukshe et al., 2008; Long et al., 2002). Because of the double mutant's inability to flower and to generate seeds (Figures S2H-S2J), we always needed to reidentify the double mutants in the segregating offspring of a self-pollinated *pip5k1*$^{-/-}$*pip5k2*$^{+/-}$ plant. We noticed that the double mutant phenotype in seedling was segregating at a proportion inferior to the expected 25% (χ_i^2=44,610, p <0,0001; Table S2)

Table 1. The *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$ mutant frequently has disconnected provascular fragments (VI), as well as a reduced complexity in the vascular tissues, i.e. reduced branching points, secondary veins and closed areoles

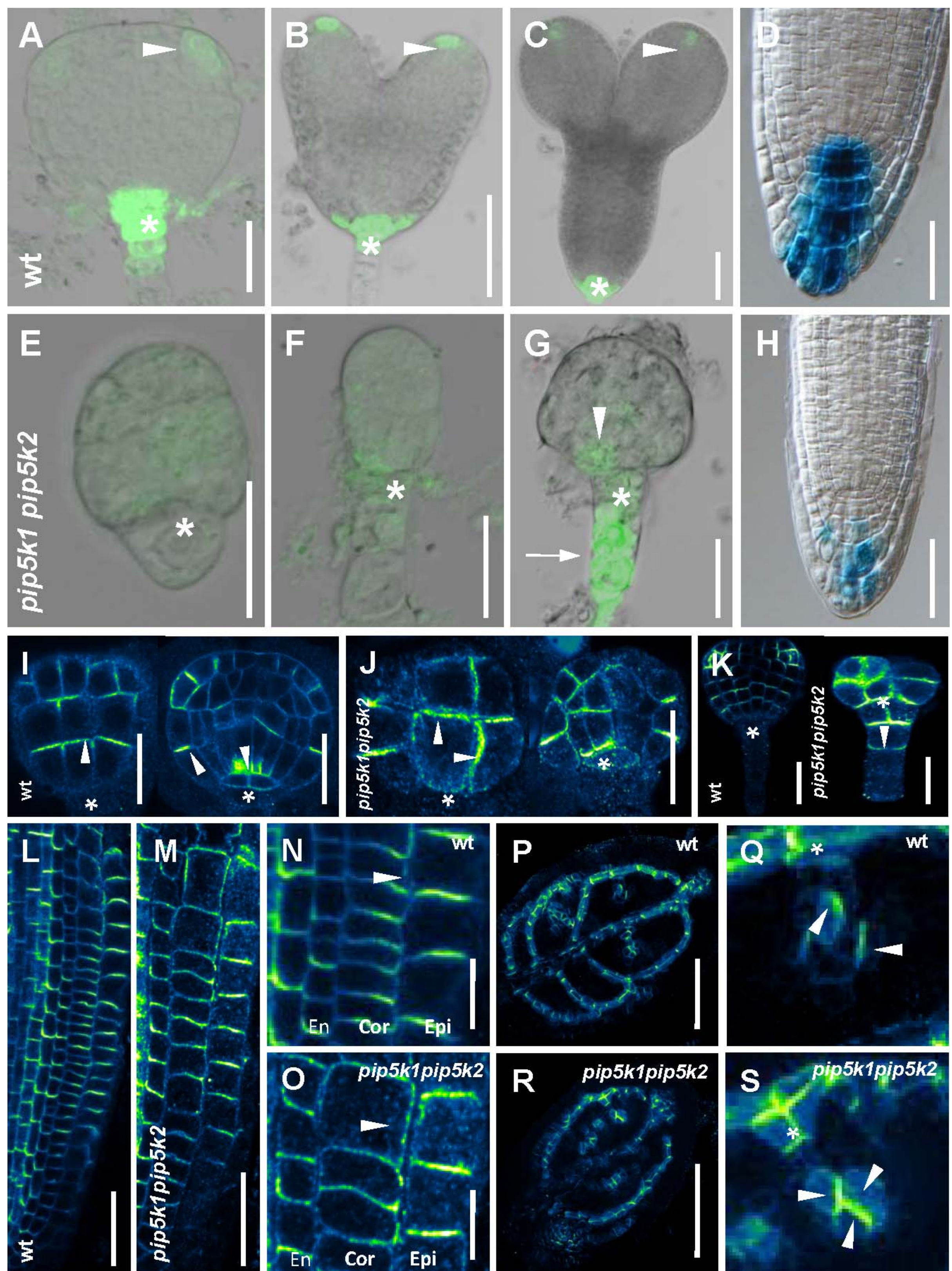
	VI (%)	**Free ends***	**Closed areoles***	**Branching points***	**Secondary veins***
wt	7.4	4.41 ± 1.2	5.55 ± 1.8	16.41 ± 2.7	6.74 ± 1.1
pip5k1$^{-/-}$*pip5k2*$^{-/-}$	72	5.64 ± 0.9	1.12 ± 0.5	3.68 ± 2.1	3.12 ± 0.9

Quantification of vascular development parameters in WT and *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$ double mutant was obtained from PIN1 immunolocalization in whole mount 7-9 DAG primary leaves. VI: Vascular Island. * Mean ± s.d. n ≥ 25.

suggesting, together with the observed cotyledon defects, a defect in embryogenesis. Therefore, we analyzed embryo morphology in siliques of *pip5k1*$^{-/-}$*pip5k2*$^{+/-}$ plants. In 12% of the embryos from the segregating population we found strong deviations from the normal developmental pattern (Figures 2Q-2V, Table S2). As early as the two-celled embryo, abnormal cell divisions could be observed (arrowhead in Figures 2T and 2U). Later in development, defects became more pronounced resulting in embryos with a not well-defined apical-basal boundary and embryos without any apparent apical-basal axis (Figure 2V). Because no aberrant embryos could be found at later stages, it seems that development of aberrant embryos arrested prior to the globular stage. These results demonstrate the fundamental requirement for PIP5K activity in various developmental processes, including those typically dependent on polarly localized PIN proteins and/or feedback regulation of PIN polarity, such as embryogenesis (Friml et al., 2003), specification of apical and basal organs identity (Dhonukshe et al., 2008), lateral root and cotyledon organogenesis (Benková et al., 2003; Okada et al., 1991), root meristem activity (Blilou et al., 2005) and vascular tissue formation (Scarpella et al., 2006).

***pip5k* mutants are defective in auxin distribution and PIN polarity**

Because many defects observed in the *pip5k* single and double mutants are reminiscent of defects in auxin distribution and signaling, we introduce the synthetic auxin response



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J
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L
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(previous page) **Figure 3. *pip5k1$^{-/-}$pip5k2$^{-/-}$*mutants have a reduced auxin response and PIN polarity defects.**

A-H. Auxin response maxima, visualized by DR5rev::GFP in WT (A-C) and *pip5k1$^{-/-}$ pip5k2$^{-/+}$* (E-G) embryos and by DR5:GUS in WT (D) and *pip5k1$^{-/-}$ pip5k2$^{-/-}$* (H) roots. The arrowheads indicate the DR5 maxima, the arrow indicates the ectopic DR5 response in the *pip5k1$^{-/-}$ pip5k2$^{-/-}$* suspensor (G), while the asterisks indicate the position of the hypophysis.

I-K. PIN1 immunolocalization in globular embryos of WT (I, surface medial views) and *pip5k1$^{-/-}$ pip5k2$^{-/-}$* embryos (J, surface and medial view). (K) In contrast to WT, *pip5k1$^{-/-}$ pip5k2$^{-/-}$* embryos displayed ectopic *PIN1* expression in the upper suspensor cells. Asterisks indicate the hypophysis and arrowheads indicate PIN polarity in I and J and ectopic PIN1expression in K.

L-O. Immunolocalization of PIN1 and PIN2 in WT (L, N) and p *pip5k1$^{-/-}$ pip5k2$^{-/-}$* (M, O) root tips, 7 DAG. In WT, stele, endodermis and epidermis cells show a clear basal or apical PIN localization. Young cortex cells have a basal PIN polarity, which shifts gradually to the apical cell side when cells mature. In *pip5k1$^{-/-}$ pip5k2$^{-/-}$* root tips, PIN2 accumulated abnormally in the lateral sides of mature cortex cells (O, arrowhead) En: endodermis; Cor: cortex; Epi: epidermis.

P-S. Immunolocalization of PIN1 in WT (P, Q) and *pip5k1$^{-/-}$ pip5k2$^{-/-}$* (R, S) primary leaves, 7-9 DAG. PIN1 shows a clear polarity in the developing vascular tissue toward the base of the leaf. Newly forming, not yet connected, provascular cells show PIN1 polarization toward existing vascular strands (Q). In *pip5k1$^{-/-}$ pip5k2$^{-/-}$*, PIN1 polarity does no longer polarize toward existing vascular strands (S). The arrowheads indicate PIN polarity and the asterisks indicate the existing/older vascular strand.Bar sizes: B, C, D-H 50 µm; A, E, F, G, I-K, N, O 20 µm.

reporter DR5 (Ulmasov et al., 1997) to indirectly visualize the auxin response distribution. During embryogenesis, DR5 maxima are typically observed near the future root pole, in the uppermost suspensor cell and in the hypophysis. Later they also appear in the incipient cotyledons and provascular strands (Figures 3A-3C; Friml et al., 2003; Benková et al., 2003). In contrast, mutant embryos from the *pip5k1$^{-/-}$pip5k2$^{+/-}$* population showed weak DR5rev::GFP activity throughout embryogenesis and often failed to establish localized auxin maxima (Figures 3E-3G). In some mutant embryos (16%, n = 30), ectopic DR5 maxima were seen in patches in the protodermal layer (Figure 3G, arrowhead) and high in the whole suspensor (6.6%, n = 30; arrow in Figure 3G). Postembryonically, in contrast to the typical DR5 staining in the central root meristem which can be broadened and increased by the polar auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) (Figure S3A; Friml et al., 2002; Moreno-Risueno et al, 2010; Sabatini et al., 1999), the root tips of *pip5k1$^{-/-}$pip5k2$^{-/-}$*mutants were devoid of

DR5::GUS (Figure 3H) activity and could not be rescued by NPA treatment (Figure S3B), suggestive of low auxin signaling levels in the *pip5k1*$^{-/-}$ *pip5k2*$^{-/-}$ root tips.

These data from both embryo and roots suggest that *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$mutants have defects in auxin distribution dependent on the activity and localization of the PIN efflux carriers. During wild-type embryogenesis, PIN1 localization is apical in the protodermal layer, gradually pointing toward the incipient cotyledons, meanwhile in the provascular tissues it is basally localized, directing the auxin fluxes to the forming root pole (Figure 3I; Friml et al., 2003). In contrast, in *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$embryos this polarity was severely compromised: PIN1 showed either an entirely apolar distribution or a rather uncoordinated, random polar distribution (100% of mutant embryos, n = 35; Figure 3J). Moreover, while in the wild type, *PIN1* expression was restricted to the apical cell lineage (proembryo), we regularly observed *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$embryos that showed *PIN1* expression also in suspensor cells (Figure 3K, arrowhead), confirming defects in cell specification along the apicalbasal axis, as suggested by the morphological analysis (Figures 3E-3G).

Postembryonically, in wild-type meristems, PIN2 is located apically in epidermis cells and displays a basal localization in young cortex cells. When cortex cells start to elongate and differentiate, their PIN2 localization becomes gradually more apical (Kleine-Vehn et al., 2008). In *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$seedlings that completed embryogenesis that and germinated, we observed an abnormally strong lateral signal in the cortex cells (Figures 3M and 3O), suggesting defects in PIN polarization also in the root meristem. As a typical example of the processes depending on feedback regulation of auxin transport, we chose to examine the development of vascular venation patterns (Berleth and Mattson 2000). In wild type, PIN1 in the newly forming vascular strand dynamically points basipetally and toward the previously formed vascular tissues (Figures 3P and 3Q;

Scarpella et al., 2006). However, in the *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$ mutant, this coordinated PIN1 polarization did not occur, failing to connect most of the vascular strands (Figures 2A and 3R, Table 1). This failure is reflected in the formation of vascular islands lacking a polarized PIN1 distribution (69%, n = 48; Figure 3S).

Together, these observations suggest that the defects of *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$ are linked to a defective differential auxin distribution and that PIP5K activity is required for dynamic PIN polarization during different processes in embryonic and post-embryonic development.

PtdIns4-P, PtdIns(4,5)P_2 and PIP5K are polarly localized

Our analysis shows that *PIP5K1* expression is required for regulation of PIN polarity, auxin distribution and downstream developmental processes. The PIP5Ks produce PtdIns(4,5)P_2 by phosphorylating PtdIns4-P in the position 5 of the inositol ring. To get some insights in the localization of these two phosphoinositides in roots we used fluorescent markers based on the binding specificity of different pleckstrin homology domains (PH) of two animal proteins: the PH domain of the phospholipase C1 (PH-PLCδ1-YFP) specifically binds to PtdIns(4,5)P_2, while the PH of the human FAPP1 (PH-FAPP1-YFP) binds to PtdIns4-P (van Leeuwen et al., 2007; Vermeer et al, 2009). In root tips of Arabidopsis lines expressing PH-PLCδ1-YFP, the fluorescence was detected at the PM where it preferentially accumulated at the apical and basal polar domains of cells (Figure 4A). The PtdIns4-P marker PH-FAPP1-YFP also localized preferentially to the PM at apical and basal cell sides (Figure 4B), and to some endomembranevesicules proposed to be part of the transGolgi network (Vermeer et al, 2009). Both phosphoinositide markers colocalize with PIN2 at the PM in epidermal cells (Figure 4C and 4D). This phosphoinositide localization pattern at the apical basal PM was confirmed

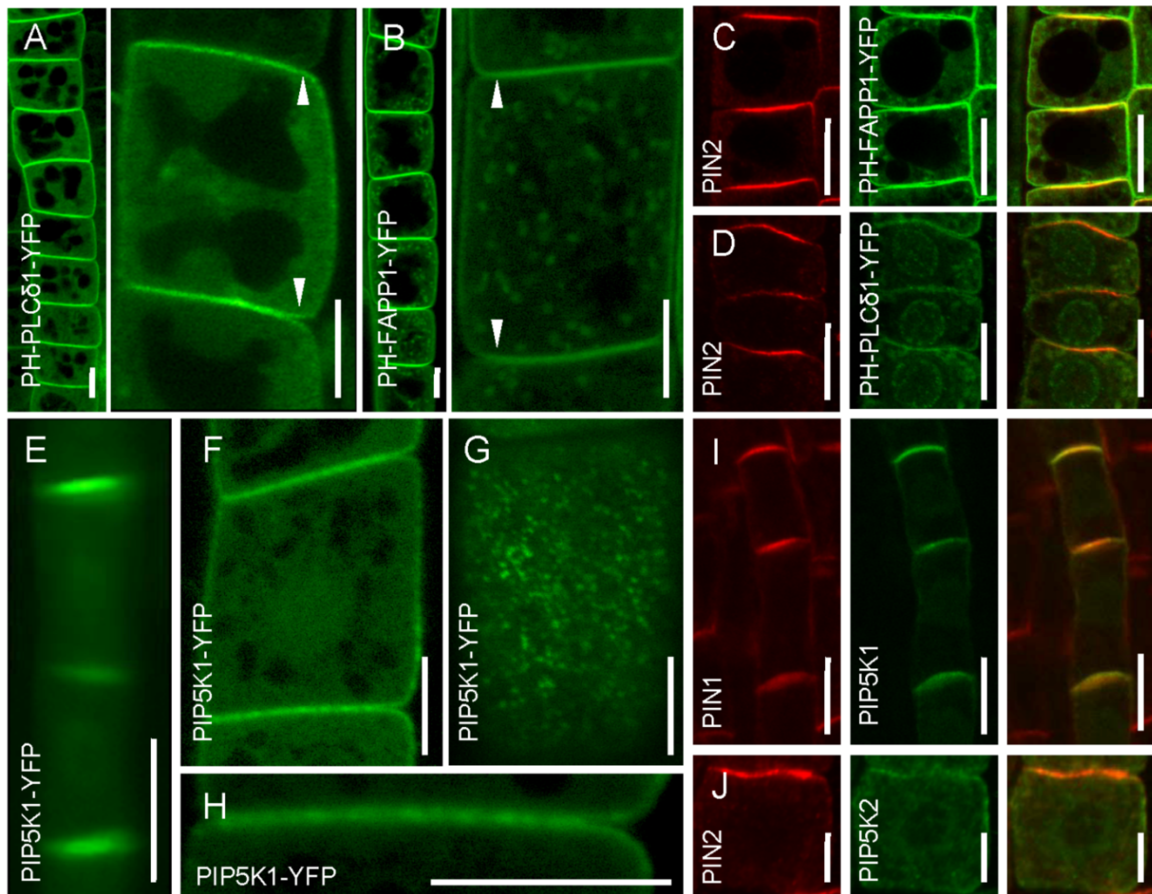

Figure 4. Phosphoinositides and PIP5K polar localization in root cells.
A, B. In vivo visualisation of PtdIns(4,5)P2 and PtIns4-P via biosensor marker lines 35S::YFP-PH-PLCδ1 (A) and 35S::YFP-PH-FAPP1 (B). 35S::YFP-PH-PLCδ1 fluorescence was enriched at the apical and basal sides of the PM (A), while the PtdIns4-P marker 35S::YFP-PH-FAPP1 was enriched at the PM (B), and in some internal vesicles.
C, D Colocalization between PIN2 and YFP-PH-FAPP1 (C) and YFP-PH-PLCδ1 (D) in root epidermal cells.
E-H. Live imaging of inducible YFP-PIP5K1 in procambial (E) and epidermal cells (F-H)
(F, G) Colocalization between PIN1 (red) and YFP-PIP5K1 (green) procambial cells (I) and YFP-PIP5K2 and PIN2 in epidermal cells (J). Bar sizes: A, B, J, H 10 µm; C, D, I, J, E 20 µm
I, J. PIP5K1 colocalizes with PIN1 in procambial cells (I) and PIN2 colocalizes with PIP5K2 in epidermal cells (J). Immunolocalizations using anti PIN1 and anti PIN2 antibodies (I, J, respectively, red pictures) and anti GFP antibodies in the bacground of inducible lines XVE>YFP-PIP5K1 (I) and XVE>YFP-PIP5K2 (I, J, respectively, green pictures)

using antibodies raised against liposomes enriched in these kind of phospholipids (Figure S4).

The enrichment of phosphoinositides at certain PM domains is achieved by the local synthesis or degradation by phosphatases, phospholipases and kinases, and they function as site-specific signals on the membranes to regulate trafficking or signaling processes. Then we tested if the polar localization of PtdIns 4-P and PtdIns(4,5)P_2 are correlated

with the localization of PIP5K using a translational fusion to YFP under the estradiol inducible promoter. After overnight induction, PIP5K1 and PIP5K2 could be found most prominently at the PM, enriched at the apical and basal membranes in procambial (Figure 4E) and epidermal cells (Figure 4F) in some cluster-like agglomerations (Figure 4G and 4H). Moreover, PIP5K1 and PIP5K2 colocalized with basal PIN1 in the stele and apical PIN2 in the epidermis (Figure 4I and 4J).

These results suggest that PMs have a higher PtdIns(4,5)P_2 content at apical and basal cell sides than at lateral cell sides which coincides with the localization of PIP5K and PIN, implicating a role of PtdIns(4,5)P_2 and PIP5K function in cell polarity.

Auxin modulates cellular phosphoinositide content and distribution

As we identified *PIP5K1* as an auxininducible gene in the context of PIN polarity regulation, it is tempting to speculate that auxin could, via regulating the expression of *PIP5K*, change the ratio of different phosphoinositides in cells and thus modulate trafficking processes, including polar targeting of PIN proteins.

To test this hypothesis, we analyzed the auxin effect on PtdIns(4,5)P_2 levels and the subcellular distribution of phosphoinositides. We treated wild-type seedlings with auxin and quantified the total PtdIns(4,5)P_2 content. Upon treatment with 10 μM IAA, we observed a transient increase in PtdIns(4,5)P_2 after 1 h (Figure 5A). When challenged with 0.1 μM IAA, an increase after 1 h was also observed (Figure 5A). Next we adressed the possibility that auxin might be changing specifically the phosphoinositide content at the PM using the PtdIns markers (Figure 4). We treated these lines with auxin for different time periods and quantified the fluorescence intensities at the PM. The fluorescence of the PtdIns(4,5)P_2 marker, PH-PLCδ1-YFP, as well as that of the PtIns4-P marker, PH-FAPP1-YFP, was not changed within 1 hour of NAA treatment. However, already after 2 hours of NAA treatment, the PH-PLCδ1-YFP signal was significantly

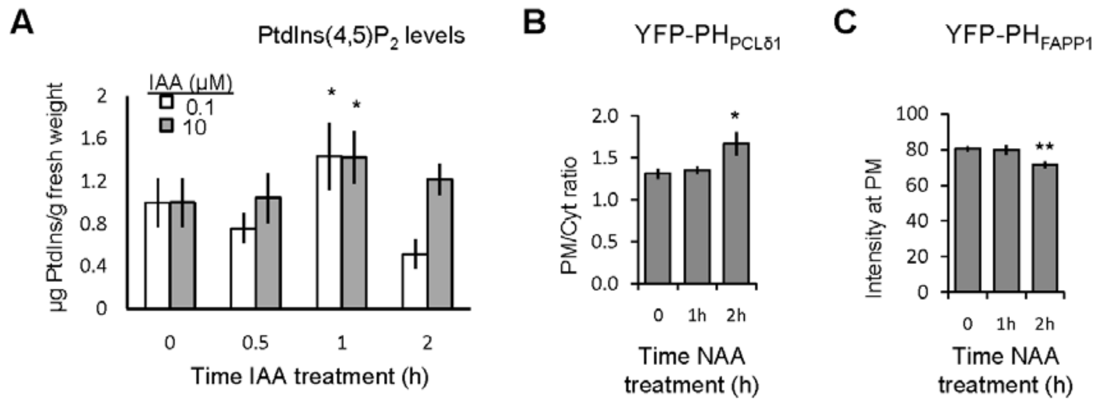

Figure 5. Auxin affects phosphatidylinositol pools at the PM.
(A) Total PtIns(4,5)P_2 quantification in seedlings after treatment with 10 or 0,1 µM auxin, 5 DAG.
(B, C) Quantification of the effect of auxin on PtdIns(4,5)P_2 (B) and PtdIns4-P (C) as visualized by the PtdIns markers (Figures 4A and 4B). Seedlings were treated for 2 hours with 10 µM NAA, imaged in the confocal microscope and the fluorescence intensity at the PM was measured. For 35S:YFP-PH-PLCδ1, the fluorescence at the apical side of the PM was quantified relative to the internal fluorescent signal per cell. For 35S:YFP-PH-FAPP1,only the apical/basal PM intensity was measured. Two tailed student test, * $p < 0.05$, ** $p < 0.01$.

increased (Figure 5B) and PH-FAPP1-YFP fluorescence at the PM was significantly reduced (Figure 5C), suggesting that auxin decreases PtdIns4-P (a PIP5K substrate) and increases PtdIns(4,5)P_2 (a PIP5K product) at the PM. While the data obtained by two very different experimental approaches suggest increased levels of PtdIns(4,5)P_2, it must be noted that the timing of PtdIns(4,5)P_2changes observed by monitoring fluorescence reporters in intact Arabidopsis roots cannot directly be compared with the biochemical data from seedlings challenged with exogenous IAA on agar plates.

These combined results show that auxin can regulate the levels of PtdIns(4,5)P_2 and other phosphoinositides at the PM, presumably via regulation of *PIP5K* expression, activity and localization, and moreover suggest the existence of functionaly different phosphoinositides pools.

DISCUSSION

Our results reveal a role for phosphoinositides in cell polarity and in auxin-mediated cell and tissue polarization in plants. We have identified PIP5K1 and its homolog PIP5K2 as

downstream effectors of the SCF^{TIR1}-Aux/IAA-ARF signaling pathway and essential factors for polar localization of PIN auxin transporters. Auxin can affect the global leves of PtdIns(4,5)P_2 and in particular those at the PM. We describe that *pip5k1pip5k2* double mutants have phenotypes typical of mutants affecting polar auxin transport and auxin signaling and could link those to aberrant DR5 maxima and PIN polarity defects. We show that PtdIns4-P and PtdIns(4,5)P_2 as well as PIP5K1 and PIP5K2 are asymetricaly distributed at the axial side at the PM where they presumably afect PIN polarity.

In animal system, polarized cell types use phophoinositide asymmetry at their plasma membrane as a way to establish and mantain their polar constitution (Shewan et al., 2011). PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are key determinants of the apical baso-lateral membrane specification in animal ephitelia (Gassama-Diagne et al., 2006; Martin-Belmonte et al., 2007). PtdIns(3,4,5)P_3 is enriched at the basolateral domain in Madin-Darby canine kidney (MDCK) cells and excluded from the apical plasma membrane domain where the PtdIns(3,4,5)P_3 3-PPtase PTEN mediate the enrichment of PtdIns(4,5)P_2 at this domain. The change in phosphoinositides composition is sufficient to change the identity of the plasma membrane. Taken toghether these studies highlight a critical function of phosphoinositides compartimentalization in generating spatial cues important for polarity generation. It is not clear yet to which extent the mechanism described above for epithelia polarity can be extrapolated to plant systems. Yet, the strong localization of PtdIns(4,5)P_2 in the apical domain of tip growing cells (e.g. root hairs and pollen tubes, van Leeuwen et al., 2007; Braun et al., 1999; Dowd et al., 2006; Helling et al., 2006; Kost et al., 1999; Stenzel et al., 2008) suggests that it might act as a signal for cellular polar domains also in plant cells.

In human cells, PtdIns(4,5)P_2 is involved in many critical cellular functions, such as cytoskeletal attachment, endocytosis and exocytosis (McLaughlin et al., 2002). the

importance of PtdIns4-P and PtdIns(4,5)P_2, as well as different PIP5Ks, both in the control of secretion (Di Paolo et al, 2004) and in clathrin-mediated endocytosis (Antonescu et al., 2011; Jost et al., 1998), is established. In plants, several PIP5Ks have also been implicated in exocytosis (Ischebeck et al., 2008 and 2010), as well as endocytosis and membrane recycling (Mei et al., 2012; Sousa et al., 2008; Zhao et al., 2010; König et al., 2008). The fact that auxin can modulate PtdIns(4,5)P_2 content at the PM is suggestive of an auxin-induced reprogramming of or a positive effect on mantaining cellular polarity via PtdIns(4,5)P_2 metabolism. This strongly favors a scenario in which auxin, by controlling transcription of *PIP5K*, modulates PtdIns(4,5)P_2 levels at the PM and thus PIN polar localization in individual cells, eventually leading to coordinated polarized development. PIN polarity is highly dependent on various trafficking processes (Geldner et al., 2001; Dhonukshe et al., 2007 and 2008; Kitakura et al., 2011), however, at which level PIP5K1/2 mediates PIN polarity is not established in the present work. However, at which step(s) of trafficking pathways in plants or in animals PtdIns(4,5)P_2 acts to influence cell polarity remains to be elucidated.

Our findings suggest that the set of molecules that govern polarity are not that different between plants and animals, despite the fundamental differences in how polarity is established and used in development. The regulatory input of the plant hormone auxin into these processes would then represent the plant-specific evolutionary innovation to mediate selforganizing, coordinated polarity rearrangements that are typical for plant development.

ACCESSION NUMBERS

Raw microarray data from this article can be found in the EMBL ArrayExpress repository under accession number E-MEXP-3283. ATIs: AT1g21980 (PIP5K1); AT1g77740 (PIP5K2).

MATERIAL AND METHODS

Plant material and growth conditions

All lines are in the Columbia background of *Arabidopsis thaliana*. The lines *pip5k1* and *pip5k2* are SALK insertional mutants SALK_146728 and SALK_012487, respectively, and were obtained from the ABRC; *arf7 arf19* double mutant as published before (Okushima et al., 2005). The marker lines DR5::GUS (Ulmasov et al., 1997), DR5rev::GFP (Friml et al., 2003), 35S:YFP-PH-PLCδ1 (van Leeuwen et al., 2007) and 35S:YFP-PH-FAPP1 (Vermeer et al., 2009)were obtained from the respective authors and introgressed (when appropriate) into the double mutant *pip5k1pip5k2* by crossing.

Seeds were sterilized overnight by chlorine gas, sown on solid Arabidopsis medium (0.5X Murashige and Skoog (MS) basal salts, 1% sucrose and 0.8% agar, pH 5.9) and stratified at 4°C for at least two days prior to transfer to a 16:8 light:dark 18°C growth regime. The seedlings were grown vertically for 4 to 12 days prior to analysis.

Microarray experiment

Seedlings of Col-0 and *HS::axr3-1* were grown for 5 days and the plates were then exposed to 37ºC for 40 minutes and allowed to recover for 1.5 hours in normal growth conditions, then the seedlings were transferred to liquid culture medium supplemented with 10 µM NAA or the equivalent amount of DMSO as control for 4 hours. The lower third of 100-130 roots per biological replicate was cut and total RNA extracted with a quiagen RNAeasy mini kit. RNA was hybridized to Affymetrix ATH1 microarrays according to the manufacturer´s instructions. Raw data was pairwise analyzed using the logit-t Algorithm (Lemon et al., 2003)with a cutoff of p=0.05.

Immunolocalization, GUS staining and live cell imaging

Primary root, embryo and leaf vasculature PIN immunolocalizations were performed as described by Sauer et al., 2006. In all cases the antiPIN1 antibody was diluted 1 to 1000,

and the secondary antibody anti rabbit coupled to Cy3 was diluted 1 to 600. GUS staining was done as published before (Swarup et al., 2008). The root tips were mounted in chloral hydrate and visualized using the DIC microscope Olympus BX51. For live imaging of the phosphatidylinositol markers, seedlings 4 DAG were mounted in a drop of Arabidopsis media and visualized immediately. All the confocal pictures were taken with the confocal microscope Zeiss CSLM 710. The measurements in the pictures were done using the ImageJ software.

Auxin treatments and PtdIns(4,5)P_2 analysis

Wild-type (Col-0) plants were grown on vertical petri dishes on solid medium containing halfconcentrated MSsalts and 1% (w/w) sucrose continuously illuminated by 7,000 lux. Five-day-old seedlings were transferred to solid media additionally containing 0.1 µM IAA or 10 µM IAA and incubated for different time periods. For each time point, 50 - 60 seedlings were harvested and the total fresh weight was determined. PtdIns(4,5)P_2 was extracted and analyzed as previously described by König et al. (2008).

The phosphoinositide changes at plasma membrane upon auxin treatments was done as follow. Seedlings 4 days after germination were incubated for the indicated times in liquid AM containing 10 µM NAA adn inmidiatly imaged under the confocal microscope using the same settings for all pictures. The pictures were then measured using ImageJ software. For the PtdIns4-P marker, the PM intensity was measured using a 3 pixels wide line covering the whole axial membrane of epidermal cells. The PtdIns(4,5)P_2 marker is partially silenced in roots so the measurement was a bit tricky. All the pictures were collected and a ratio between the PM intensity (measured as described before) and the internal citoplasmic fluorescence intensity (measured using a small circle covering an area with even fluorescence. Like this, we normalize the PM intensity by the internal signal. Then for both phosphoinositide markers, all cells in one root were pooled to

calculate the average per root and then the mean of ten roots was calculated for each time point. The experiment was done in three biological replicates.

Transcript level analysis

Total RNA was extracted with the RNeasy Mini kit (Qiagen, Venlo, The Netherlands). For the quantitative RT-PCR, poly(dT) cDNA was prepared from 1 µg total RNA with an iScript cDNA synthesis kit (Biorad) and quantified on a LightCYCler 480 (Roche) with the qPCR LightCYCler 480 SYBR Green I Master (Roche). PCR was performed on 384well reaction plates, which were heated for 10 min to 95°C, followed by 45 cycles of denaturation for 10 s at 95°C and annealing and extension for 15 s at 60°C and 72°C, respectively. Target quantifications were performed with specific primer pairs designed with the Beacon Designer 4.0 (Premier Biosoft International). Expression levels were normalized to CBP20 (AT5g44200) and eIF4A (AT1g54270) which showed constitutive expression across samples. All PCRs were done in three technical repeats and the data were processed using qBase v1.3.4 (Hellemans et al., 2007). The primers used are listed in Table S3.

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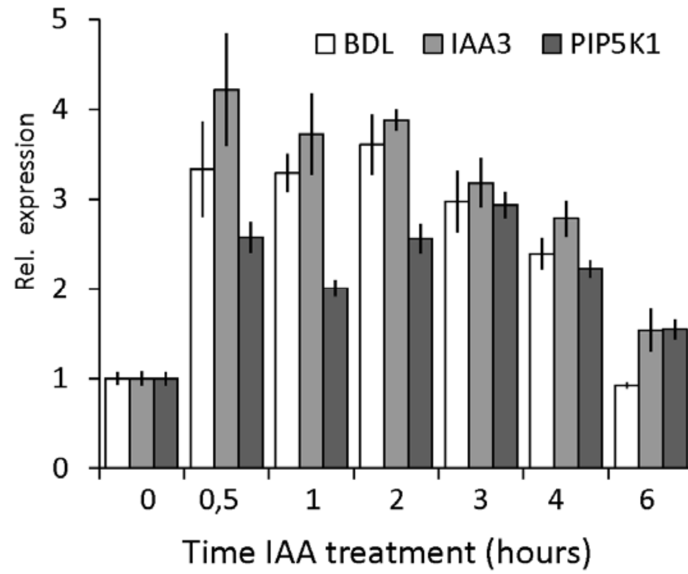

Figure S1. PIP5K1 transcription is auxin regulated.
Time-course of auxin-regulated (10 µM IAA) expression levels of early auxin responsive genes (BDL/IAA12, SHY2/IAA3) and PIP5K1 in roots of 10 day old seedlings, determined by Q-RT-PCR.

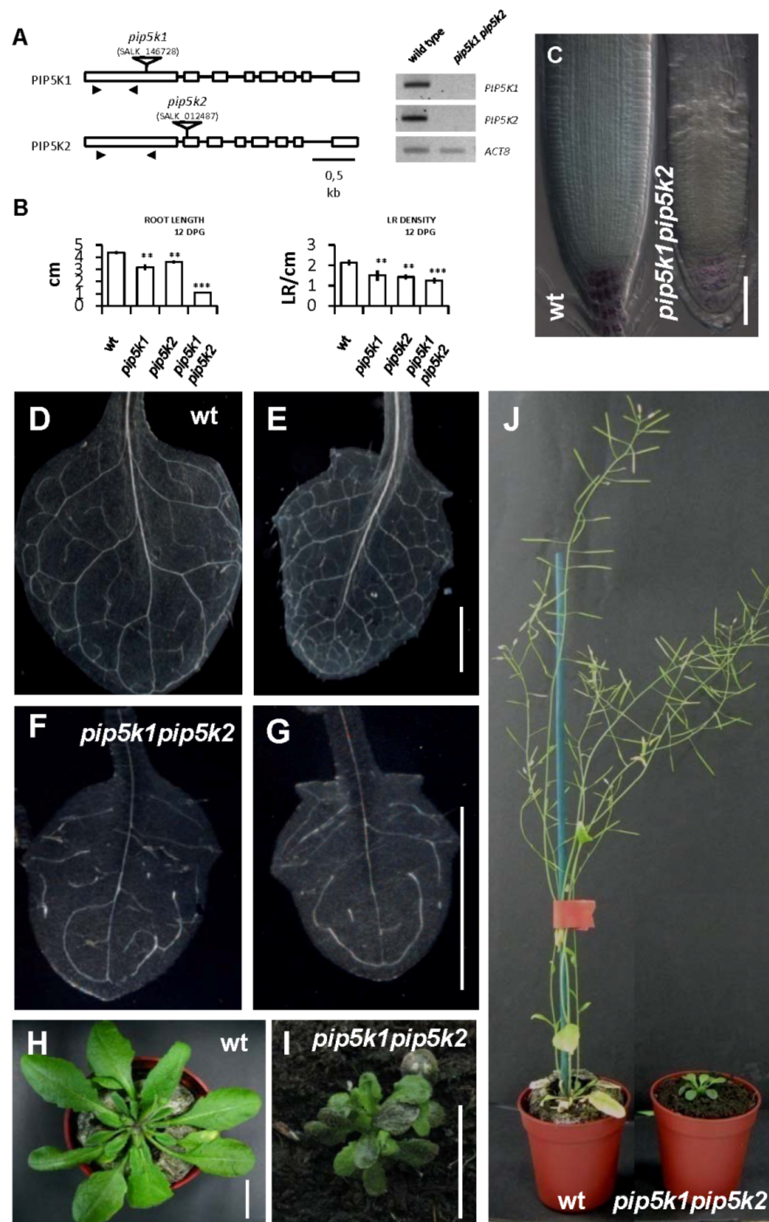

Figure S2. Phenotypes of knock out *pip5k1* and *pip5k2* mutats

A. Intron -exon structure of PIP5K1 and PIP5K2 genes with indication of the position of the T-DNA insertion sites (Exons indicated as boxes, introns as lines). The gel pictures show that no cDNA can be amplified from pip5k1−/− pip5k2 −/+ double mutants, suggesting that these mutant alleles represent full knock-outs. ACTIN8 (ACT8) was used as a constitutive expressed gene control. The arrowheads indicate the positions of the primers used for the analysis.

B. Root length and lateral root density in single and double mutant seedlings 12 DAG. Graphs show Mean ± s.d.. The asterisks indicate significant differences compared to wild type. Two tailed Student's t-test; ** $p < 0,05$, *** $p < 0,001$. $n > 20$.

C. *pip5k1 pip5k2* root apical meristems are smaller and have reduced starch staining in the columella than wild type.

D-G. Vascular tissues in leaves from 2 week old plants in wild type (D, E) and *pip5k1 pip5k2* mutant (F, G).

H-J. Wild type (H, J right) and *pip5k1 pip5k2* (I, J left) adult plants. Bar sizes: C 50 µm; H, I 1 cm

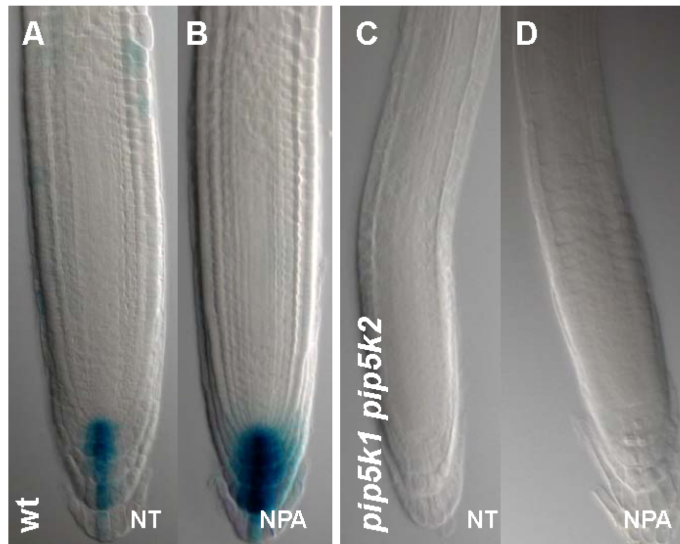

Figure S3. Auxin response in *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$roots is not rescued by inhibition of polar auxin transport by naphthylphthalamic acid (NPA).

A-B. Seven days old wild type (A, B) and *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$ (C, D) seedlings were incubated for 2 hours in 20 µM NPA (B, D) or mock treatment (A, C) and the auxin response was monitored by DR5-GUS.

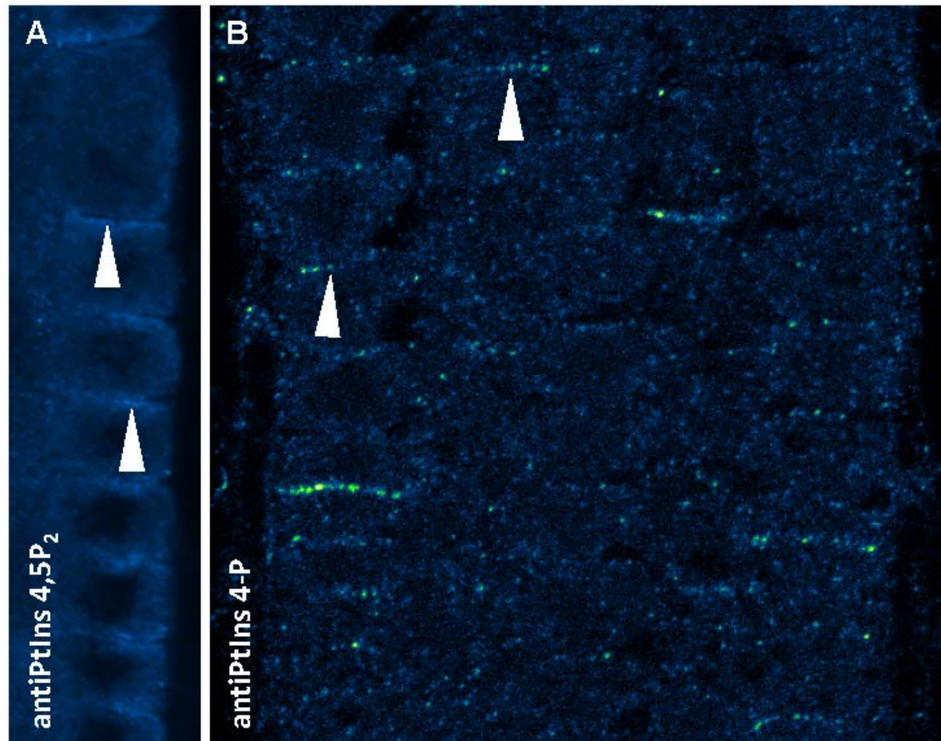

Figure S4. Phosphoinositide immunolocalization in root tips.

A-B. Phosphoinositides were detected in root tips in wild type seedlings seven days after germination. Both antibodies recognizing PtdIns(4,5)P2 (A) and PtdIns4-P (B) label axial plasme membranes in epidermal cells (arrowheads)

Supplementary Table S2. Mutant phenotype segregation in seedlings and embryo in wild type and *pip5k1*$^{-/-}$ *pip5k2*$^{+/-}$ double mutant.

GENOTYPE	PHENOTYPE							
	SEEDLING				EMBRYO			
	observed		expected*		observed		expected*	
	wild type	mutant	wild type	mutant	wild type	mutant	wild type	mutant
wt	200 (100%)	0 (0%)	200 (100%)	0 (0%)	150 (100%)	0 (0%)	150 (100%)	0 (0%)
pip5k1$^{-/-}$*pip5k2*$^{+/-}$	162 (82.2%)	35 (17.8%)	150 (75%)	50 (25%)	106 (88%)	15 (12%)	112.5 (75%)	37.5 (25%)

* Expected phenotypes under the assumptions of a full mutant phenotype penetrance, no gametophytic defects, and a Mendelian segregation.

Supplementary Table S3. List of primers used for transcripts level analysis

Gene	Sequence	Primer Orientation	Application
EIF4A	ACGGAGACATGGACCAGAAC	Fw	qPCR
EIF4A	GCTGAGTTGGGAGATCGAAG	Rv	qPCR
CBP20	GATTACGGTACTGGCTCATTGG	Fw	qPCR
CBP20	GATTACGGTACTGGCTCATTGG	Rv	qPCR
PIP5K1	GGAACATTGTGAATCGAGGACTG	Fw	qPCR
PIP5K2	CCGTCTCGTCTCTCTACTTCTT	Rv	qPCR
IAA3	CAACCCAAGCACAGACAGAG	Fw	qPCR
IAA3	TGATTGGATGCTCATTGGTG	Rv	qPCR
BDL	TTGGGTCTAAACGCTCTGCT	Fw	qPCR
BDL	AAGCCCTGAACTTTCGGATT	Rv	qPCR
ACTIN8	ACCTTGCTGGTCGTGACCTTACTG	Fw	RT-PCR
ACTIN8	GATCCCGTCATGGAAACGATGTCTC	Rv	RT-PCR
PIP5K1	ATGAGTGATTCAGAAGAAG	Fw	RT-PCR
PIP5K1	ATCACACCACTACGCCACTCT	Rv	RT-PCR
PIP5K2	ATGATGCGTGAACCGCTTG	Fw	RT-PCR

Chapter 3

Phosphatidylinositol 4-phosphate 5-kinase function during reproductive development

Ricardo Tejos, José Manuel Ugalde, Jiří Friml and Gabriel León

SUMMARY

Pollen develop after meiosis in anther pollen mother cells. A series of complex structural changes follows and generates mature pollen grains that will be able to perform their task during ovule fertilization. Several signaling pathways have been involved in the regulation and appropriate control of the different processes during pollen development. Auxin and phosphoinositides are the major signaling pathways controlling pollen formation. While auxin has been characterized as important in the synchronization of pollen maturation with flower development, which is essential for proper autofertilization in Arabidopsis, phosphatidylinositol synthesis has proven functions during microgametogenesis and pollen tube elongation. Here, we characterize PtdIns(4,5)P_2 synthesis by phosphatidylinositol 4-phophate 5-kinase (PIP5K) as a downstream component of auxin signaling, important for early pollen development. We show that *PIP5K1* and *PIP5K2* are auxin inducible genes in pollen. A double knockout mutant *pip5k1pip5k2* shows male gametophytic lethality that we could trace back to the tetrad stage . We also observed defects in vacuole formation during later stages of pollen maturation. These results implicate auxin signaling as an important cue for early developmental stages and vacuole formation during late microgametogenesis.

The gametophytic part of the angiosperm life cycle is represented by only a few cells formed during gametogenesis in specialized organs within the flowers. The male gametophytes emerge after meiosis of a diploid pollen mother cell within the anthers, generating a tetrad of haploid cells. All these cells are then released as free microspores and undergo a series of complex processes that finally produce the mature pollen grains (MPGs). In *Arabidopsis thaliana* a MPG consists of two generative cells encapsulated in the cytoplasm of a larger vegetative cell (Figure 1). While the last one contributes to the pollen survival and pollen tube formation, the two smaller germline cells will participate in ovule fertilization and produce the zygote and the surrounding endosperm and seed tissues (Hamamura et al., 2012).

Phosphatidylinositol (PtdIns) are important structural lipids present in all eukariots. PtdIns can be phosphorylated at different positions at the cytosol-facing inositol ring to form a versatile group of negatively charged lipids, collectively called phosphoinositides. Phosphoinositides participate in a variety of signaling cascades during stress responses and plant development (Meijer and Munnik 2003; Xue et al., 2009; Chapter 2). Phosphoinositides regulate pollen function at two levels: during early pollen formation and at later stages of pollen tube elongation (Figure 1, Table S1). In particular, PtdIns3-P/PtdIns(3,5)P_2 homeostasis regulated by the PtdIns 3-kinase AtVPS34 (Lee et al., 2008a) and the PtdIns3-P 5-kinases FAB1A/B (Whitley et al., 2009) had been shown to be crucial in maintaining vacuole function trough pollen development. Moreover, the modulation of PtdIns3-P contents in endosomal compartments by the lipid phosphatase PTEN1 is crucial for later stages of pollen development (Gupta et al., 2002) and proper autophagy and tube elongation in pollen tubes (Zhang et al., 2011). The other phosphoinositide specie important for pollen function is PtdIns(4,5)P_2, generated by the phosphatidylinositol 4-phosphate 5 kinases (PIP5K). Out of the eleven genes codifing

PIP5K, five are pollen-specific and only three of them are not expressed in pollen. PIP5K4, 5, 6, 10, and 11 are localized in a subapical region in elongating tubes and they regulate tip-focused secretion by modulating clathrin-mediated endocytosis and exocytosis of cell wall material (Ischebeck et al., 2008; Zhao et al., 2010). Knockout *pip5k* mutants or PIP5K overexpression generate defects in pollen tube growth and morphology as well as in pollen germination and fertilization defects.

Auxin has been indicated as a master regulator for tissue patterning and polarity generation in numerous aspects of plant development (Grunewald and Friml 2010), including flower and pollen development (Sundberg and Østergaard 2009). As soon as the floral meristem primordia are getting established in the shoot apical meristem (SAM), cycles of auxin maxima and minima are involved in the formation of the floral organs which depend on PIN1-driven polar auxin transport (Heisler et al., 2005; Reinhardt et al., 2003; Heisler et al., 2005). In later stages, auxin contributes to several other aspects of flower development like anther dehiscence, anther filament elongation and pollen maturation (Feng et al., 2006; Cecchetti et al., 2008; Ishiguro et al., 2001; Nagpal et al., 2005). Although components of auxin synthesis, homeostasis and auxin response (Feng et al., 2006; Cecchetti et al., 2008; Ding et al., 2012) are transcribed in several steps during pollen development, neither the role of auxin signaling, nor the downstream components of the anther-derived or pollen-produced auxin, are fully understood, specially for early developmental stages in male gametogenesis.

Here, we present evidence linking auxin signaling to the production of PtdIns(4,5)P_2 during early pollen development. We show that *PIP5K1* and *PIP5K2* are auxin inducible genes during pollen development. The double null mutant *pip5k1pip5k2* displays defects during early microgametogenesis which generate aborted pollen grains. In addition, we observed that a proportion of pollen grains from the *pip5k1pip5k2* double mutant proceed

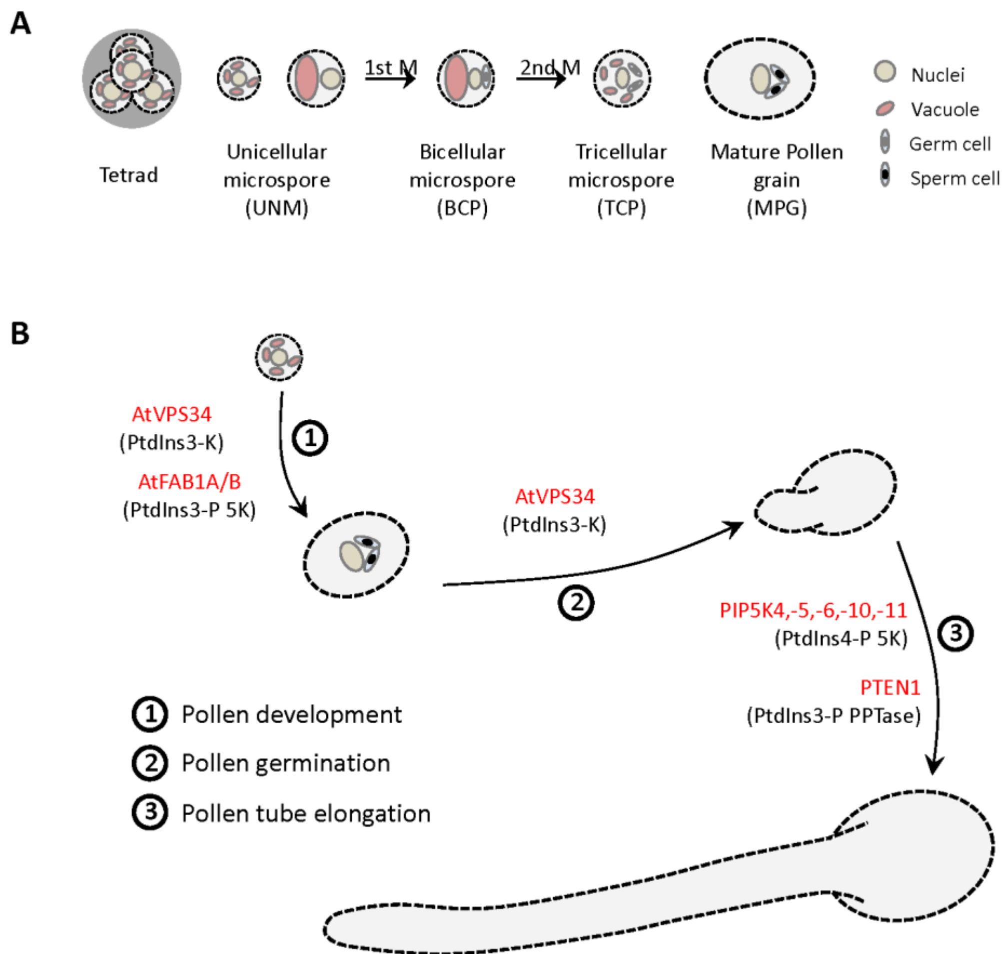

Figure 1. Pollen development.
A. A tetrad of haploid cells is formed after a meiotic division of pollen mother cells in anther pollen sacs. These haploid unicellular microspores (UNMs) follow a stage of vegetative growth driven by the growth of the central vacuole. The first mitotic division (1st M) generates a small germ cell which is encapsulated in the cytoplasm of the bigger vegetative cell (bicellular pollen, BCP). After this stage or in the process following the first mitotic division, the unique central vacuole is divided in smaller vacuoles, which will stay small in size but will change their fate during the rest of pollen development (for details, see Yamamoto et al., 2003). A second mitotic division (2nd M) will divide the germ cell to generate two sperm cells that will further maturate together with the vegetative cell to form the mature pollen grain (MPG).
B. Phosphoinositide metabolizing enzyme functions during pollen development and pollen tube growth. Over the cycle of pollen development and pollen tube formation several kinases of phosphatidylinositols have been characterized as being involved in vacuole formation in pollen (1), in pollen germination (2) and in pollen tube elongation (3).

to late developmental stages and these show defects in vacuole formation and fail to germinate. We propose PIP5Ks as downstream targets of auxin signaling important for early pollen development, and as important players during vacuole morphogeneisis in later stages of pollen formation.

Table 1. Mutant phenotype segregation in the *pip5k1*$^{+/-}$*pip5k2*$^{+/-}$ double mutant

	EMBRYO@		SEEDS#			
	normal	**mutant**	**normal**	**aborted**	**%**	**Un Ov&**
wild type	199	0	45.4 ± 9.78	0.6 ± 0.70	1.3	4.2 ± 3.52
pip5k1$^{+/-}$*pip5k2*$^{+/-}$	111	17(13%)	11.9 ± 3.54	0.5 ± 1.08	4	27.9 ± 5.11

Average of seeds from 10 siliques (mean ± SD); @ Total amount of embryo counted from 14 siliques. + WT allele; − mutant allele, & Un Ov: Unfertilized Ovules, counted as aborted ovules within the siliques.

RESULTS

The *Arabidopsis thaliana* genome contains eleven genes that can be categorized as phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks). They can be divided in type A and B depending on the overall protein structure (Mueller-Roeber and Pical, 2002). We found PIP5K1 in a microarray approach designed to identify new regulators of cell polarity. Because a single null *pip5k1* mutant did not exhibit any evident defect, we created a double mutant with its closest homolog PIP5K2 (Chapter 2). We observed a variety of phenotypes appearing in the double heterozygous *pip5k1pip5k2* mutant which were explained by defects in PIN polarity establishment and hence auxin-dependent development (Chapter 2). We further showed that the double homozygous *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$ mutants, characterized as smaller seedlings with dark cotyledons or as aborted embryos, were segregating in a proportion inferior to the one expected in a double heterozygous mutant (Table 1). As well, besides the shown embryo lethality that could be spotted as white ovules or shrunk seeds in opened siliques, we observed aborted ovules indicative of a failure during the fertilization. Furthermore, as we did not observe any defect in ovule number nor during its development in *pip5k1*$^{+/-}$*pip5k2*$^{+/-}$ mutants, we decided looking in detail at pollen development.

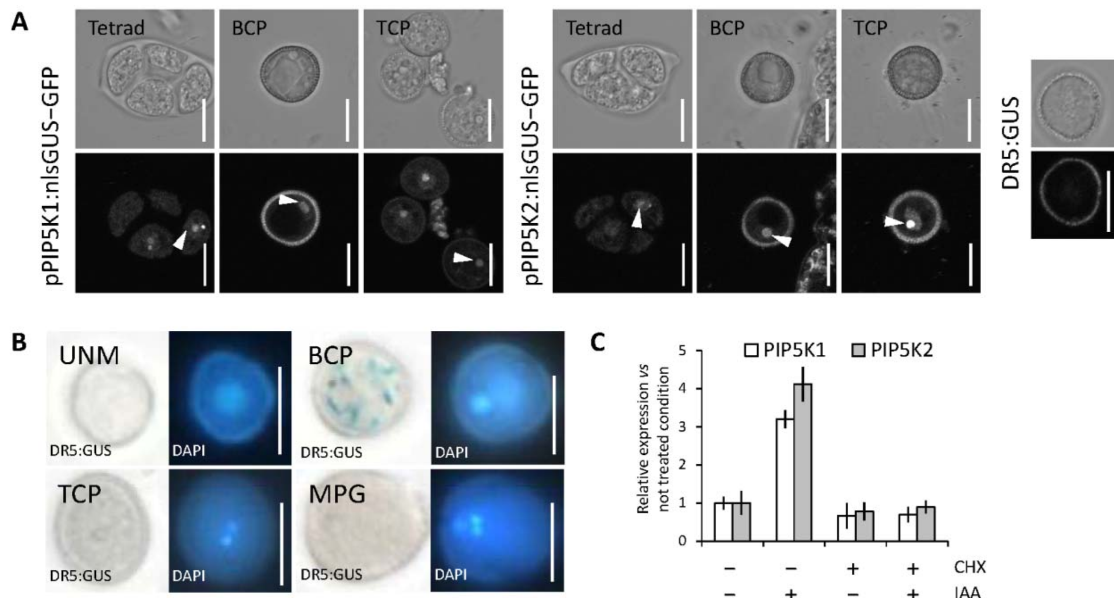

Figure 2. ***PIP5K1/2*** **are expressed early during pollen development and are auxin inducible genes.**
A. *PIP5K1* and *PIP5K2* are expressed early and during pollen development. 2000 base pairs upstream of the ATG start codon of *PIP5K1* and *PIP5K2* were fused to a nuclearly localized GUS-GFP chimera and pollen in tetrad, bicellular pollen (BCP) and tricellular pollen (TCP) stages were extracted from the respective plants and observed under confocal microscope. DR5:GUS pollen is shown as a control for pollen autofluorescence. Arrowheads indicate the GFP positive nuclei.
B. Auxin response during pollen development is higher in the BCP. Inset in each picture is a DAPI staining of each picture after staining for GUS activity.
C. Auxin response for PIP5K1 and PIP5K2 in pollen grains. Pollen was purified from open flowers and treated for 4 hours with 10 µM indole 3-acetic acid (IAA) with or without the pre-incubation of 50 µM cycloheximide (CHX). The mean ± SE of three independent experiments is indicated. Bar size 10 µm.

PIP5K1 and *PIP5K2* are auxin-regulated genes in pollen

We looked for *PIP5K* expression during pollen development using available microarray data (Honys and Twell 2004). From the eleven PIP5Ks, only *PIP5K3*, *PIP5K7* and *PIP5K9* are not expressed in pollen (Table S2). *PIP5K1, PIP5K2* and *PIP5K11* are expressed earlier in microgametogenesis. *PIP5K1* appears to be expressed only in bicellular pollen (BCP) and it is also expressed together with *PIP5K2* in unicellular microspores (UNMs) (Table S2). The pollen expression for *PIP5K1* and *PIP5K2* was confirmed using a promoter transcriptional fusion to a nuclear localized GUS-GFP chimera (nlsGUS-GFP) showing expression as early as the tetrad stage of pollen development (Figure 2A). *PIP5K1* appeared as an auxin inducible gene in a microarray approach (Chapter 2) and because auxin has important functions during pollen

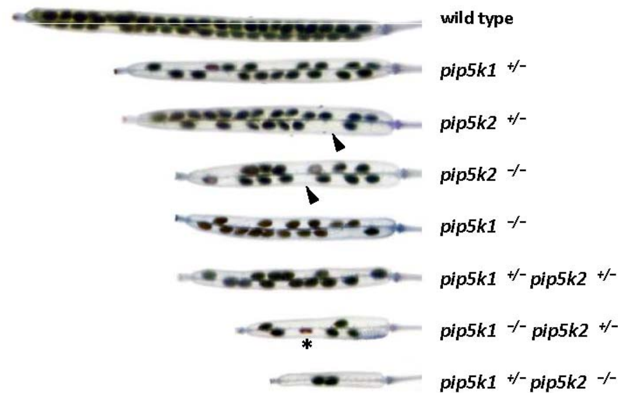

Figure 3. Seed set in single and double *pip5k1pip5k2* mutants.
Mature siliques were cleared in ethanol:acetic acid (9:1) and photographed under a binocular. Arrowheads indicate empty spots indicative of failed ovule fertilization while the asterisk indicates a shrunken seed due to embryo abortion.

development (Sundberg and Østergaard 2009) and auxin response could be detected in BCP (Figure 2B), we checked if *PIP5K1* and *PIP5K2* are auxin inducible in pollen. Pollen was isolated from wild-type flowers and we measured the relative expression of both genes in auxin-treated and non-treated pollen. Both genes appeared to be auxin inducible. Moreover, we observed that the auxin inducibility is depending on a newly synthesized co-factor because the auxin effect disappeared after incubation with an inhibitor of protein biosynthesis, cycloheximide (CHX, Figure 2C). This data strongly suggest that PIP5K1 and PIP5K2 are redundantly regulating pollen development and form part of a so far uncharacterized auxin response machinery during early male gametophytic development.

A double mutant pip5k1pip5k2 displays lethality early during microgametogenesis

As mentioned earlier, a silique of a double heterozygous *pip5k1*$^{+/-}$*pip5k2*$^{+/-}$ mutant showed empty spaces in siliques that we presumed to be an indication of a failure during fertilization. We further characterized in more detail the seed set in several combinations of single and double homozygous mutants (Figure 3). At some degree the single heterozygous mutants showed empty spaces, but we observed a reduction in seed set with

an increasing number of mutant alleles (Figure 3). Moreover, we observed a difference in the phenotype penetration when *pip5k1* is homozygous and *pip5k2* is heterozygous compared with the opposite situation, suggesting only a partial redundancy most probably due to differences in expression patterns or enzymatic activities.

To test the hypothesis that the observed phenotypes arise as a consequence of a defect during pollen formation, we isolated fresh pollen from opened flowers. We could easily observe completely collapsed pollen grains using differential interference contrast (DIC) microscopy in a *pip5k1*$^{+/-}$*pip5k2*$^{+/-}$ mutant (Figures 4A and 4B). Pollen derived from those mutant plants was further analyzed for viability using Alexander's stain, which will stain the cytoplasm of viable pollen purple, in contrast to dead pollen that will remain green (Figures 4C and 4D). We used this method to count the proportion of pollen grains that were aborted in the combination of one, two or three mutant alleles (Figure 4E). Consistent with what we observed for the seed set (Figure 3) we could see a positive correlation between the number of mutant alleles and the of dead pollen grains. In heterozygous single mutants, around 10% of the pollen grains were not viable, while this number increased to around 30% in the single homozygous mutants (Figure 4E). Moreover, these numbers are inferior to what could be expected if a single mutant allele was 100% penetrant (see Table S3 for data comparison) and also the fact that we could easily obtain homozygous mutants is indicative of a partial penetrance of the phenotype probably due to a functional redundancy as the expression profile (Table S2) and previous studies (Ischebeck et al., 2008 and 2010; Chapter 2) suggest. We could further see this phenomenon when increasing the number of mutant alleles to two in a *pip5k1*$^{+/-}$*pip5k2*$^{+/-}$ mutant (35.1% unviable pollen) and three mutant alleles in a *pip5k*$^{+/-}$*pip5k2*$^{-/-}$ plant (36.5% unviable pollen). In all cases, the pollen abortion appeared in a frequency that is in between a full single mutant allele penetrance and the two allele penetrance situation

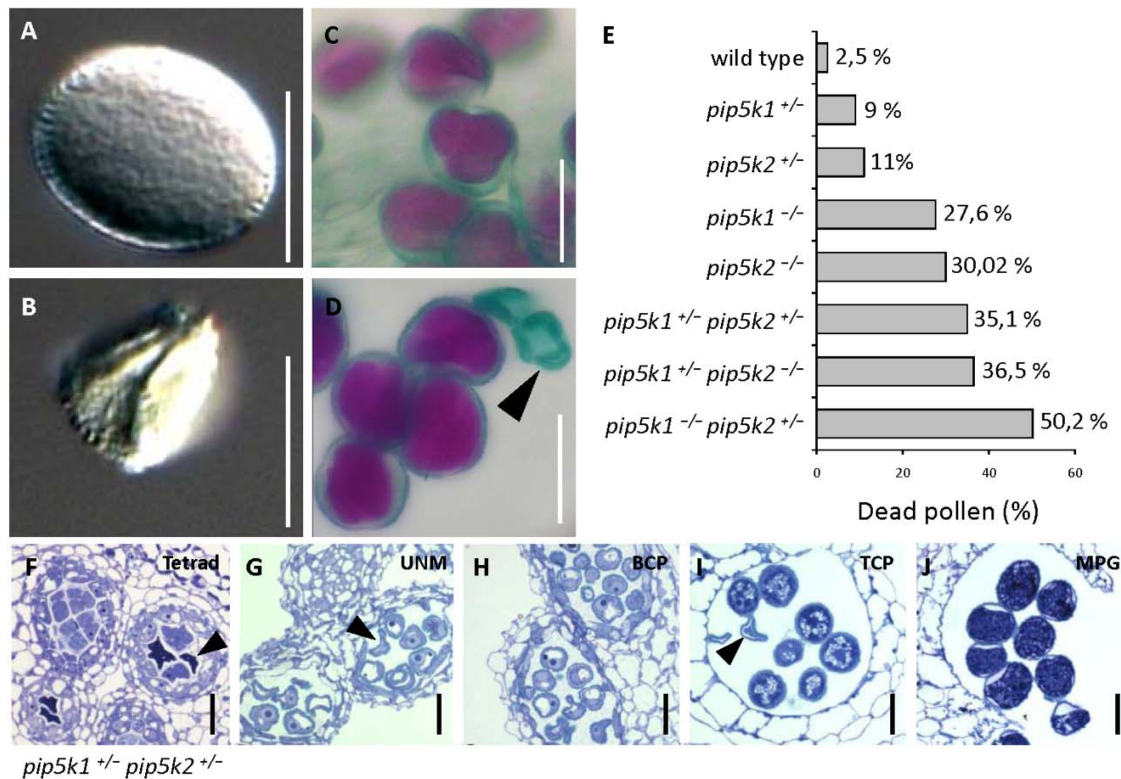

Figure 4. Phenotypic analysis of pollen development in *pip5k1pip5k2* mutants.
A-B. DIC visualized pollen grains extracted from wild-type (A) and *pip5k1*$^{+/-}$ *pip5k2*$^{+/-}$ (B) flowers.
C-E. Alexander staining in pollen grains from wild-type (C) and *pip5k1*$^{+/-}$ *pip5k2*$^{+/-}$ (D) flowers. The arrowhead indicates an unviable pollen grain which was not stained purple due to the absence of cytoplasm. Using this staining we quantified the percentage of dead pollen grains from ten flowers of the indicated genotypes (E).
F-J. Histological analysis of anthers of *pip5k1*$^{+/-}$ *pip5k2*$^{+/-}$ flowers using 2 µM-thick toluidine blue stained transversal sections. Arrowheads indicate aborted pollen grains which appear as early as the tetrad stage. Developmental stages: Tetrad (F) Unicellular microspore (G), Bicellular pollen (H), tricellular pollen (I) and Mature pollen grain (J). Bar size 10 µm (A-D, F-J)

(Table S3). It is important to notice the enhanced penetrance of the pollen lethality (Figure 4E) and the seed set (Figure 3) phenotypes in *pip5k1*$^{+/-}$*pip5k2*$^{-/-}$ double mutants compared to *pip5k1*$^{-/-}$*pip5k2*$^{+/-}$ which migh suggest additional functions for PIP5K2 in supporting pollen development or pollen tube elongation or during ovule development.

In order to assess at which stage of pollen development the phenotype arises, we chemically fixed floral tissues from *pip5k1*$^{+/-}$*pip5k2*$^{+/-}$ plants, sectioned and stained with toluidine blue, which stains polysaccharides and nucleic acids (Figures 4F-4J). We observed abortion of microspores as early as the tetrad stage, spotted as darker stained

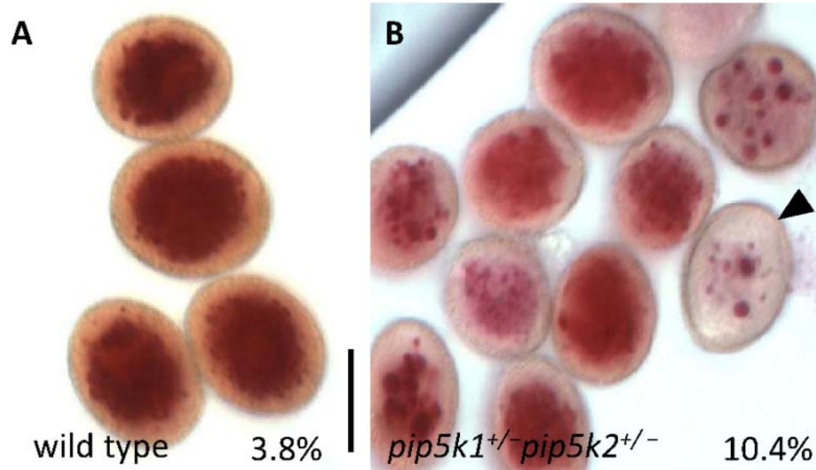

Figure 5. Abnormal vacuole morphology in pollen from double *pip5k1pip5k2* mutants.
Neutral red stained pollen from wild-type (A) and *pip5k1*$^{+/-}$ *pip5k2*$^{+/-}$(B) flowers. The percentage indicates the amount of pollen grains found with abnormal vacuoles, which are indicated with an arrowhead. Bar size 10 µm.

structures (arrowhead in Figure 4F) and these defects were also seen in the following developmental stages (Figures 4G-4J). All together this data indicates that there is a redundant function of PIP5K1/2 during early stages of microgametogenesis, essential for proper pollen development.

pip5k1 pip5k2 pollen displays abnormal vacuoles and have a reduced pollen germination rate

Phosphoinositides have been shown to influence the formation of vacuoles during pollen development (Lee et al., 2008; Whitley et al., 2009). To further investigate *pip5k1*$^{+/-}$*pip5k2*$^{+/-}$ pollen lethality, fresh mature pollen, derived from *pip5k1*$^{+/-}$*pip5k2*$^{+/-}$ plants, was stained with Neutral Red, which rapidly stains vacuoles and other vesicles. During pollen formation, vacuoles follow a characteristic pattern (Figure 1; Yamamoto et al., 2003). As early as the microspore is released from the tetrad, a large vacuole appears which is produced by fusion of pre-existing vacuoles. The vacuole growth is thought to be the driving force for the early vegetative expansion of the unicellular microspore. This unique very large vacuole will get fragmented after the first mitotic division and theses

resulting small vesicles will stay till the end of pollen and further change their structural characteristics (Yamamoto et al., 2003). Mature pollen grains stained with neutral red appear as having numerous small vacuolar structures (Figure 5A). In formation contrast, pollen grains from *pip5k1*$^{+/-}$*pip5k2*$^{+/-}$ display less and abnormal vacuoles in 10.4% of the cases observed (Figure 5B). We observed similar vacuolar defects in the root tips of mutant seedlings. Double mutants were preselected based on seedling phenotype (Figure S1C), stained with Lysotracker Red, which stains acidic compartments such as vacuoles, lysosomes and prevacuolar compartments, and later observed under confocal microscope. All double mutant seedlings analyzed (n = 28) showed smaller fragmented vacuoles in root tips (Figure S2A and S2B), which is an additional indication that PIP5Ks are involved in vacuole morphogenesis.

DISCUSSION

The phytohormone auxin participates in multiple aspects of plant development, including flower and pollen formation. Although it had been characterized that components of auxin biosynthesis, homeostasis and signaling are present throughout pollen development, the auxin' role in pollen remains elusive. Auxin biosynthetic genes are expressed early during anther development and during later stages members of the TIR/ABF auxin receptors are being expressed together with an increased auxin activity measured by DR5 reporter (Cecchetti et al., 2008, Feng et al., 2006). Therefore, most probably auxin acts at the transcriptional level to control a subset of genes transiently expressed during microgametogenesis. However, to date there is not a single example of downstream components of auxin response that could give a glimpse about auxin function during microgametogenesis. We described here that PIP5K functions downstream of auxin. We had found that auxin is able to induce the expression of PIP5K1 and PIP5K2 in pollen and this transcriptional effect seems to depend on the synthesis of an additional transcriptional

factor. It would be interesting to analyze if any component of auxin synthesis or signaling has defects in early pollen development or during vacuole biogenesis or to find the unknown transcription factor involved in the auxin transcriptional response of PIP5Ks.

Phosphoinositides are minor but important lipid species present in all eukaryotes. They are formed by phosphorylation in the inositol ring in phosphatidylinositol, generating a variety of signaling molecules that are involved in almost every aspect of development in higher organisms. PtdIns(4,5)P_2 is produced by phosphorylating PtdIns4-P at the position five in inositol by PIP5Ks. In the Arabidopsis genome, there are eleven of those enzymes that form a highly redundant class of proteins. For instance, PIP5K4/5 have redundant functions during pollen tube elongation (Ischebeck et al., 2008), as well PIP5K10/11 (Ischebeck et al., 2011). PIP5K1/2 also form a pair of functionally redundant proteins during auxin-dependent development (Chapter 2) and here we have characterized their redundant role during pollen development. We described an increasing number of aborted pollen with the increase in mutant alleles in single and double mutants. This defect generated a defect in seed set and in the transmission of mutant alleles, as visualized by the phenotype segregation in seedlings. Moreover, the fact that it is possible to obtain double homozygous mutants highlights the partial penetrance of the phenotypes and the further redundancy in function with other pollen expressed PIP5Ks (Table S2).

Vacuole biogenesis has been pointed out as an important structural change in the developing male gametophyte where vacuoles of different size and structural characteristics have been observed (Figure 1; Yamamoto el al., 2003). It had been previously reported that the synthesis of PtdIns3-P and PtdIns(3,5)P_2 is essential for vacuole morphogenesis and that Arabidopsis mutant pollen lacking components in the biosynthetic pathway of those phosphoinositides show severe pollen developmental defects (Qin et al., 2007; Lee et al., 2008; Whitley et al., 2009; Xu et al., 2011). By itself,

PtdIns3-P is a signal for targeting to the lumen of the vacuole (Kim et al., 2001) but it is also the precursor for the formation of PtdIns(3,5)P2 which also plays an important role in yeast vacuolar morphogenesis (Efe et al., 2005). Out of the four PtdIns3-P 5 kinase genes present in the Arabidopsis genome (Mueller-Roeber and Pical 2002), two of them, FAB1A and FAB1B are expressed in pollen and the double mutant *fab1a/b* showed pollen lethality as a consequence of a failure in vacuole rearrangement (Whitley et al., 2009). A hemizygote AtVPS34/*atvps34* mutant segregates pollen grains that contain abnormally large vacuoles after the first mitotic division that later lead to completely collapsed pollen grains (Lee et al., 2008). AtVPS34, the only gene encoding a PtdIns 3-kinase present in Arabidopsis, synthesizes PtdIns3-P from phosphatidylinositol which is essential for normal plant growth (Welters et al., 1994). In yeast, PtdIns 3-P regulates autophagy or vacuolar targeting depending on additional components of the protein complex (Strahl and Thorner 2007). The yeast ortholog of AtVPS34 interacts with two other proteins, VPS15p and VPS30p, to form the core of a complex involved in PtdIns 3-P synthesis. Arabidopsis AtVPS15 and AtVPS30 are also involved in male gametophyte development showing similar defects as the one appearing in atvps34 pollen grains (Xu et al., 2011; Qin et al., 2007).

Here, we described that enzymes involved in the synthesis of the phosphoinositide PtdIns(4,5)P_2 also display abnormalities in vacuolar morphogenesis. Taking in account the similitude of the vacuole phenotype of *pip5k1pip52* mutant pollen grains with those observed in mutants involved in the biosynthesis of PtdIns(3,5)P_2 there is the possibility that PIP5Ks also use PtdIns3-P as a substrate. However, based on structural characteristics it is plausible only phosphorylation of 4-phophate phosphoinositides and not of PtdIns3-P (Mueller-Roeber and Pical 2002). Moreover, using *in vitro* assays it was possible to show that PIP5K1 is able to synthesize only PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3

lipids (Elge et al., 2001). Therefore, most probably the vacuolar phenotype is a direct effect of the lack of PtdIns(4,5)P_2 or the over accumulation of PtdIns4-P, although it is not possible to directly link the defect seen in vacuoles with the early pollen lethality seen in *pip5k1pip5k2*.

MATERIAL AND METHODS

Plant material and growth conditions

All lines are in the Columbia background of *Arabidopsis thaliana*. The lines *pip5k1* and *pip5k2* are SALK insertional mutants SALK_146728 and SALK_012487, respectively, and the transcriptional promoter reporter constructs PIP5K1:nlsGUS-GFP and PIP5K2:nlsGUS-GFP were described in Chapter 2. Seeds were sterilized overnight by chlorine gas, sown on solid Arabidopsis medium (0.5X Murashige and Skoog basal salts, 1% sucrose and 0.8% agar, pH 5.9) and stratified at 4°C for at least two days prior to transfer to a 16h:8h light:dark 18°C growth regime. The seedlings were grown vertically for 4 to 12 days prior to analysis. For pollen analyses, one-week-old seedlings germinated on solid media were transferred to soil and grown for additional 6-10 weeks in a growth chamber with controlled temperature at 22°C and a ligh/dark cycle of 8h/16h.

Pollen analysis

For the histological analysis, flowers were collected and fixed overnight in 3% glutaraldehyde solution in 0.1 M sodium cacodylate (pH 7.2), then dehydrated in acetone series and embedded in Epon resine Embed 812 following manufacturer's instructions (EMS). Anther transverse sections (2 μM) were stained with 1% toluidine blue prior to analysis. Alexander staining (Malachite green 0,05% (w/v), acid fuchsin 0,05% (w/v), orange G 0.005% de (w/v), phenol 5% (w/v), acetic acid 2% (v/v), glycerol 25% (v/v), and ethanol 50% (v/v)) and neutral red staining (0,01 % (w/v) in 8% sucrose w/v) were performed on slides incubating whole flowers for 5 min in the respective stains and

followed by immediate imaging. Stained pollen and anther cross sections were viewed using an Olympus IX81 microscope and bright-field photographs were taken using a Micro Publisher 3.3 RTV digital camera.

Expression analysis

Pollen grains were collected from 50 to 100 complete inflorescences by softly grinding them in a mortar in the presence of a solution of Mannitol 3M taking care to open all closed flower buds. The pollen extract was filtered using two sequential Nilon mesh (Nitex) of 80 μm and 35 μm. Then the filtered pollen suspension was centrifuged for 7 min at 14000 rpm. Glass beads were used to grind the pollen grains and RNA extraction was performed using the RNeasy Plant mini RNA extraction kit (Quiagen). RNA was treated with DNaseI (Roche). cDNA was synthetized using 1 μg of RNA using the SuperScript II First Strand Synthesis Kit (Invitrogen) and quantitative PCR was performed with the system Fast Evagreen qPCR master mix (Biotium) using 95 °C for 10 min and 40 cycles of 95 °C for 10 sec, 60 °C for 15 sec, and 72 °C for 20 sec. For normalizing the expression levels, we used At5g55840, a pentatricopeptide protein coding gene. The results were analyzed using the software MxPro – Mx3000P v4.1 Build 389 (Schema 85). All the primer used for expression analyses as well genotyping of *pip5k* mutants are listed in Table S4.

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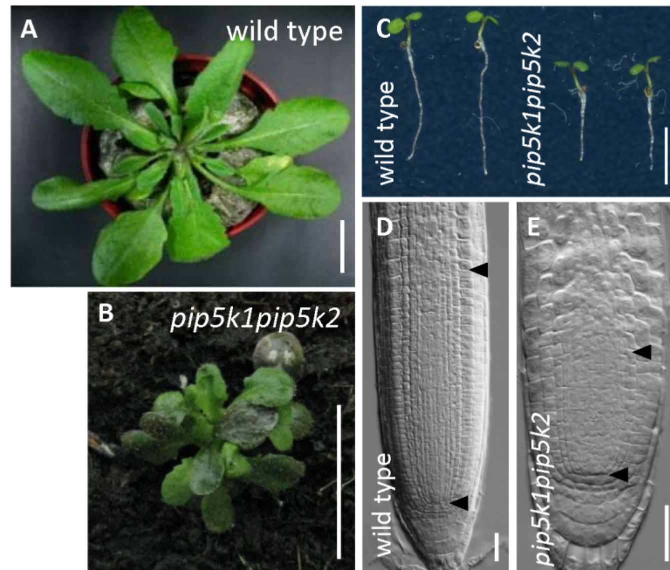

Figure S1. Mutant phenotypes described for *pip5k1pip5k2* double homozygous mutants.
A-B. Phenotype in adult plants for wild type (A) and *pip5k1pip5k2* double homozygous mutants (B). The *pip5k1pip5k2* double mutant do not develop any inflorescence. Bar size 1 cm.
C. Seedling phenotypes. The *pip5k1pip5k2* double mutants are clearly shorter than the wild type seedlings.
D-E. Root apical meristem (RAM) in wild type (D) and *pip5k1pip5k2* double mutants (E). Note that the mutant RAM is considerably shorter than the wild type RAM. The arrowheads indicate the size of RAM that goes from the quiescent center to the differentiation zone. Bar size A, B 1 cm; D, E 50 µm; C 0.5 cm

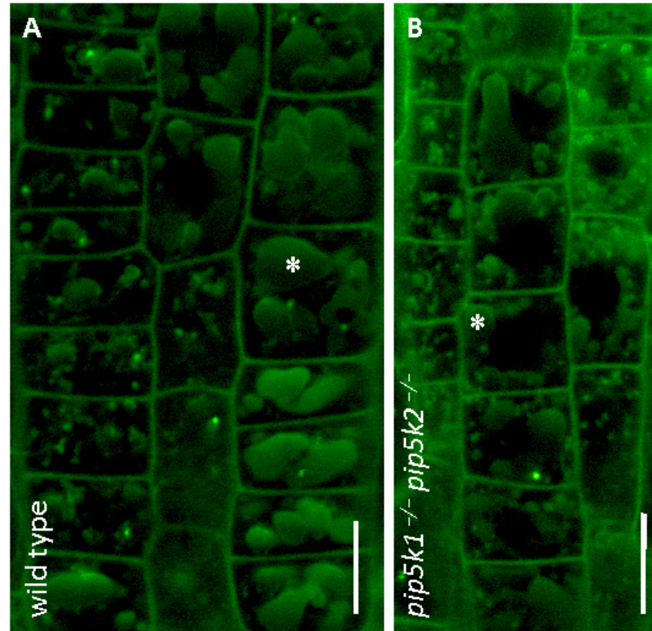

Figure S2. Abnormal vacuole morphogenesis in *pip5kpip5k2* root tips.

Seven days post germination seedling of wild type (A) and *pip5k1pip5k2* double mutants (B) were stained for 2 hours in Lysotracker Red and visualized under the confocal microscope. The Lysotracker fluorescence is in green. Both pictures are displayed in the same magnification. Asterisks indicate examples of vacuoles in trichoblast cells. Bar size 20 µm

Table S1: Phosphoinositide kinases and phosphatases involved in pollen development and pollen tube growth

Enzyme	Enzymatic Activity (S/P[&])	Expression Pattern*	Characteristics	References
atVPS34	PtdIns/PtdIns3-P	Endothecium, epidermis, tapetum, pollen mother cells and immature pollen grains	Localized at endosomal vesicles in roots. Knock out mutant shows male gametophyte lethality and vacuole morphology in pollen as well as reduction male transmission and germination rates and pollen tube elongation. Mutants in other components of a stereotypical PtdIns 3K complex like AtVPS15 and AtVPS30 are also exhibit male gametophytic defects.	Lee et al., 2008a, Qin et al., 2007, Xu et al., 2011
PTEN1	PtdIns3-P/PtdIns	Pollen specific	Dual lipid-protein phosphatase specifically expressed in pollen. Regulates autophagy in pollen tubes by modulating PtdIns3-P content in endosomal compartments.	Gupta et al., 2002; Zhang et al., 2011
FAB1A/B	PtdIns3-P/PtdIns(3,5)P_2	ND	Localized at endosomes in roots. A double heterocigous*fab1a fab1b* mutant show collapsed pollen grains. Mutant pollen display completely disorganized vacuoles after first mitotic division, generating at the tricellular stage a extremely large vacuole which produce a total collapse of pollen grains.	Whitley et al., 2009
PIP5K4/5	PtdIns4-P/PtdIns(4,5)P2	Pollen	Pollen expressed type B PtdIns 4-P 5-kinase. A double *pip5k4pip5k5* show reduced pollen germination and pollen tube abnormalities while overexpressing them generated multiple branching events due to defects in apical pectin secretion and/or vesicle trafficking events but not callose deposition neither cytoskeleton abnormalities.	Ischebeck et al., 2008 Ischebeck et al., 2010b; Souza et al., 2008
PIP5K6	PtdIns4-P/PtdIns(4	Pollen	Type B PtdIns 4-P 5-kinase. It is localized to subapical membrane in pollen tube. Knock down inhibits clathrin mediated endocytosis while overexpression induces massive aggregation of membrane at the pollen tube tip. The later can be overcome by inhibiting the endocytosis using the dominant negative clathrin dominant or by modulating the PtdIns4P/PtdIns(4,5)P_2 balance.	Zhao et al., 2010
PIP5K10/11	PtdIns4-P/PtdIns(4	Pollen	Pollen specific type A PtdIns4P 5-K localized to the subapical plasma membrane domain in pollen tubes. Overexpression in tabaco pollen generates severe tube swelling and defects in actin cytoskeleton partially by regulating the Rac5/RhoGDI2 interaction.	Ischebeck et al., 2011

[&] S/P: Substrate/Product based on protein homology to known enzymatic activities.* expression pattern in the context of pollen development

Table S2. PIP5K expression levels in Unicellular microspore (UNM), Bicellular pollen (BCP), tricellular pollen (TCP) and Mature pollen grain (MPG). The data represents normalized absolute values as described in Honys and Twell 2004.

		Developmental Stage			
		UNM	**BCP**	**TCP**	**MPG**
PIP5K1	**At1g21980**	**0**	**166**	**0**	**0**
PIP5K2	**At1g77740**	**127**	**132**	**0**	**0**
PIP5K3	At2g26420	0	0	0	0
PIP5K4	At3g56960	66	180	1496	1281
PIP5K5	At2g41210	144	254	2048	1585
PIP5K6	At3g07960	309	349	1470	1609
PIP5K7	At1g10900	0	0	0	0
PIP5K8	At1g60890	441	417	470	538
PIP5K9	At3g09920	0	0	0	0
PIP5K10	At1g01460	0	0	1250	3103
PIP5K11	At4g01190	224	226	0	0

Table S3. Comparison of observed and expected segregation of male gamete abortion in two scenarios: one in which each mutant allele is fully penetrant by its own (A) and other where only the combination of the two mutant alleles generates pollen lethality (B). Values are expressed in percentage.

		Observed		Expected A	Expected B
pip5k1	***pip5k2***	**normal**	**aborted**	**aborted**	**aborted**
+/+	+/+	**97.5**	**2.5**	0	0
−/+	+/+	**91**	**9**	50	0
+/+	−/+	**89**	**11**	50	0
−/−	+/+	**72.4**	**27.6**	100	0
+/+	−/−	**69.98**	**30.02**	100	0
+/−	+/−	**64.9**	**35.1**	75	25
+/−	−/−	**63.5**	**36.5**	100	50
−/−	+/−	**49.8**	**50.2**	100	50

Table S4. Primers used

Primer Name	Sequence	Use
PIP5K1-1	AAGATGGGTGCATGTACGAAG	Genotyping
PIP5K1-2	TTCCACCTGAAATCCACTGAC	Genotyping
PIP5K2-1	GGAAGTTTGACTGGGGAGAAG	Genotyping
PIP5K2-2	TCATACTGGCAGACGTGTTTG	Genotyping
LBb1	GCGTGGACCGCTTGCTGCAACT	Genotyping
PIP5K1-7	GCGGGAAGAGTAAAAAGGTAAATC	qPCR
PIP5K1-8	CCTCCCCAACCCAAAAACTT	qPCR
PIP5K2-7	GCATTAGGGGCAAAAGGGT	qPCR
PIP5K2-8	AAATAAGTAATCCCCCACCCAA	qPCR
PPR-F	AAGACAGTGAAGGTGCAACCTTACT	qPCR
PPR-R	AGTTTTTGAGTTGTATTTGTCAGAGAAAG	qPCR

Chapter 4

PATELLIN family of phosphatidylinositol transfer proteins regulates embryo patterning

Ricardo Tejos, Maciej Adamowski, Michael Sauer, and Jiří Friml

SUMMARY

The phosphatidylinositol transfer proteins (PITPs) regulate the interphase between lipid metabolism and phospholipid synthesis and have essential roles in mammals, yeast and plants. *Arabidopsis thaliana* contains 32 PITPs, from which the PATELLINs (PATLs) subgroup was identified in a microarray designed to find new regulators of auxin-mediated cell polarity. Here, we show that *PATL* expression patterns are developmentally regulated and partially overlapping. Using reverse genetics we have found that PATLs regulate embryogenesis, root patterning and gravitropic response. Furthermore, we show that PATLs are plasma membrane associated proteins, which are asymmetrically localized.PATL4 colocalizes with polarly localized PIN auxin efflux carriers, suggesting a link between phospholipid synthesis and signaling and auxin-dependent processes.

Multicellular organisms rely on a countless number of signaling molecules that participate in intracellular, cell-to-cell and long distance communication and allow the organism to integrate a variety of cellular responses and processes to tissue contexts (Robert and Friml 2009). Phosphatidylinositols (PtdIns) are signaling lipid molecules commonly present in all eukaryotic membranes (Balla et al., 2009). They have a dual cellular function as scaffold lipids to recruit cytosolic proteins and as precursors of other lipid or soluble second messengers participating in a variety of signaling processes, including stress responses to the environment and during development. Their synthesis is temporally and spatially controlled by metabolic enzymes, such as phosphatases, kinases and phospholipases allowing a fine-tuned response. An intriguing group of phosphatidylinositol related proteins, which are proposed to play a role between PtdIns metabolism and signaling, are the PtdIns/Phosphatidylcholine (PtdCho) transfer proteins (PITPs), defined by the yeast Sec14p protein (Bankaitis et al., 1990). In *in vitro* assays, PITPs are able to transfer PtdIns or PtdCho between membranes (Aitken et al., 1990), to stimulate Ca^{+2}-triggered exocytosis (Hay and Martin 1993), to regulate budding and vesicle formation at the trans Golgi network (TGN; Simon et al., 1998) and to assist phospholipase C-mediated PtdIns(4,5)P_2 hydrolysis (Cunningham et al., 1995). Because of these activities, PITPs are placed in a central position at the interphase between phosphoinositide metabolism and signaling (Cockcroft 2001), however which one reflects their *in vivo* function is not clearly established yet.

As sessile organisms, plants have developed unique specific signaling molecules, in order to cope with the changing environment by triggering polar cellular or tissue responses and to generate asymmetry and patterns during development. Auxin is a key plant hormone involved in most aspects of plant life, including directional growth responses and formation of new polarity axes (Grunewald and Friml 2010). Auxin is characteristically

distributed in concentration gradients as consequence of local biosynthesis, polar cell-to-cell transport (polar auxin transport, PAT) and intracellular catabolism (Ruiz-Rosquete et al., 2012). The direction of PAT is determined by the polarly localized auxin exporters of the PINFORMED (PIN) family (Petrásek et al., 2006; Wisniewska et al., 2006). PINs are subcellularly regulated by auxin at multiple levels (transcription, trafficking, localization and degradation), all of them involving different subsets of auxin receptors and molecular components. Auxin inhibition of PIN endocytosis (Paciorek et al., 2005) is mediated by putative extracellular signaling via the auxin receptor AUXIN BINDING PROTEIN1 (ABP1; Robert et al., 2010). Moreover, auxin-mediated regulation of PIN localization is specific for each PIN protein and for the cell where it is expressed. It relies on the nuclear auxin signaling pathway mediated by the auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1; Sauer et al., 2006). Whereas non-genomic ABP1 signaling relies on clathrin-mediated endocytosis (Robert et al., 2010), the nuclear TIR1/auxin-mediated signaling regulation of PIN localization depends on the function of a so far unknown modulator which is under the transcriptional control of the repressor AXR3 (Sauer et al., 2006).

In order to unravel this AXR3-dependent pathway for PIN localization regulation, a microarray-based approach was designed to fish out new regulators of PIN polarity (Chapter 2). Among the candidate genes, several PATELLIN (PATL) genes were spotted as auxin regulated and differentially responding in the background of the dominant negative version of AXR3 (HS:*axr3-1*, Figure S1), making them good candidates for AXR3-mediated PIN polarity regulation. PATLs belong to a protein family characterized by a Sec14p-like domain, suggesting an interesting link between auxin signaling and phosphatidylinositol metabolism regulation. Current research suggests that PATLs would play a very essential role in plants due to their participation in several signaling pathways

such as those in response to brassinosteroid (Deng et al., 2007; Tang et al., 2008), cytokinin (Černý et al., 2011), and pathogen attack (Benschop et al., 2007; Elmore et al., 2012; Kiba et al., 2012), as well as in some basic cellular processes like cytokinesis (Peterman et al., 2004). However, a clear characterization of the function of the Sec14p-like subfamily of PATELLIN proteins in plants is still lacking. Here, we present an analysis of the PATLs' function in the context of auxin-dependent development and cell polarity in Arabidopsis.

RESULTS

Arabidopsis PITPs

In order to identify all Arabidopsis PITPs, we used the yeast Sec14p protein sequence as bait to search in the TAIR database for proteins having significant homology and found a total of 32ArabidopsisSec14p-like proteins (Table S1) that can be grouped into two phylogenetic clades (Figure 1A). While some of them (14/32) consist solely of a Sec14p-like domain, similar to the yeast Sec14p protein organization, others have incorporated a second protein domain (Figure 1B; Mousley et al., 2007).With a relatively high homology to the yeast Sec14p (37-43% homology, Table S1), 14 Arabidopsis thaliana Sec fourteen homologs(AtSFHs) group in a single cluster (Figure 1A). In addition to the Sec14p domain, 12 AtSFHs contain at their C-terminal end a 100 amino acids long Nodulin domain. This domain was initially characterized in the Nodule specific protein Nlj16 and defines a plasma membrane (PM) targeting module (Kapranov et al., 2001). The second cluster is a heterogeneous group of proteins with a variable homology to Sec14p (between 21-32% homology, Table S1). We have called them *Arabidopsis thaliana* Phosphatidylinositol transfer proteins (AtPITPs). This cluster contains the PATL protein family, a subclade of 6 proteins containing a Golgi dynamic domain (GOLD, Figure 1B)

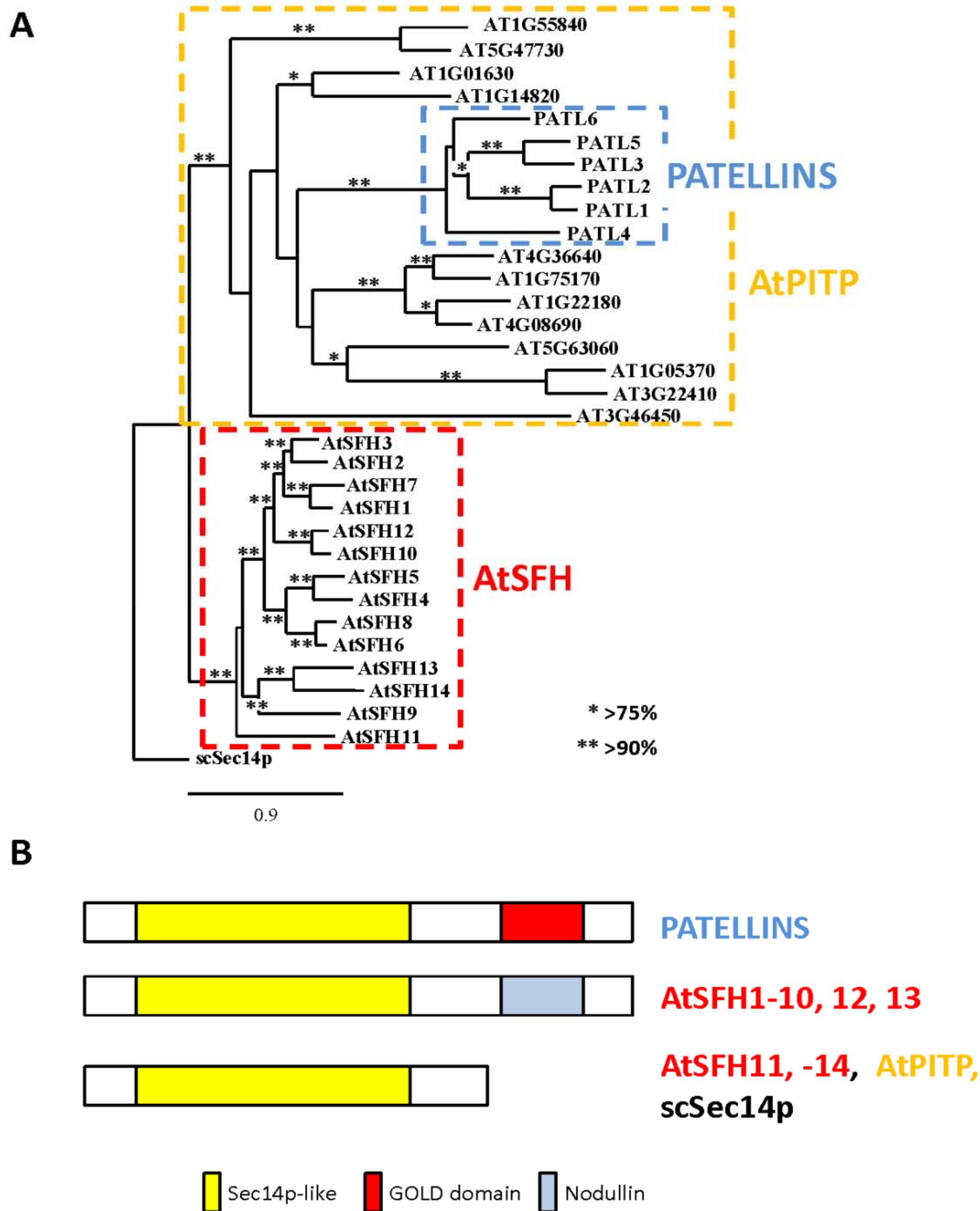

Figure 1. Sec14-like proteins in *Arabidopsis thaliana*.
A. Phylogenetic tree of Sec14p-like proteins from Arabidopsis. *Saccharomyces cerevisiae* Sec14p (scSec14p) was used as outgroup. The proteins group in two clades, one highly homologous to scSec14p containing 14 proteins (AtSFH1-14), which seem to be evolutionarily older and less diverse although two of them do not contain an extra nodulin like domain (AtSFH11 and 14). The second clade contains a more diverse group of PITPs in terms of homology to scSec14p. Within this group appears a small cluster of six proteins that contain an additional GOLD domain, the PATELLINS (PATLs).
B. Schematic representation of the protein configuration found among Arabidopsis PITPs. A Sec14p-like domain (yellow box) can be found in tandem with a GOLD domain (Red box), a Nodullin-like domain (light blue box), or it can be the only distinguishable protein domain, as it the case for yeast Sec14p. See text for an extended description.

tandem with the Sec14p-like domain. The GOLD domain is widely present among mammal proteins that are associated to membranes by hydrophobic interactions and

participate to vesicle formation at the ER/Golgi. It is also found in proteins that modulate membrane homeostasis (Anantharaman et al., 2002). As this is the case for many other protein families, PITPs in plants have greatly expanded in number and diversified their function. The few published studies on plant PITPs implicated them in responses to abiotic (Kearns et al., 1998, Monks et al., 2001) and biotic (Kiba et al., 2012) stress as well as during nodule formation (Kapranov et al., 2001), cell division (Peterman et al., 2004), and subcellular trafficking (Vincent et al., 2005; Böhme et al., 2004), which suggests that a broad spectrum of functions are regulated by PITPs in plants.

Developmentally regulated PATELLIN expression patterns

The function of a protein is naturally linked to its expression pattern during plant development. To get an insight into PATELLIN function, we generated Arabidopsis transgenic lines carrying PATL promoter fusions (2000 base pairs upstream of the ATG codon) to a nuclear-localized GUS-GFP chimera (*PATL3*, *PATL4*) or transcriptional fusion to GFP (*PATL2*, *PATL4*), and supported by *in silico* analysis (*PATL5*, *PATL6, eFP* browser, Winter et al, 2006). The *PATL1* expression pattern was analyzed by immunolocalization using an anti-PATL1 antibody (Peterman et al., 2004).

PATL genes are expressed in roots and cotyledons in a predominantly overlapping pattern. Their expression is in general linked to the cytokinesis and to post-cytokinetic events. PATL1 localizes to the PM of newly divided cells in the proximal root apical meristem (RAM) (Figure 2A; Peterman et al., 2004), and in the cytoplasm and PM of stele cells in the distal border of the RAM, where the cells stopped dividing and start to differentiate in vascular tissues (Figure 2B). *PATL2* is expressed in differentiated vascular tissues in roots, in the central cylinder (Figure 2C). In embryos, *PATL2* is active in provascular cells in root and cotyledons (Figure 2D). *PATL3* expression is restricted to a

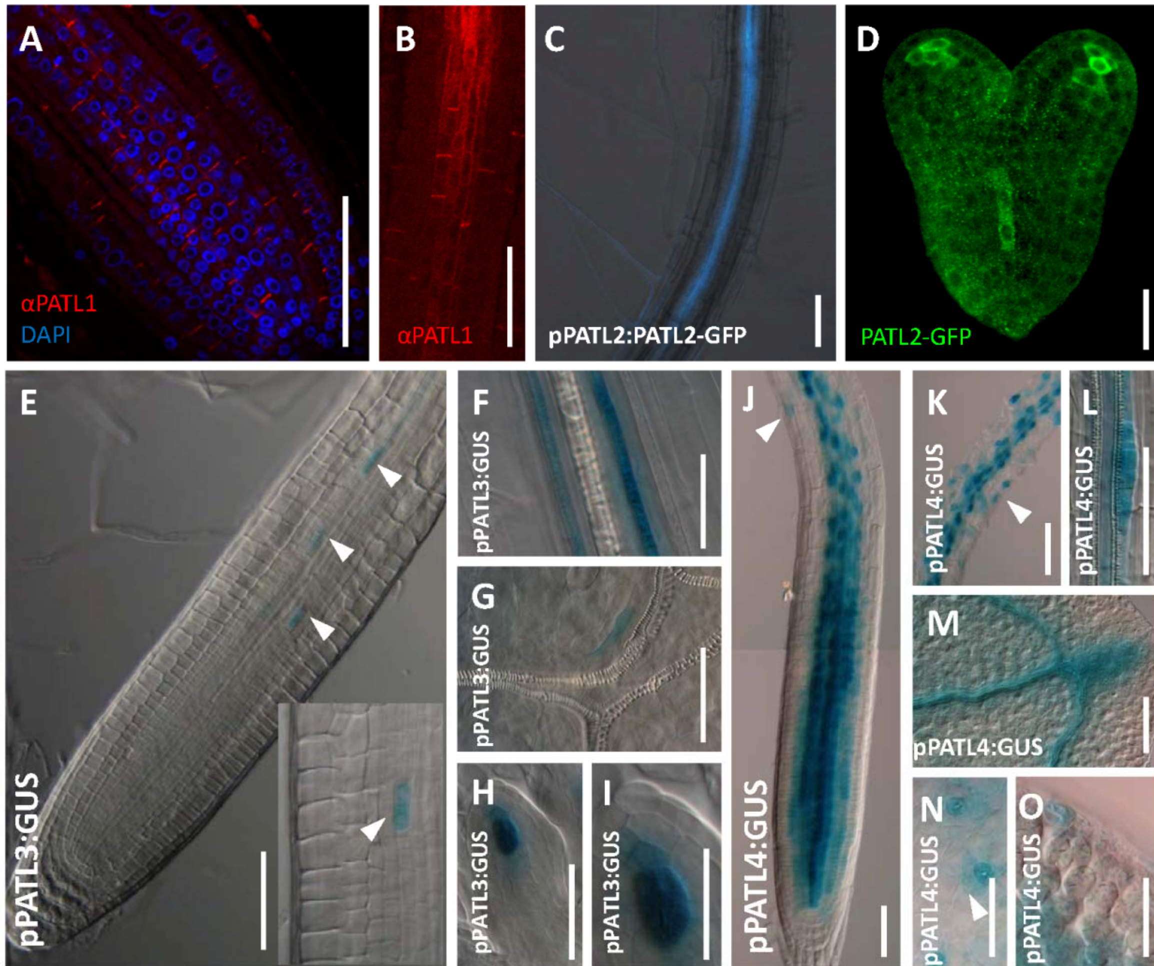

Figure 2. PATELLIN expression patterns.
A-B. *PATL1* expression in root apical meristem (A) and in inner vascular tissues in the differentiation zone in root tips (B) detected by anti-PATL1 antibodies.
C-D. *PATL2* is expressed in vascular tissues in mature roots (C)and in provascular tissues during embryogenesis (D) as indicated by GFP fluorescence (C) or anti-GFP immunolocalization (D) in the *pPATL2:PATL2-GFP* line.
E-O. *PATL3* and *PATL4* expression was monitored using transcriptional fusions of *PATL3* and *-4* promoters with a nuclear localized GUS-GFP chimera (nlsGUS-GFP) and detected by GUS staining. *PATL3* is expressed during vascular tissue differentiation in the root tip (E), cotyledon (F), mature root (G) and leaf primordia (H and I) in a subset of cells. *PATL4* is localized in all cell layers in root tips (J) and older roots and in a differentiation zone presumably in trichoblast cells (arrowheads in J and K). It is also expressed in lateral root primordia (L), vascular tissues in cotyledons (M) and stomata (O) and stomata companion cells (arrowheads in N). Bar size: A-C, E, F, J-O 50 µm; D 20 µm; J-I 25 µm

subpopulation of vascular cells inside the transition zone in roots, between the RAM and the differentiation zone (Figure 2E), in mature primary roots (Figure 2F), in cotyledons (Figure 2G), and in early differentiating vascular tissues in leaf primordia (Figure 2H and 2I). PATL4 is present in all cell types of the RAM, strongly in inner tissues (Figure 2J),

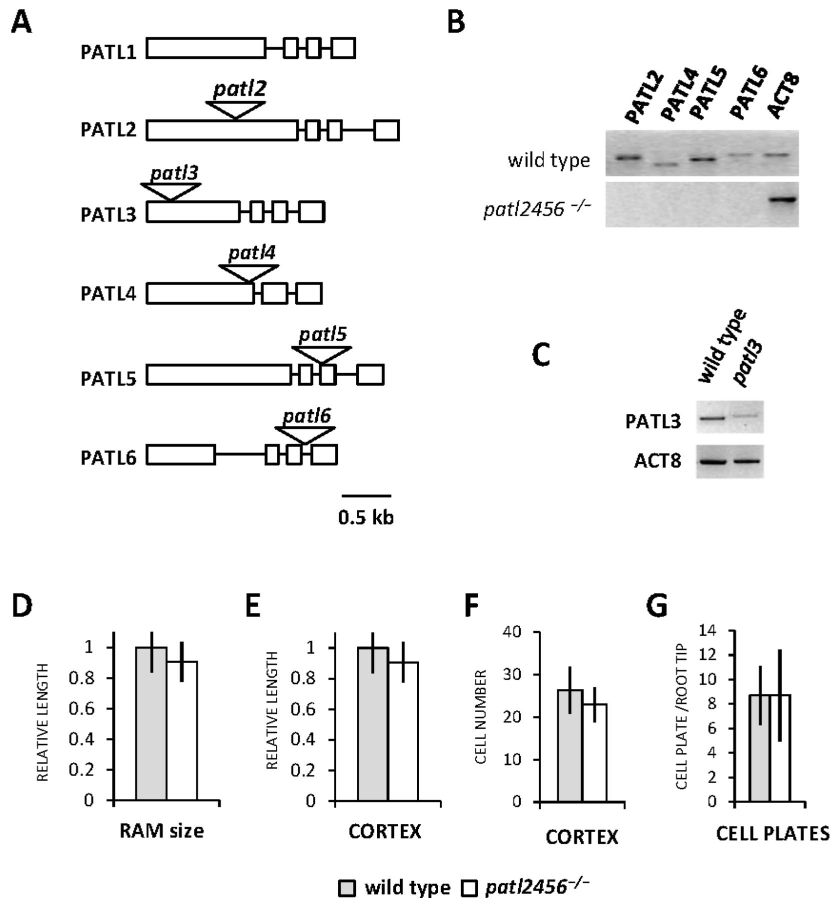

Figure 3. PATELLIN quadruple mutant *patl2456*$^{-/-}$ has a shorter RAM due to a reduced cell size and cell number.

A-C. Scheme of *PATL* genes representing exons (boxes) and introns (lines) and the insertion sites of the T-DNA insertions in the *patl* mutants (A). RT-PCR analysis for *PATL* gene expression in wild type and *patl2456*$^{-/-}$ quadruple knockout mutants (B) and *patl3* knockdown mutant (C).

D-G. Quadruple *patl2456*mutant has a shorter RAM size as a combined consequence of smaller and less cells (E and F, respectively), but most probably not by a defect in cytokinesis event number (G).

including trichoblast cells (Figure 2J and 2K) and lateral root primordia (Figure 2L). In cotyledons, *PATL4* is expressed in the inner vascular tissues (Figure 2M), in the stomata and stomata companion cells (Figure 2N and 2O). *PATL5* and *PATL6* are also expressed in a subset of root cells as suggested by publicly available microarray data (Figure S2). These patterns point to a developmentally regulated expression especially in highly dividing tissues as previously reported (Peterman et al., 2004), but also suggest their involvement in differentiation processes of vascular tissues, root hairs and stomata.

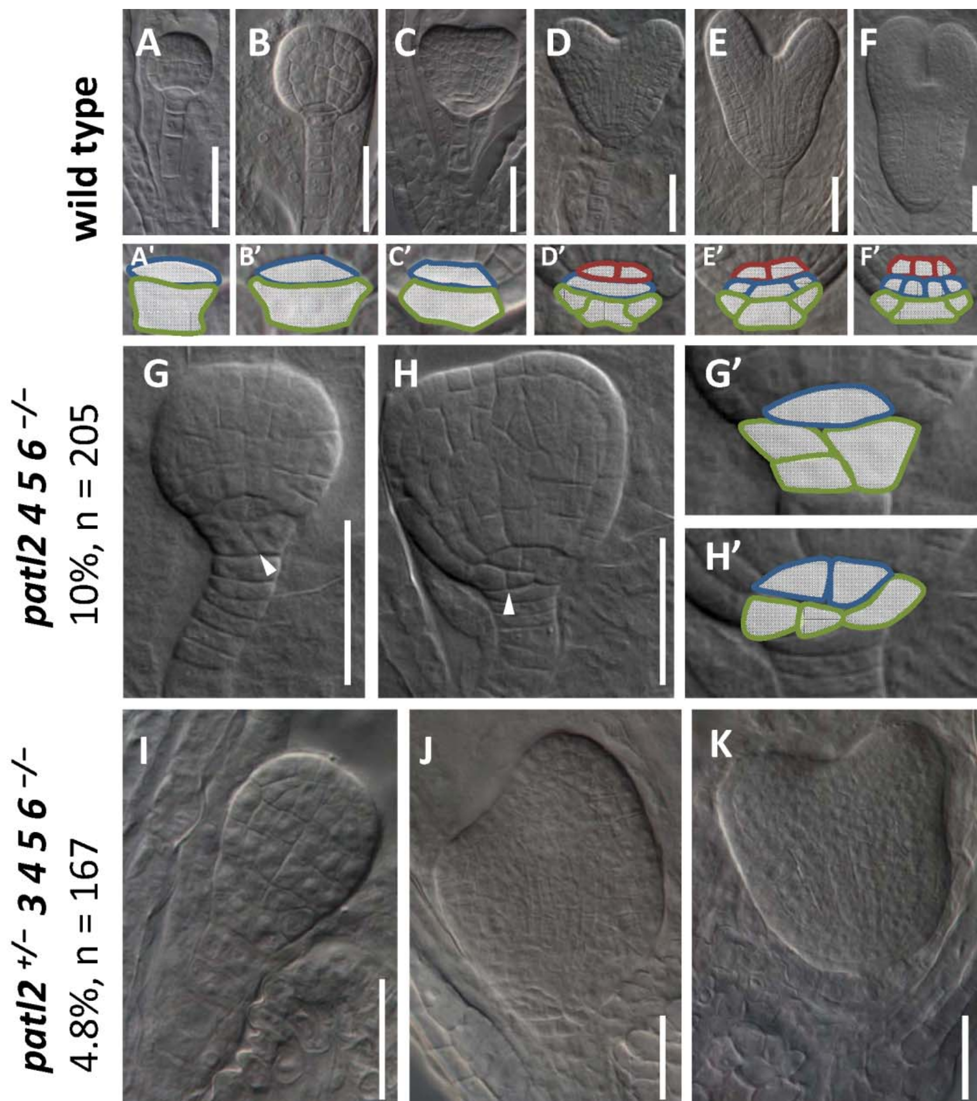

Figure 4. PATELLINs regulate embryo patterning and development.

A-F'. Embryo development in wild type (A-F) and an inset of the division patterns during basal pole formation (A'-F').

G-H'. *patl2456*$^{-/-}$ mutant embryos displaying abnormal division planes. Compare G' with B' and H' with C'. Notice that there are extra divisions in both cases (indicated with arrowheads in G and H).

I-K. Mutant embryos segregating from a *patl2*$^{+/-}$*patl3456*$^{-/-}$ plant. They show defects in basal (I) or apical poles (J), as well as a general abnormal shape (K). Bar size: 20 µm

PATELLINs redundantly regulate embryo patterning

To address the role of PATELLINs during plant development, we isolated knockout mutants for PATL2/4/5/6 and a knockdown mutant for PATL3 (Figure 3A and 3C) from the Salk collection (Alonso et al., 2003). Unfortunately we were not able to obtain any knockout or knowckdown insertional line for PATL1. No developmental abnormality was

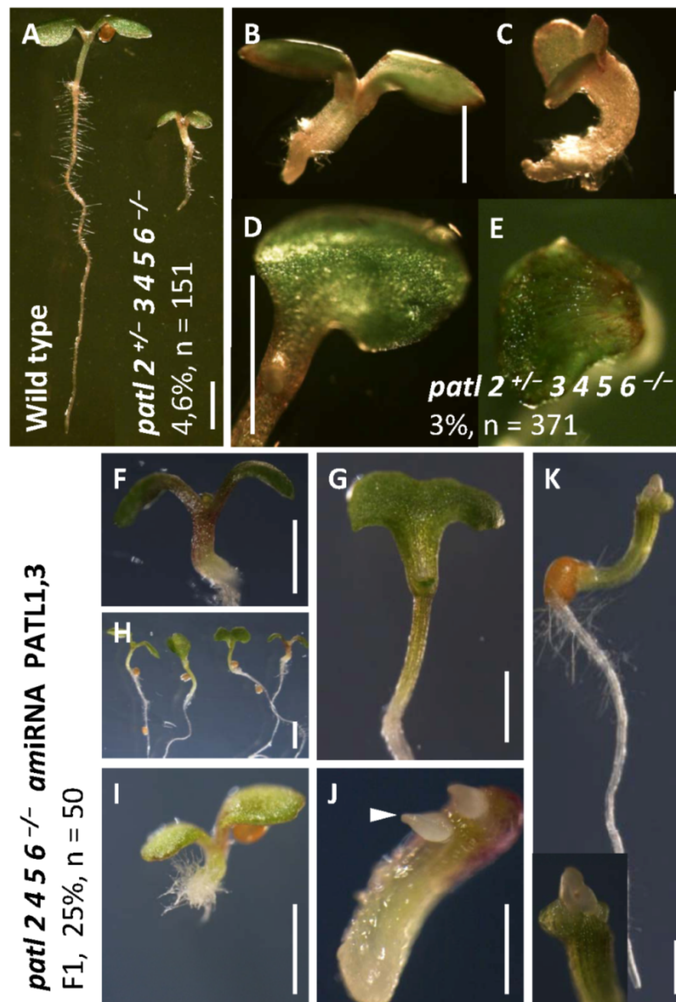

Figure 5.Seedling phenotypes in multiple *patellin* mutants.
A-E. Abnormal seedlings segregating in the progeny of a *patl2*$^{+/-}$*patl3456*$^{-/-}$ plant. 4 days post germination, wild-type Col-0 seedlings (A, left seedling) are noticeably bigger than 4.6 % (n = 151) of *patl* mutants (A, smaller seedling at the right). Besides, in 3% of the cases(n = 371) some rootless seedlings (B, C), and seedlings with cotyledon phenotypes such as tri-cotyledons (C) and mono-cotyledons (D and E), appeared in the progeny of a *patl2*$^{+/-}$*patl3456*$^{-/-}$ plant.
F-K. Mutant seedlings resulting from a cross between two homozygous plants (*patl2456*$^{-/-}$ amiPALT1 and *patl2456*$^{-/-}$ amiPATL3). We observed small seedlings (F), some seedlings displaying defects in cotyledons (H and G), rootless seedlings (I), plants without cotyledons (K), as well as a totally deformed seedling with nothing but a couple of leaf primordia-like structures(arrowhead in J). Bar size: 0.1 cm.

detected in roots in single, double and triple mutant combinations, indicating a strong functional redundancy among the *PATL* gene family. In the quadruple *patl2patl4patl5patl6* mutant (*patl2456*$^{-/-}$) the RAM was reduced by 25% in size (Figure 3D), likely due to a reduced cell number and cell size (Figure 3E and 3F). As the number of mitosis event is unaffected (Figure 3G)

Table 1. Segregation of the *patl2* allele in the progeny of a *patl2* $^{+/-}$ *patl3456* $^{-/-}$ plant.

patl2	n_O	f_O	n_E	f_E	
+ / +	14	0.1944	18	0.25	χ^2 = 13.861
+ / −	51	0.7083	36	0.5	p = 0.001
− / −	7	0.0972	18	0.25	
total	72	1	72	1	

n_O observed amount; f_O observed frequency; n_E expected amount; f_E expected frequency. Expected values under de assumption of Mendelian segregation of the traits and no linkage among the different alleles.

As *PATL2* and *PATL4* (not shown) are expressed during embryo development, we checked the quadruple mutant siliques for defects during reproductive development. Arabidopsisembryogenesis follows a highly controlled division pattern. After zygote formation, an initial asymmetric division generates a small apical cell and a larger basal cell. While the basal cell further divides into a cell file, the suspensor, the apical cell develops into an embryo body with clearly defined embryonic leaves, shoot and root poles (Figures 4A-4F). Ten percent of *patl2456* $^{-/-}$ embryos showed aberrant cell divisions of the hypophysis (compare Figures 4A'-4F' with Figures 4G' and 4H'), the future root meristem, which results in defective roots (Figure 5). As defects were observed only in the basal part of the embryo, we introduced the *patl3* mutation into the quadruple *patl2456* $^{-/-}$ mutant to further impair PATL function during embryogenesis. Interestingly 4.8% of the embryos of a segregating *patl2* $^{+/-}$ *patl3456* $^{-/-}$ Arabidopsis plant displayed polarity aberrance at basal (Figure 4I) and/or apical (Figures 4J and 4K) poles. Segregation distortion for *patl2* in the progeny of a *patl2* $^{+/-}$ *patl3456* $^{-/-}$ plant was repeatedly observed when compared to the Mendelian segregation (Table 1). So we hypothesized that the quintuple mutant was partially lethal. Indeed, 7.6% of *patl23456* $^{-/-}$ aberrant embryos managed to get through development producing very tiny seedlings (Figure 5A) as well as rootless (Figures 5B and 5C) and mono- or triple-cotyledon seedlings (Figures 5C-5E).

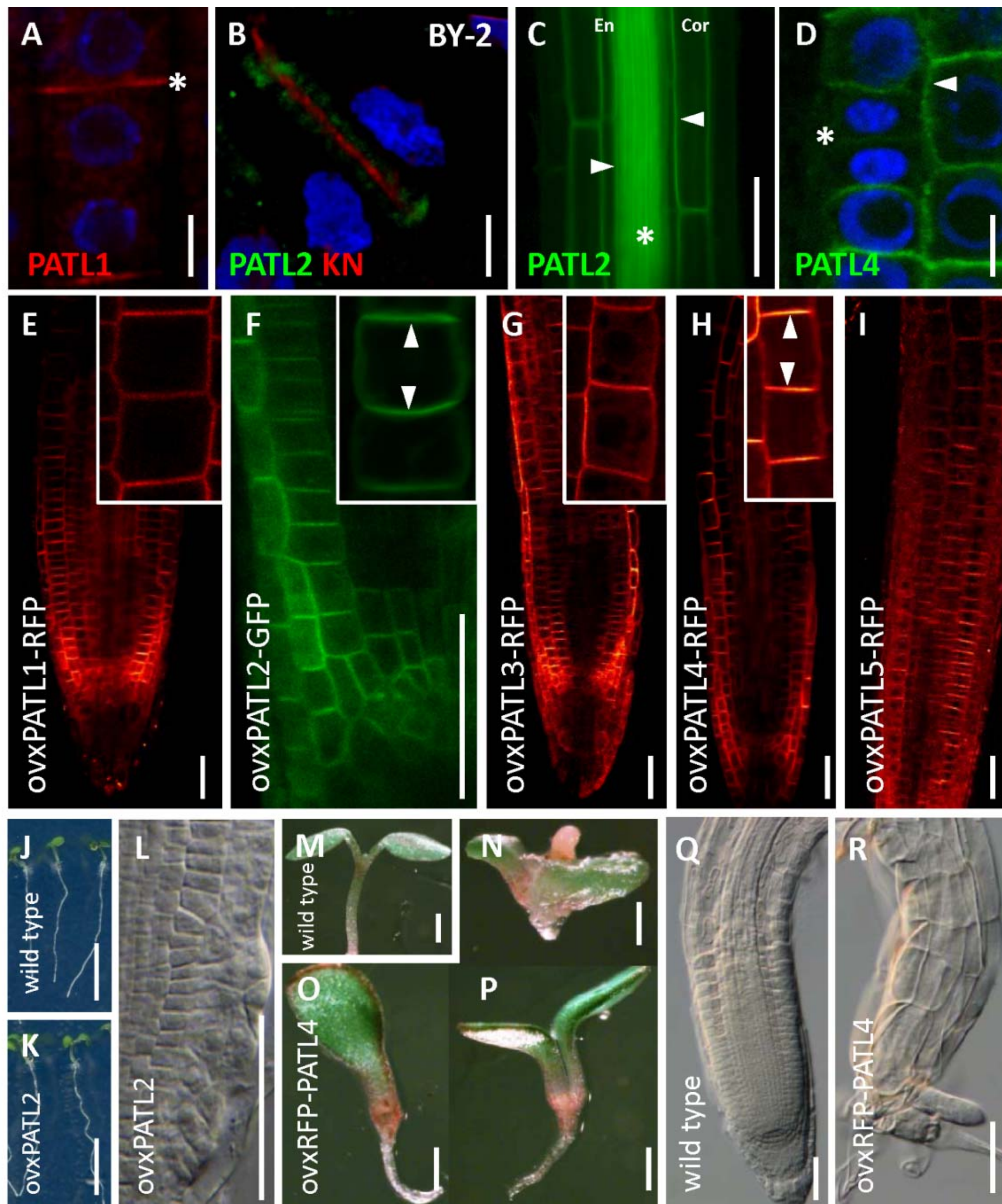

Figure 6. PATLs' subcellular localization.

A-D. PATL1 is localized to the cell plate during late stages of cytokinesis (asterisk in A). When expressed in tobacco BY-2 cells, PATL2-GFP localizes to the periphery of the forming cell plate (green signal in B) surrounding the KNOLLE (KN) labeling the cell plate (red signal in B). When expressed under its native promoter, PATL2-GFP localizes exclusively to the PM in cortex root cells (C, arrowhead) and inner vascular tissues (asterisk in C). PATL4-GFP is localized to the forming cell plate (D, asterisk) and the PM in cells that are not dividing (arrowhead in D).

E-I. PATLs polarly localize to the PM when overexpressed in the RAM. 35S:PATL1-RFP (E), 35S:PATL2-GFP (F), 35S:PATL3-RFP (G), 35S:PATL4-RFP (G), 35S:PATL5-RFP (I).

J-R. Seedling phenotypes in *PATL2* overexpressing (J, K, L) and *PATL4* overexpressing (M-R) lines.

PATL immunolocalization (A, B, D) or live cell imaging of PATLs tagged with a fluorescent protein (C, E-I). Endogenous PATL1 protein was detected using an antiPATL1 antibody (A) and PATL4-GFP (B) and PATL2-GFP (D) were detected using antiGFP antibodies. In blue is the DNA stained by DAPI (A, B, D).Bar size: A, D 10 µm; C, E-1, M, O-R 50 µm; J, K 0.5 cm; N 25 µm.

We sought to completely abolish the PATL function by generating artificial microRNAs (amiRNA) targeting *PATL1* and *PATL3* into a quadruple *patl2456*$^{-/-}$ mutant. Already in the first generation, 25% of *patl2456*$^{-/-}$*patl1/3*^{amiRNA} seedlings showed similar phenotypes compared to the above described ones for *patl2*$^{+/-}$ *patl3456*$^{-/-}$ mutants, including small seedlings (Figure 5F), rootless seedlings (Figure 5I), cotyledon defects (Figures 5G, 5H and 5K) and seedlings with ectopic structures (Figure 5J). All together these observations suggest a very crucial and redundant function for PATELLINs in root development and embryo patterning.

PATLs are membrane associated proteins

PATL1 is a membraneassociated protein located in small vesicles, at the PM and in forming cell plates (Figures 2B and 6A; Peterman et al., 2004). PATL1 clusters together with PATL2 (Figure 1) and as expected by their high functional redundancy, PATL2 is similarly able to localize to the cell plate in tobacco BY-2 cells (Figure 6B; Peterman et al., 2004). However, the *PATL2* gene is not endogenously expressed in actively dividing tissues like *PATL1*, *PATL3* and *PATL4*, but rather to differentiated tissues (Figure 2). In Arabidopsis roots, PATL2 is located at the PM in vascular tissues and in endodermis and cortex cells with certain enrichment at the cell side facing toward the stele (Figure 6C). On the other hand, PATL4 is localized to the forming cell plate in dividing cells and stays at the PM after cytokinesis (Figures 6D and S3). These observations point to common roles for PATLs linked to their PM association. Indeed, when overexpressed, *PATL1* to *5* localized to the PM with some degree of apical/basal asymmetry (Figures 6E-6I). Moreover, *PATL2* overexpression generated seedlings with wavy roots at a low frequency (5%, n = 50; Figure 6K) that could be explained by abnormal division patterns in the

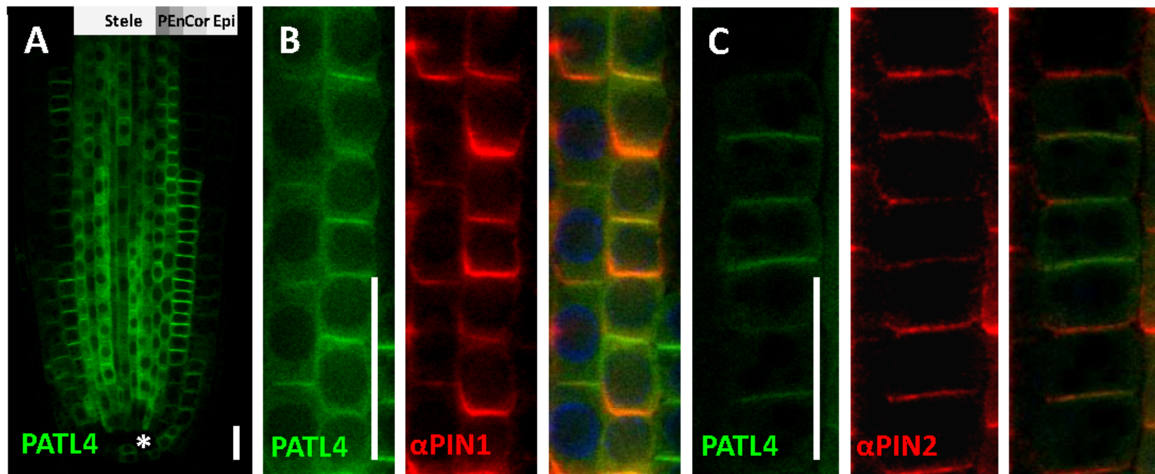

Figure 7. PATL4 is polarly localized at PM.
A. *PATL4-GFP* is expressed in all cell layers in root tips, without any distinction in the inner cell layers of the stele, the pericycle (P) and the endodermis (En). At some degree its expression is down regulated in older cells in the cortex (Cor) and epidermis (Epi), as well as in the QC (asterisk).
B, C. PATL4-GFP colocalizes with basal PIN1 in pericycle and endodermis (B) and apical PIN2 in epidermis (C). Bar size: 20 µm.

lateral root cap (Figure 6L), a tissue important for the signals transduction during root gravitropism (Perrin et al., 2005). On the other hand, *PATL4* overexpression generated strong seedling phenotypes (Figures 6M-6P) and root meristem collapse (Figure 6R), resembling the defects seen in multiple *patl* mutants (Figure 5). These results indicate that an imbalance in *PATL* expression perturbs root patterning, root gravitropism and embryogenesis. Moreover, together these observations suggest that all members of the PATL family associate to the PM, with specificity due to expression levels and tissue-and cell type-specific factors.

PATL4 polarly localizes at the plasma membrane with PIN proteins

All the defects described for *patl* loss- and gain-of-function mutants (Figures 4, 5 and 6) suggest a crucial function for PATLs in regulating polarity formation/establishment at different levels during plant development. We therefore analyzed in more detail PATL localization in Arabidopsis root tips using a pPATL4:PATL4-GFP marker line to include any transcriptional regulation and cell-specific determinants. PATL4-GFP is present in

actively dividing cells within the RAM and the initial cells surrounding the quiescent center (QC), but is totally absent from the QC (Figures 7A and S4). In the differentiation and elongation zones, *PATL4-GFP* expression is lost in epidermis and cortex, while still active in the inner cell layers of the root (Figure 7A). Similarly to PATL1, PATL4 is also associated to the cell plate and PM (Figures 6D and S3). Furthermore, *PATL* overexpression pointed out to asymmetric PM localization for PATL2 and PATL4 (Figures 6C, 6F, and 6H). A closer inspection revealed an asymmetric apical-basal distribution of PATL4-GFP at PMs of endodermis and cortex cells, where it colocalizes with polar auxin transporters PIN1 (endodermis) and PIN2 (cortex, Figures 7B and 7C). All these observations open the interesting possibility that PATL could be regulating PIN polar localization and PIN-mediated polar cell-to-cell auxin transport, thereby modulating plant development.

CONCLUSIONS

This work is the first attempt to describe the function of the family of GOLD-containing Sec14p-like proteins, the PATELLINs. The results presented here indicate that *PATL*s' overlapping expression patterns are developmentally regulated. *PATL*s are expressed in tissues with high cytokinesis rates (RAM) or entering a differentiation program (vascular tissues, root hairs and stomata). Therefore, in addition to the described localization and putative function of PATL1 during late cell plate formation (Peterman et al., 2004), they may be involved in regulating other lipid-based signaling pathways at the PM implicated in or linked to cell differentiation.

Our genetic analysis suggests that PATLs have redundant functions in plant development. In addition to their partially overlapping expression patterns, knocking out four of the six *PATL* genes (*patl2456*$^{-/-}$) produces mild defects during embryonic root patterning at low frequency. When only one *PATL* gene remained expressed in the quintuple mutant

background, stronger phenotypes in apical-basal patterning were observed. In addition, all PATLs shown here share a PM localization when expressed under a constitutive promoter and are localized to the cell plate when expressed in heterologous systems (for instance PATL2 in BY-2 cells). Overall, these data indicate that PATELLINs are a highly redundant class of plant PITPs with potential signaling functions during plant development.

PATLs are phosphoinositide binding proteins (based on PATL1 binding affinity) that are related to key enzymes involved in the interphase between lipid synthesis and lipid signaling, the PITPs. There are just a couple of examples of Arabidopsis PITPs described, and only one, the AtSfh1/COW1 had been functionally characterized (Vincent et al., 2004; Böhme et al., 2004). AtSfh1/COW1 gene is expressed mainly in roots, strongly in RAM and root hairs. *Atsfh1* knockout mutants display wavy and branched root hairs. Mutations in AtSfh1/COW1 were shown to enhance the root hair phenotypes described in AGD1, a phosphoinositide-binding ARF-GAP protein (Yoo et al., 2008). Interestingly, the mutant in the gene encoding for the PtdIns4-P 5-kinase 3 (PIP5K3) also enhanced the phenotypes in the *agd1* mutants and, on the contrary, mutations in the PtdIns4-P 4-PPtase RHD4 was epistatic to *arg1* (Yoo et al., 2012). Overall, these results indicate that phosphoinositide metabolism is somehow modulated by PITPs, such as COW1, hence regulate the phosphoinositide-dependent recruitment of ARF-GAP proteins and active small GTPases to specific membrane domains.

These result also suggest a positive effect of COW1 on PtdIns(4,5)P_2 metabolism and indicate a functional and/or physical interaction between PITP proteins and PIP5Ks. Taken together all those observations may shed light in the future directions of research to functionally characterize other PITP like the PATLs. PATLs are associated to the PM, where they may regulate the levels and/or localization of phosphoinositides. In this way,

PATLs would influence signaling cascades controlling directly or indirectly different PM proteins, such as PIN auxin efflux carriers. The extent of this regulation and the implications of these effects might help to explain the defects seen during plant development and extend the knowledge of PITP in plants.

MATERIAL AND METHODS

Plant material

All lines are in the Columbia background of Arabidopsis thaliana. Insertional mutants *patl2* (SALK_086866), *patl3* (SALK_093994), *patl4* (SALK_139423), *patl5* (SALK_124448) and *patl6* (SALK_099090) were obtained from ABRC and genotyped for homozygosity using the primers listed in Table S2. Seeds were sterilized overnight by chlorine gas, sown on solid Arabidopsis medium (0.5X Murashige and Skoog basal salts, 1% sucrose and 0.8% agar, pH 5.9) and stratified at 4°C for at least two days prior to transfer to a 16h:8hlight:darkgrowth room kept at 20°C. The seedlings were grown vertically for 4 to 12 days prior the analysis.

Cloning procedures and artificial microRNA generation

Coding sequences for PATELLINs as well as promoter sequences corresponding to 2000 bp upstream of the ATG codon were amplified using iPROOF DNA polymerase (BioRad), cloned using pENTR™ Directional TOPO® Cloning Kit (Invitrogen) or Gateway® BP Clonase (Invitrogen) and recombined into destination expression vectors as previously described (Karimi et al., 2007). All forward primers for cloning coding sequences contain the attB1 sequence GGGGACAAGTTTGTACAAAAAAGC AGGCTTC upstream of the gene-specific sequence, and all reverse primers contained the attB2 sequence GGGGACCACTTTGTACAA GAAAGCTGGGTC upstream of the gene-specific sequence. Similarly, forward primers for cloning promoter sequences contained the attB4 sequence GGGGACAACTTTGTATAG AAAAGTTGGA, and

reverse primers the attB1r adapter sequence GGGGACTGCTTTTTG TACAAACTTGC. All sequence-specific primers used for cloning are listed in Table S2.

Artificial microRNAs (amiRNA) were designed using the "Web microRNA designer" (Ossowski et al., personal communication; http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl). Briefly, we used full *PATL1* and *PATL3* coding sequences, selected for each gene two amiRNA sequences from the list of sequences suggested by the web designer (PATL1a TATAGTGTAGTTTGCTGGCGG; PATL1b TCGAATTGTTTAACAGCCCGT; PATL3a TGTCTTATTATAAAGCTCCGT; PATL3b TACACATAAGATATCTCGCTT) and generated two amiRNAs following a PCR-based approach using the primers indicated in Table S3 to generate point mutations in the microRNA precursor MIR319a (plasmid template pRS300). Then, the amiRNA sequences generated were cloned using Gateway technology and recombined to expression vectors containing the strong constitutive promoter RPS5a and transformed by floral dip into the quadruple homozygous PATELLIN mutant ($patl2456^{-/-}$). After selection of the transgenic plants, $patl2456^{-/-}$ $patl1^{amiRNA}$ and $patl2456^{-/-}$ $patl3^{amiRNA}$ were crossed to generate a sextuple mutant abolishing the PATL function in plant.

Expression analysis

Total RNA was extracted with the RNeasy Mini kit (Qiagen).Isolated RNA was treated with DNase I recombinant (Roche) to remove contaminating genomic DNA. For the RT-PCR reactions, Poly(dT) cDNA was prepared from 1 µg of total RNA with an iScript cDNA synthesis kit (Biorad). PCR conditions were as follows: PCR mix was heated for 5 min to 95°C, followed by 30 cycles of denaturation for 30 s at 95°C and annealing at 57°C for 30 s and extension for 60 s at72°C. As housekeeping gene, the expression of the constitutive gene ACTIN 8 (AT1G49240) was used.All the primers used are listed in Table S2.

GUS staining, immunolocalization and microscopy

Primary root and embryo immunolocalizations were performed as described by Sauer et al. (2006) using an automatized alternative. Antibodies were diluted at 1:1000 for rabbit anti-PIN1 and rabbit anti-PATL1 (Peterman et al., 2004), at 1:600 for rabbit anti-GFP (Molecular Probes) and the secondary rabbit anti-IgG conjugated to Cy3. GUS staining was performed as published (Swarup et al., 2008). The root tips were mounted in chloral hydrate and visualized using the DIC microscope Olympus BX51. For live imaging, seedlings at four days after germination were mounted in a drop of liquid Arabidopsis medium and visualized immediately. All the confocal pictures were taken with the confocal microscope Zeiss CSLM 710. All the quantifications were done using the ImageJ software (http://imagej.nih.gov/ij/).

Phylogenetic analysis

All *Sec14p-like* genes in Arabidopsis were identified using the BLAST tool from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/Blast/index.jsp) with the yeast Sec14p (scSec14p; YMR079W) as query sequence. 32 Arabidopsis proteins appeared as having some degree of homology to scSec14p (Table S1).The phylogenetic analysis was performed using the web-based tool freely available at http://www.phylogeny.fr/ using Muscle alignment and Neighbor-Joining method with 100 bootstraps.

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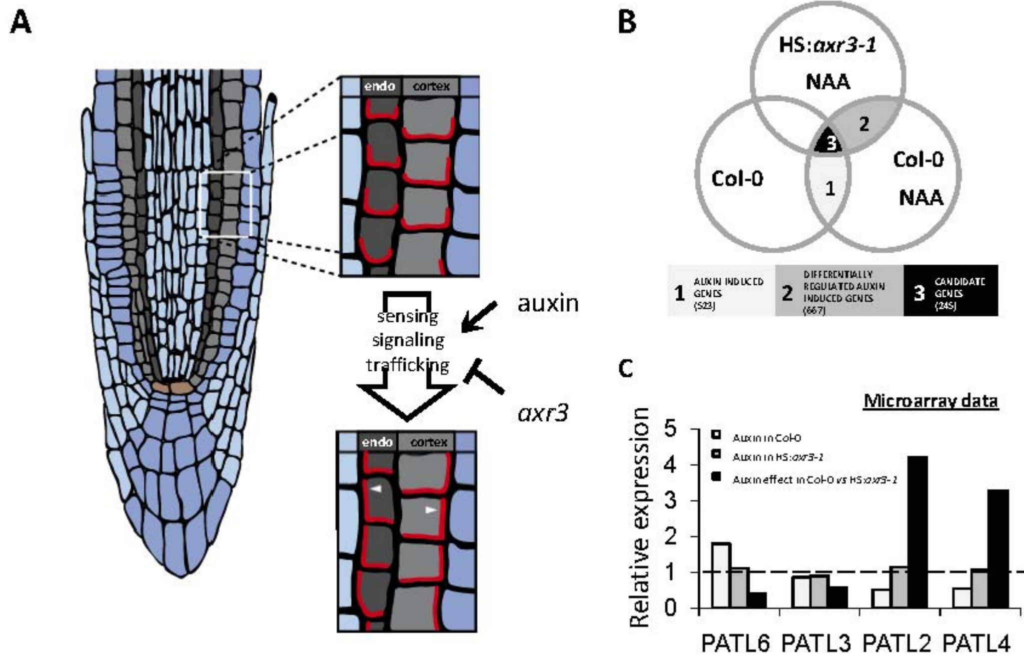

Figure S1. Microarray for auxin effect on PIN lateralization identifies PATELLINS as candidate genes.

A. PIN1 and PIN2 are basally localized in endodermis and young cortex cells, respectively, pumping auxin in the direction of the root tip. Auxin is able to modify PIN1 and PIN2 localization to baso-lateral. This effect is dependent on the AXR3-mediated transcription of so far unknown components.

B. Microarray results. Among the auxin-regulated genes in wild type, we selected 245 gene candidates as being auxin inducible but differentially expressed in the dominant negative version of AXR3 (HS:*axr3-1*).

C. PATELLINs are auxin-regulated genes in an AXR3-dependent fashion. PATL 6 is auxin inducible depending onAXR3. On the other hand, PATL2 and PATL4 are negatively regulated by auxin and in the HS:*axr3-1* background this effect is lost.

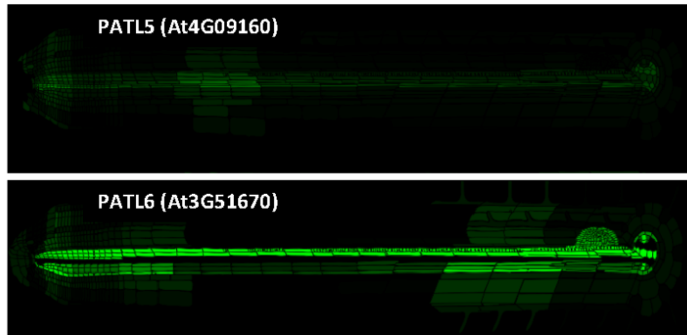

Figure S2. *PATL5* and *PATL6* expression levels.
Expression data for *PATL4* and *PATL6* using the Arabidopsis eFP browser.

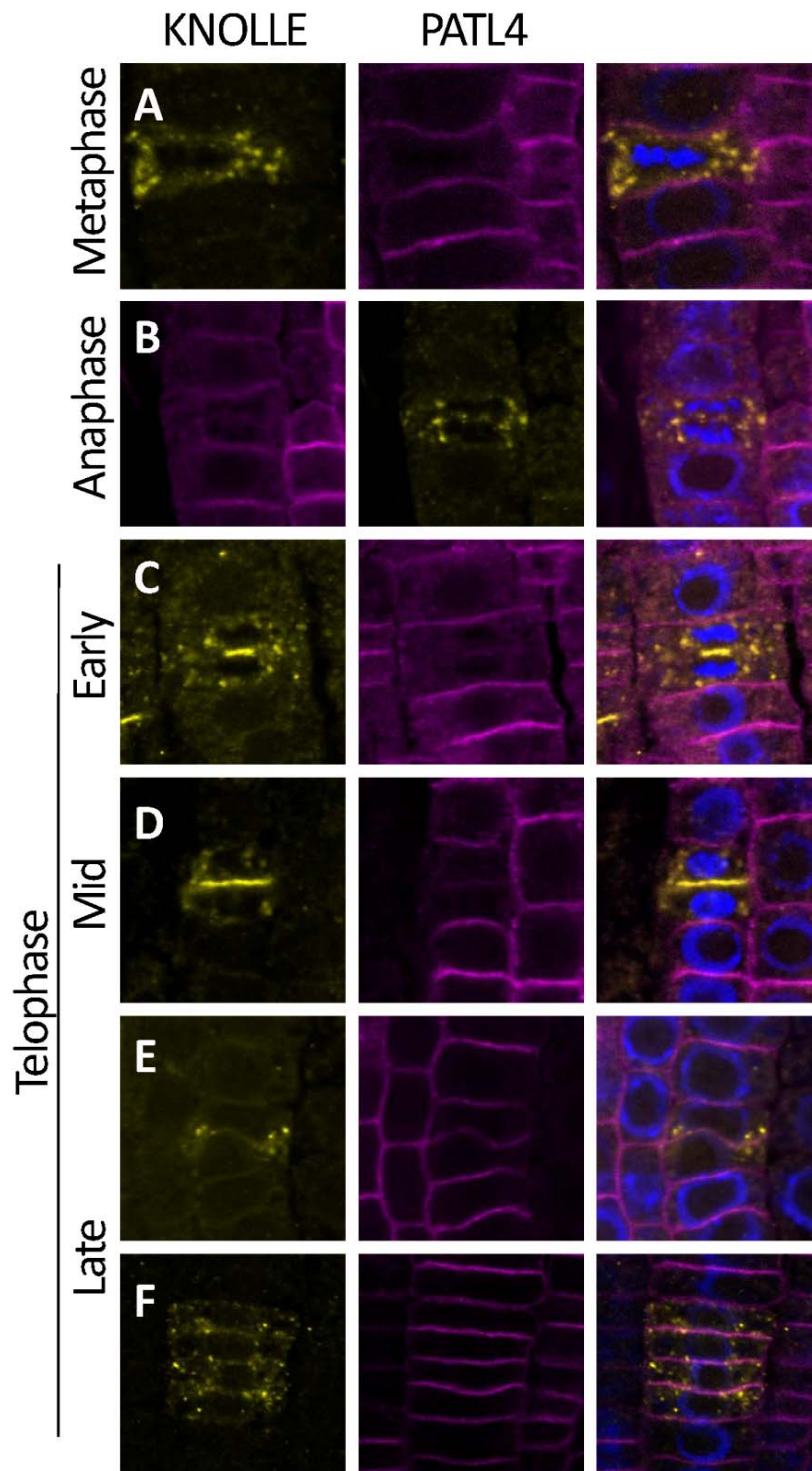

Figure S3. PATL4 localization dynamically switches between PM and cell plate in cells ongoing cytokinesis.

A-F. The different stages of root cortex cell cytokinesis are shown: Metaphase (A), Anaphase (B), and early Telophase (C), mid Telophase (D) and late Telophase (E, F). Whole mount root immunolocalization using anti-KNOLLE (in yellow) antibody in the background of the *pPATL4:PATL4-GFP* transgenic line (GFP fluorescence in magenta). We used DAPI to stain nucleic acids (blue).

Table S1. Sec14-like proteins in Arabidopsis.

Gene model	ATI code	Sec14p homology			Other protein domains
		Identities	+	E value	
AtSFH1/COW1	AT4G34580	102/242	152/242	1e-53	Nodullin[a]
AtSFH2	AT4G39180	102/236	146/236	2e-53	Nodullin[a]
AtSFH3	AT2G21540	104/250	150/250	1e-52	Nodullin[a]
AtSFH4	AT1G19650	103/241	150/241	1e-51	Nodullin[a]
AtSFH5	AT1G75370	101/241	147/241	3e-48	Nodullin[a]
AtSFH6	AT4G39170	97/239	149/239	1e-50	Nodullin[a]
AtSFH7	AT2G16380	102/248	149/248	1e-49	Nodullin[a]
AtSFH8	AT2G21520	97/243	150/243	7e-49	Nodullin[a]
AtSFH9	AT3G24840	100/242	141/242	2e-47	Nodullin[a]
AtSFH10	AT2G18180	89/232	140/232	1e-47	Nodullin[a]
AtSFH11	AT5G47510	95/236	133/236	2e-46	ND
AtSFH12	AT4G36490	91/243	146/243	4e-47	Nodullin[a]
AtSFH13	AT1G55690	90/237	141/237	5e-40	Nodullin[a]
AtSFH14	AT5G56160	90/236	142/236	4e-43	ND
PATL1	AT1G72150	58/221	100/221	2e-11	GOLD; CRAL/Trio
PATL2	AT1G22530	69/221	103/221	3e-11	GOLD; CRAL/Trio
PATL3	AT1G72160	62/216	106/216	4e-17	GOLD; CRAL/Trio
PATL4	AT1G30690	68/224	106/224	2e-16	GOLD; CRAL/Trio
PATL5	AT4G09160	62/217	101/217	2e-15	GOLD; CRAL/Trio
PATL6	AT3G51670	68/232	102/232	1e-14	GOLD; CRAL/Trio
AtPITPs	AT4G36640	75/254	116/254	9e-18	ND
	AT5G47730	60/209	99/209	2e-16	ND
	AT1G55840	62/209	98/209	4e-16	ND
	AT1G01630	66/218	98/218	1e-15	ND
	AT1G75170	67/217	102/217	4e-15	ND
	AT1G22180	31/96	56/96	7e-08	ND
	AT4G08690	56218	97/218	2e-10	ND
	AT1G14820	54/222	96/222	2e-08	ND
	AT3G46450	54/213	86/213	2e-06	ND
	AT1G05370	41/182	79/182	5e-05	ND
	AT5G63060	52/214	88/214	2e-03	ND
	AT3G22410	39/183	85/183	5e-04	ND

Table S2. List of primers used for cloning procedures, *patl* mutant genotyping, amiRNA construct and transcripts level analysis.

Gene	Sequence	Orientation	Application
PATL1	ATGGCTCAAGAGGAAGTAC	Fw	Cloning coding sequence, RT-PCR
PATL1	AGTTTTGAACCTGTAGTAG	Rv	Cloning coding sequence, RT-PCR
PATL2	ATGGCTCAAGAAGAGATAC	Fw	Cloning coding sequence, RT-PCR
PATL2	TGCTTGGGTTTTGGACCTG	Rv	Cloning coding sequence, RT-PCR
PATL3	ATGGCTGAAGAACCTACTAC	Fw	Cloning coding sequence, RT-PCR
PATL3	GAGAGGTTTGACATTGAAC	Rv	Cloning coding sequence, RT-PCR
PATL4	ATGACTGCTGAAGTTAAGG	Fw	Cloning coding sequence, RT-PCR
PATL4	GGAAGAGGATTCAGTCTTG	Rv	Cloning coding sequence, RT-PCR
PATL5	ATGTCTCAAGATTCTGCAAC	Fw	Cloning coding sequence, RT-PCR
PATL5	CTCACAAGCTAAAGGCTTA	Rv	Cloning coding sequence, RT-PCR
PATL6	ATGGATGCTTCATTGTCTCC	Fw	Cloning coding sequence, RT-PCR
PATL6	GACGGTTGTAGTAGATTTCCGG	Rv	Cloning coding sequence, RT-PCR
ACTIN8	ACCTTGCTGGTCGTGACCTTACTG	Fw	RT-PCR
ACTIN8	GATCCCGTCATGGAAACGATGTCTC	Rv	RT-PCR
PATL1	GGATTTTTAACGGATCACTC	Fw	Promoter sequence cloning
PATL1	CTTTCTTGTCTGATTTTAGA	Rv	Promoter sequence cloning
PATL2	TCCGGTTTGACTGGATTTTT	Fw	Promoter sequence cloning
PATL2	GATCACTTGATTCGAAAGGG	Rv	Promoter sequence cloning
PATL3	TTTTTACTTGTGCCGTCTTG	Fw	Promoter sequence cloning
PATL3	GCAGGTTTAGGAAACAATTC	Rv	Promoter sequence cloning
PATL4	ATAACTGTTGACTTCAACTA	Fw	Promoter sequence cloning
PATL4	CTTAAAGCCTGTCATTCAGA	Rv	Promoter sequence cloning
PATL5	CCCTAATTCACATTGGTC	Fw	Promoter sequence cloning
PATL5	TTTTTTATTGTTCTTGAA	Rv	Promoter sequence cloning
PATL6	TATTTAGCCATAGTGGAAAG	Fw	Promoter sequence cloning
PATL6	TGTTTCTTGAGAGTTTTTC	Rv	Promoter sequence cloning
PATL2	GGAAAAATCTCTTGAGGCTGAA	Right	Genotyping *patl*mutants
PATL2	CTTGTTTGTCGACACCGTGAG	Left	Genotyping *patl*mutants
PATL3	GTCATTGGATCCAATTTCACG	Right	Genotyping *patl*mutants
PATL3	AACCTTCTCAAGATCATCCAC	Left	Genotyping *patl*mutants
PATL4	TCTACTGTTTTGAACCCACCG	Right	Genotyping *patl*mutants
PATL4	CTGAGGCTGTTGTTACCGAAG	Left	Genotyping *patl*mutants
PATL5	TTTGTAGCTGGTGGTGTTTCC	Right	Genotyping *patl*mutants
PATL5	GGCTTTTGTTACTCACAAGC	Left	Genotyping *patl*mutants
PATL6	CAAACCCAAGAAAGAAAACCC	Right	Genotyping *patl*mutants
PATL6	ATTTGTGCGGTTTCTTGAG	Left	Genotyping *patl*mutants
LBb1+	ATTTTGCCGATTTCGGAAC		Genotyping *patl*mutants
LBa1	TGGTTCACGTAGTGGGCCATCG		Genotyping *patl*mutants

Chapter 5

Fluorescence-based screen to identify new components in the PIN2 trafficking pathway

Ricardo Tejos, Mugurel I. Feraru and Jiří Friml

SUMMARY

The auxin distribution changes dynamically during many plant processes due to constitutive PIN recycling. This mechanism may be important for targeting auxin transporters to a new cell surface in response to environmental or developmental cues. Some of the components involved in PIN trafficking are known to date, but a complete understanding of this process and some important molecular components is still missing. The PIN2-GFP protein is expressed in the epidermal and root cap cells where it is polarly localized. The fungal toxin Brefeldin A (BFA) stops the normal PIN2 cycling to the plasma membrane, leading to its accumulation in endosomal-like structures inside the cell, the "BFA bodies". The PIN2-GFP response to BFA enabled us to screen for endocytic mutants (*endo*) after a short BFA treatment as the mutants not forming BFA bodies, and for exocytic mutants (*exo*) as those that displayed BFA bodies after a long BFA treatment. Moreover, we were able to find mutants defective in PIN2 polarity and protein stability. Here, we present the results of the screen and some initial characterization of the mutants. Overall, this fluorescence-based screen provides a powerful tool for finding new components in trafficking and polarity pathways.

The phytohormone auxin has a great impact on several developmental programs and tropism responses in plants (Grunewald and Friml 2010; Durbak et al., 2012; Sundberg and Østergaard 2009). Its accumulation or depletion in specific cell types can trigger different developmental programs in a spatio-temporal context (Lau et al., 2011; Weijers et al., 2006; Dubrovsky et al., 2008). A central mechanism controlling local auxin homeostasis is the polar cell-to-cell transport (polar auxin transport, PAT), which is generated by the concerted action of auxin transporters (Robert and Friml 2009). The polarly localized PINFORMED (PIN) proteins, which work at the plasma membrane (PM) pumping auxin outside the cell (Petrásek et al., 2004), have been shown to play a central role in the directionality of the auxin flow (Wisniewska et al., 2006) and thereby in generating and maintaining auxin gradients during embryo patterning (Friml et al., 2003), organogenesis (Benková et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Scarpella et al., 2006), gravi- and phototropism (Friml et al., 2002; Harrison et al., 2008; Kleine-Vehn et al., 2010; Rakusová et al., 2011; Ding et al., 2011) and in the re-specification of vascular tissues after wounding (Sauer et al., 2006a).

The mechanisms controlling PIN targeting to the PM and the factors determining PIN polar localization at the PM are of central importance to understand auxin response and auxin-related processes. PIN proteins are constitutively endocytosed by a clathrin-dependent mechanism (Dhonukshe et al., 2007) and they cycle from the PM to internal endosomes and back to the PM in a process dependent on the guanine nucleotide exchange factor on ADP ribosylation factor G protein (ARF-GEF) GNOM (Steinmann et al., 1999). GNOM is sensitive to the fungal drug Brefeldin A (BFA; Geldner et al., 2003) and hence, after short BFA treatments, PIN1 cycling is impaired and PIN1 is accumulated inside the cell in so-called “BFA bodies”. This effect is fully reversible: after washing out BFA from seedlings, the BFA bodies disappear due to restoration of exocytosis. On the

other hand, more than 15-h BFA treatment makes basally localized PIN1 to switch to the apical side of the cell, making the otherwise visible BFA bodies disappear (Kleine-Vehn et al., 2008a and 2008b). It had been proposed that the basal recycling pathway is fully sensitive to BFA while the apical pathway is only partially sensitive so the effect seen after long BFA treatments, which is explained by a transcytosis-like mechanism targeting the rather basal PIN1 to the apical pathway after inhibiting the basal pathway (Kleine-Vehn et al., 2008a and 2008b). The initial observation that PIN proteins cycle from the PM to internal endosomes opened a new view for PAT control and therefore big efforts had been made in characterizing PIN trafficking pathways.

The above described BFA effect on PIN trafficking was already used to screen weak mutants impaired in trafficking pathways. By using an ethyl methane sulfonate (EMS)-mutagenized PIN1:PIN1-GFP population, Tanaka and collaborators (2009) described a set of *BFA visualized endocytic trafficking defective* (*Ben*) mutants from which *Ben1* was mapped and characterized as a new ARF-GEF involved in PIN1 endosomal trafficking. The basal pathway, represented by PIN1 in root stele cells and PIN2 in young cortex cells, and the apical pathway, represented by PIN2 in epidermis or AUX1 in stele cells, are distinct in their molecular components (Kleine- Vehn et al., 2006) and BFA sensitivity (Kleine-Vehn et al., 2008a and 2008b). Therofore, we used a PIN2:PIN2-GFP AUX1:AUX1-YFP EMS-mutagenized line to screen for new regulators of PIN trafficking linked to the apical pathway, exploiting BFA sensitivity as a tool to visualize the endocytic route. We selected mutants resistant to the BFA effect in terms of PIN2-GFP BFA bodies formation after short and long treatments, as well other interesting mutants realted to PIN2-GFP or AUX1-YFP proteins levels, differential AUX1-YFP sensitivity to BFA PIN2-GFP polar localization.

RESULTS

The screen

We aimed to screen for new components of the PIN2 trafficking pathway by exploiting BFA sensitivity as a tool. PIN proteins are localized at the PM and they can be visualized either by translational fusions with green fluorescent proteins (Figures 1A-1C) or by immunolocalization using specific anti-PIN antibodies (Figures 1D-1F). A short BFA treatment induces intracellular PIN2 agglomerations ("BFA bodies", Figures 1B and 1E) which disappear after prolonged treatments (Figures 1C and 1F). We designed a forward genetic screen to identify mutants that were impaired in PIN2-GFP BFA body formation after 90 min of treatment and for mutants that were not able to efficiently remove BFA bodies after a long, 15- to 24-h treatment (Figure 1G). As endocytosis is only partially sensitive to BFA treatment and vacuolar targeting is thought to be inhibited in the screening conditions we used (e.g. BFA 50 µM; Kleine-Vehn et al., 2008c), we aimed to get new molecular components in endocytic/exocytic PIN2 trafficking pathways (Figure 1G). As short BFA treatment effectively agglomerates PIN2-GFP coming from endocytosis we called these *endo* mutants; and mutants with BFA bodies still present after long

Table 1. Overview of the screening results.

	PRIMARY SCREENING			RE-SCREENING						
					Confirmed phenotype		Agravitropic[§]			
MUTANT DESCRIPTION	N*	Total[&]	MEAN (mutant/pool)	Total**	GFP	PIN2	+	++	+++	Total
PIN2 endocytosis	96	352	3,03	94	4	1	5	17	9	31(33%)
PIN2 exocytosis	69	173	1,49	25	5	2	4	10	6	20(29%)
PIN2 localization	9	11	0,09	4	0	0	0	0	0	0(0)

* Total amount of pools which it was found one more mutant.
& Total number of mutants found.
** Total number of mutant rescreened
§ The agravitropic phenotype was recorded based on vertically grown plants and divided into weak (+), medium (++) and strong (+++) agravitropic roots. The percentage of the agravitropic plants in the total amount of mutants re-screened is shown.

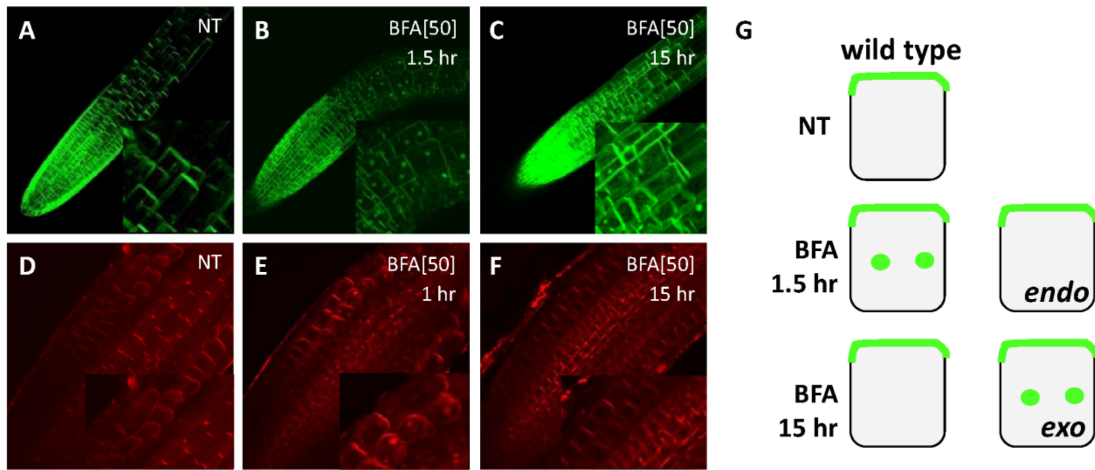

Figure 1. Overview of the screening.
A-F. Wild-type PIN2-GFP AUX1-YFP line imaged under a confocal microscope (A-C) or immunolocalized using anti-PIN1 and anti-PIN2 antibodies (E-F). Under normal, not treated (NT) conditions, (A, D) PIN2-GFP and AUX1-YFP accumulated to the PM. Upon short, 1.5-h BFA 50 µM treatment, intracellular PIN2-GFP/AUX1-YFP (B) and also PIN1 (E) agglomerations appeared. Extended BFA treatments for 15 hours or longer (C, F) made BFA induced agglomerations, so-called BFA bodies, disappear.
G. Schematic representation of the genetic screen. We used 300 pools (1,200 M1 plants) of an EMS-mutagenized PIN2:PIN2-GFP AUX1:AU1-YFP (*pin2 aux1*) line (referred to as wild type). Root epidermal cells accumulate PIN2-GFP at the apical side of the cell (drawn in green) and when exocytosis is inhibited by BFA, all endocytosed PIN2-GFP proteins get "trapped" in intracellular structures. *Endocytosis* mutants (*endo*) were designated as those that do not form intracellular agglomerations. After longer treatments, the accumulated PIN2-GFP proteins are sent back to the PM by exocytosis. *Exocytosis* mutants (*exo)* where identified here as those accumulating PIN2-GFP in BFA bodies after long incubation (15 hours and more) with BFA.

BFA treatment, which are assumed to be due to a defective exocytosis, we called *exo* mutants.

We screened an EMS-mutagenized M2 population of PIN2-GFP AUX1-YFP. After screening 30,000 seedlings coming from 1,200 M1 plants using a fluorescence stereomicroscope, we confirmed in M3 four *endo* and five *exo* mutants (Table 1). In the course of the screening, we also identified mutants with abnormal PIN2-GFP localization and AUX1-YFP hypersensitivity to BFA, and agravitropic mutants that were impaired in AUX1-YFP protein stability (Table 1). In the following sections we describe some of these mutants and the current status of characterization.

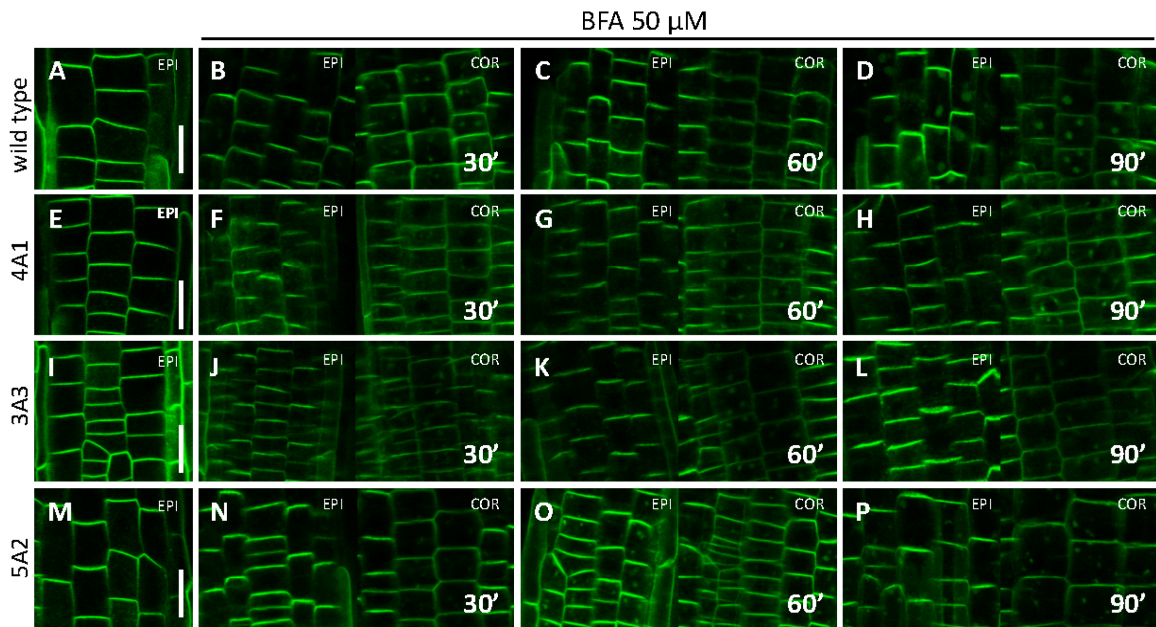

Figure 2. *Endo* mutants have different sensitivities to BFA treatment.
A-P. Seedlings 4 days after germination were treated with mock DMSO control for 90 min (A, E, I, M) or with BFA 50 μM for 30 min (B, F, J, N), 60 min (C, G, K, O) or 90 min (D, H, L, P) and then imaged in the confocal microscope. All pictures were taken with identical confocal settings. Epi: epidermis; Cor: Cortex. Bar represents 20 μm.

Endo mutants

Wild-type root cells respond to BFA as early as after 30 min by gradually forming BFA bodies, usually two or three per cell, the response in cortex and epidermis being slightly different (Figures 2A-2D). Contrary to what we expected, we did not find mutants that were totally resistant to BFA in terms of intracellular agglomeration; rather we found four *endo* mutants that were impaired in BFA body formation, creating smaller bodies similarly to what was described earlier for *ben* mutants (Tanaka et al., 2009). In *endo*-4A1, *endo*-3A3, and *endo*-5A2 mutants, BFA bodies are visible but smaller and less evident after 30 min (Figures 2F, 2J, 2N) when compared to wild type (Figure 2B) in cortex and epidermal cells, while the overall protein content seems to be the same (Figures 2A, 2E, 2I and 2M). After 60 min and 90 min, wild-type epidermis and cortex cells form bigger BFA bodies over time (Figures 2C and 2D), while *endo*-4A1 (Figures 2G and 2H), *endo*-3A3 (Figures 2K and 2L) and *endo*-5A2 mutants

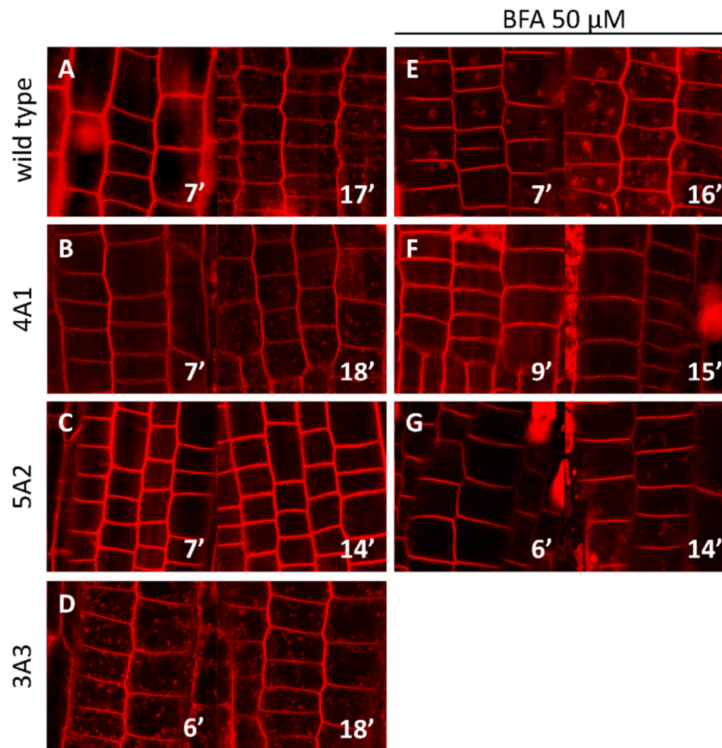

Figure 3. *Endo* mutants have an impaired FM4-64 uptake.
A-B. Time course of FM4-64 uptake in wild type (A) and *endo*-4A1 (B), *endo*-5A2 (C) and *endo*-3A3 mutants.
E-G. Seedlings of wild type (E) and *endo*-4A1 (F) and *endo*-5A1 (G) mutants were preincubated for 30 min in BFA 50 µM and then the FM4-64 uptake was followed.
Seedlings 4 days after germination (DAG) were incubated for 5 min on ice in liquid Arabidopsis media containing 1 µM FM4-64, mounted and immediately visualized under a confocal microscope. The time points correspond to the minutes that passed between removing the seedlings from the medium containing FM4-64 and mounting them for imaging.

(Figures 2O and 2P) do not aggregate them further. After 90 min, these three *endo* mutants display smaller BFA bodies in cortex cells (Figures 2H, 2L and 2P) compared to wild type (Figure 2D). Wild-type epidermal cells form big BFA bodies (Figure 2D), *endo*-4A1 (Figure 2H) and *endo*-5A2 mutants (Figure 2P) form small multiple agglomerations and *endo*-3A3 mutants do not form visible BFA bodies in the epidermis (Figure 2L). We then visualized the trafficking of endocytosed membranes using the lipid dye FM4-64, which labels the PM and is internalized and traffics through the endocytic pathway towards the vacuole. In wild-type epidermal cells, FM4-64 was incorporated into the PM and labeled intracellular vesicles (Figure 3A) that were rapidly aggregated after BFA treatment (Figure 3E). Different sensitivities

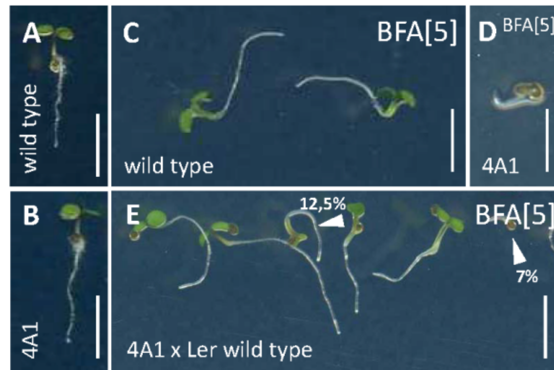

Figure 4. *Endo*-4A1 mutant is hypersensitive to BFA.
A-D. The growth of mutant *endo*-4A1 is inhibited when germinated in the presence of 5 µM BFA (D) when compared to wild type (C), in which there is only a visible effect on gravitropism. Both wild-type (A) and *endo*-4A1 (B) seedlings, germinated in media without BFA, are similar in size.
E. The hypersensitivity of *endo*-4A1 to BFA segregates in the second generation of the cross with Landsberg *erecta* (L*er*) wild type. The percentages indicate the fraction of mutant seedlings found. n = 2145.
Wild type (A, C), *endo*-4A1 mutants (B, D), and the cross between *endo*-4A1 and L*er* (E) were grown in the presence of 5 µM BFA for 4 days (C, D, E) or in a control medium (A, B). Size bars represent 0.5 cm (A-C, E) and 0.2 cm (D).

were seen for *endo* mutants. *Endo*-4A1 internalized FM4-64 dye in a normal way (Figure 3B) but this internalization was inhibited by BFA (Figure 3F); *endo*-5A2 shows a reduced FM4-64 uptake (Figure 3C) that is not totally inhibited as BFA treatment resulted in some internalized dye agglomerations (Figure 3G). Surprisingly, *endo*-3A3 showed an enhanced FM4-64 uptake (Figure 3D), which does not explain the slower formation of BFA body of PIN2-GFP in the epidermis as a defect in endocytosis, but it may point to a defect in a post-endocytic event.

An additional observation came from an enhanced sensitivity to BFA seen in the *endo*-4A1 mutant. Compared to wild type, *endo*-4A1 mutant seedlings have a comparable size (compare Figures 4A and 4B). When germinated in the presence of low amounts of BFA (5 µM), wild-type seedlings were agravitropic while their overall size did not change dramatically (Figure 4C). On the other hand, *endo*-4A1 mutants were hardly germinating and their growth was totally inhibited in the presence of BFA (Figure 4D). This phenotype segregated closer to the expected 25% in the second generation after crossing

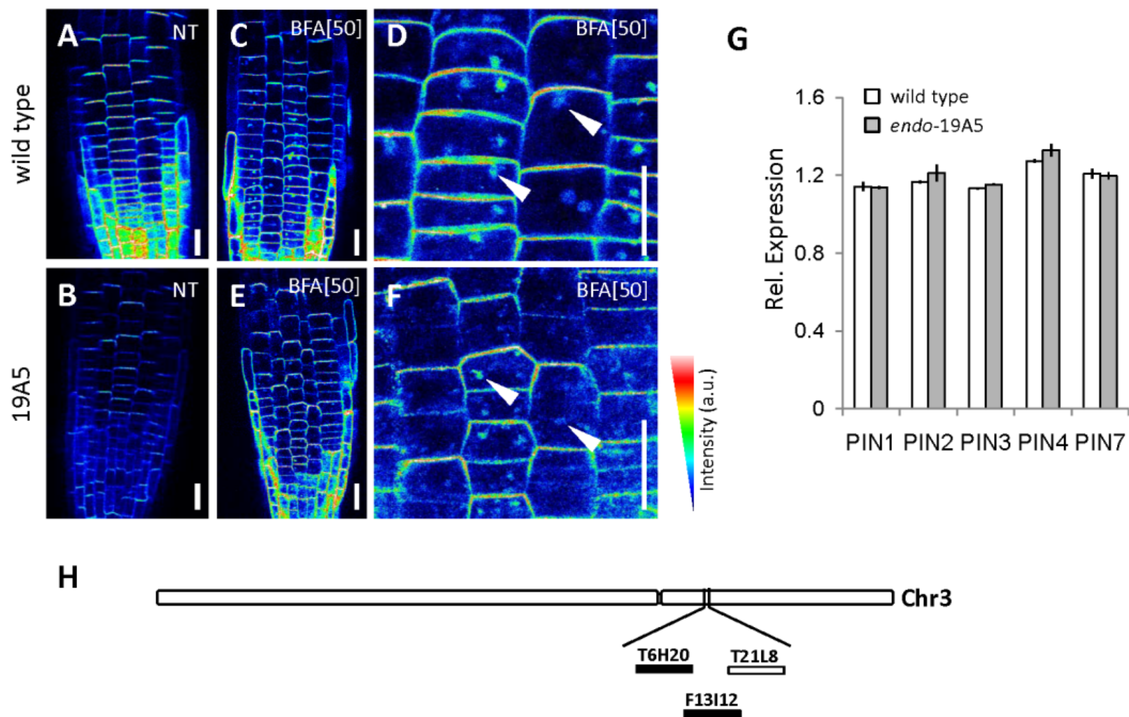

Figure 5. The *endo*-19A5 mutant has reduced amounts of PIN2-GFP at the PM due to a defect in an unknown posttranscriptional mechanism.
A-F. Root epidermal cells of wild type (A, C, D) and *endo*-19A5 (B, E, F) treated with 50 µM BFA for 60 min (D, C, E, F), or of not treated controls (A, B). The *endo*-19A5 mutant accumulated less PIN2-GFP at the PM (B) compared to wild type (A). BFA bodies in wild type (C, arrowheads in C) are considerable bigger than in *endo*-19A5 mutants (E, arrowhead in F). Bar size 20 µm.
G. Quantitative PCR (qPCR) for PINs in root tissues of wild type and *endo*-19A5 mutant. There is not a significant change in transcript levels. The mean of three independent experiments and SE is shown.
H. The *endo*-19A5 mutant has been partially mapped between two markers T6H20-XbaI and F13I12-18 to a region containing 32 genes (Table 2).

to Landsberg *erecta* (L*er*) wild type (Figure 4E), indicating that it is linked to a single mutant allele, and every BFA hypersensitive seedling was also showing the subcellular mutant phenotype (Figures 2E-2H). This could be used for further efforts in establishing mapping populations for cloning the *endo*-4A1 gene.

The *endo*-19A5 mutant was also found due to a reduced BFA formation, but this defect was more likely a consequence of reduced amounts of PIN2-GFP and AUX1-YFP proteins at the PM (Figures 5A and 5B) rather than to a defect in trafficking. Nonetheless, BFA body sizes were clearly smaller in the *endo*-19A5 mutant compared to wild type (Figures 5C-5F). We then tested the hypothesis whether the reduced amount of

Table 2. Genes within the mapping interval of *endo*-19A5.

Gene Locus	Description	Seq.[%]
AT3G46700	UDP-Glycosyltransferase superfamily protein	–
AT3G46710	NB-ARC domain-containing disease resistance protein;	–
AT3G46720	UDP-Glycosyltransferase superfamily protein	–
AT3G46730	NB-ARC domain-containing disease resistance protein; FUNCTIONS IN: ATP binding; INVOLVED IN: defense response, apoptosis; LOCATED IN: endomembrane system	–
AT3G46740	Component of the translocon outer membrane (TOC) complex. Forms the outer envelope translocation channel (beta-barrel)	–
AT3G46750	unknown protein	–
AT3G46760	Protein kinase superfamily protein	–
AT3G46770	AP2/B3-like transcriptional factor family protein;	–
AT3G46780	plastid transcriptionally active 16 (PTAC16);	–
AT3G46790	Encodes a member of a PCMP (plant combinatorial and modular protein) family (PCMP-H subfamily) with 9 pentatricopeptide (PPR) repeats. The protein is involved the intergenic processing of chloroplast RNA between rps7 and ndhB, which is essential for ndhB translation.	–
AT3G46800	Cysteine/Histidine-rich C1 domain family protein; FUNCTIONS IN: zinc ion binding; Involved in intracellular signaling pathway;	–
AT3G46810	Cysteine/Histidine-rich C1 domain family protein; FUNCTIONS IN: zinc ion binding; Involved in intracellular signaling pathway;	–
AT3G46820	Encodes the catalytic subunit of a Type 1 phosphoprotein Ser/Thr phosphatase, expressed in roots, shoots and flowers.	N.M.
AT3G46830	RAB GTPase homolog A2C (RABA2c)	N.M.
AT3G46840	Subtilase family protein;	N.M.
AT3G46850	Subtilase family protein	N.M.
AT3G46860	Predicted to encode a PR (pathogenesis-related) peptide that belongs to the PR-6 proteinase inhibitor family. Six putative PR-6-type protein encoding genes are found in Arabidopsis	N.M.
AT3G46870	Pentatricopeptide repeat (PPR) superfamily protein;	N.M.
AT3G46875	pre-tRNA; tRNA-Ser (anticodon: AGA)	N.M.
AT3G46880	unknown protein	N.M.
AT3G46890	unknown protein	N.M.
AT3G46900	encodes a member of copper transporter family and functionally complements a high affinity copper transporter mutant in yeast	N.M.
AT3G46901	unknown protein	N.M.
AT3G46904	This gene encodes a small protein and has either evidence of transcription or purifying selection.	N.M.
AT3G46910	Cullin family protein; ubiquitin protein ligase binding	N.M.
AT3G46911	pseudogene of CUL4 (protein binding / ubiquitin-protein ligase)	N.M.
AT3G46920	Protein kinase superfamily protein with octicosapeptide/Phox/Bem1p domain	N.M.
AT3G46930	Protein kinase superfamily protein	N.M.
AT3G46940	DUTP-PYROPHOSPHATASE-LIKE 1 (DUT1)	N.M.
AT3G46950	Mitochondrial transcription termination factor family protein;	N.M.
AT3G46960	The gene encodes a DExD/H box RNA helicase, involved in the regulation of K+ deprivation stress response.	–

[%] **:** Genes were fully sequenced in the background of *endo*-19A5 and compared to wild type for finding mutations. N.M.: no mutations found in *endo*-19A5

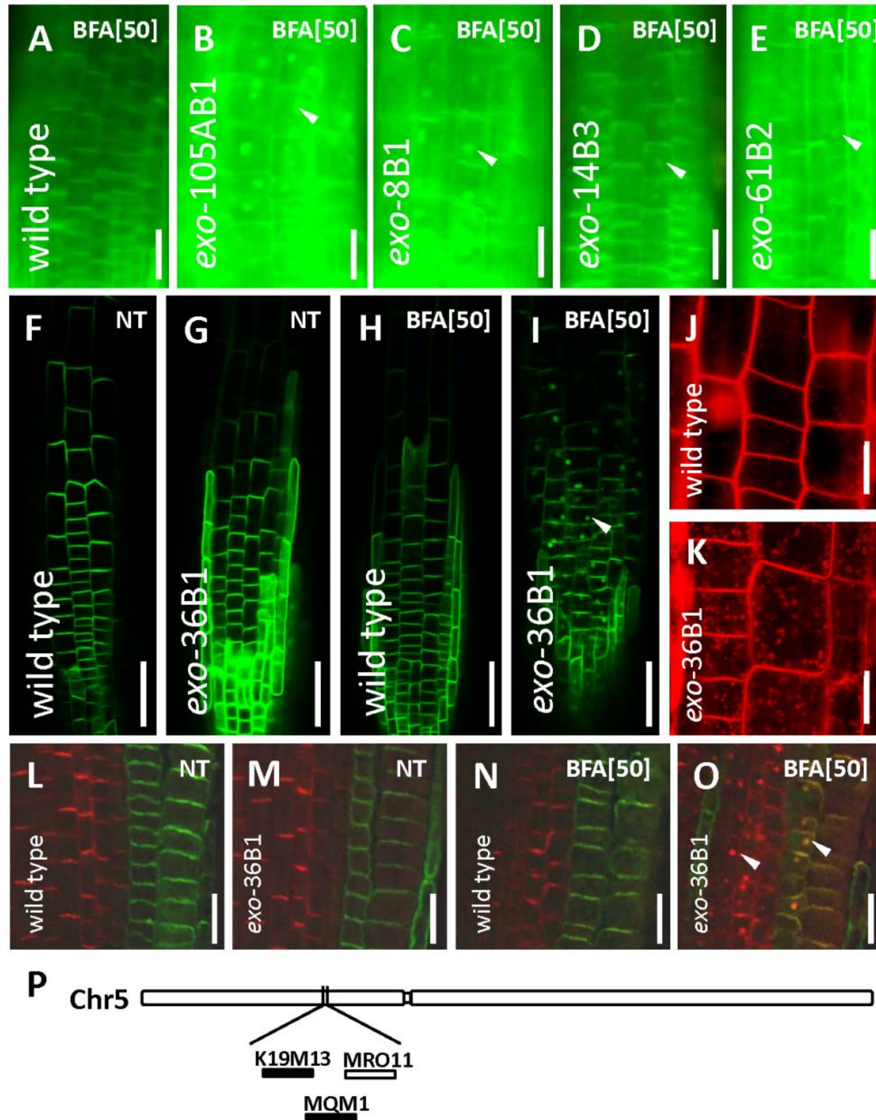

Figure 6. ***Exo*** **mutants display PIN2-GFP BFA bodies after long BFA treatments.**
A-E. Epifluorescence microscope pictures of wild-type (A) and mutants *exo*-105AB1 (B), *exo*-61B2 (C), *exo*-14B3 (D) and *exo*-8B1 (E) root epidermal cells after treatment with BFA for 15 hours. Arrowheads indicate BFA bodies. Bar size 20 µm.
F-I. Confocal pictures of wild type (F, H) and *exo*-36B1 mutants (G, I) treated with 50 µM BFA for 15 hours (H, I) and the mock treatments (F, G). Arrowheads indicate BFA bodies. Bar size 50 µm.
J-K. FM4-64 uptake in wild type (J) and *exo*-36B1 (K). An image is shown after 10 min of incubation with 2 µM FM4-64. Bar size 20 µm.
L-O. Immunolocalization of PIN1 (red pseudocolor) in wild type (L, N) and *exo*-36B1 mutant (M, O) after 15-h treatment with 50 µM BFA (N, O) or the mock treatment (L, M). The green signal is the PIN2-GFP fluorescence. Arrowheads indicate BFA bodies. Bar size 20 µm.
P.*exo*-36B1 was partially mapped to a region between the markers K19M1-Hpy8I and MQM1.

PIN2-GFP protein was due to a defect in transcription. We performed quantitative RT-PCR for all root-expressed PINs and we found no difference in transcript levels (Figure

5G), pointing to a defect in a posttranscriptional mechanism to explain the observed phenotype. This mutant was partially mapped to an interval containing 30 genes, from which 18 where sequenced and showed no mutations (Table 2). Further work is needed to sequence the rest of the candidates.

These observations indicate that under the same conditions these *endo* mutants had a diminished internalization and BFA bodies formation as well as a decreased FM4-64 uptake and BFA sensitivity. Moreover, slight differences in phenotypic characteristics among these *endo* mutants could indicate that they are not allelic, however this only can be ruled out based on reciprocal crosses and testing for mutual complementation and with previously characterized *Ben* mutants (Tanaka et al., 2009).

***Exo* mutants**

Long BFA treatment (12 hours, 50 μM) makes basal PIN1 in stele cells and basal PIN2 in young cortex cells to be targeted to the apical cell side in root cells by transcytosis-like translocation (Kleine-Vehn et al., 2008a and 2008b). We also observed that BFA-induced PIN2-GFP agglomerations disappeared after 15 hours of treatment (Figures 6A, 6F and 6H). Most probably such a prolonged inhibition of the apical BFA-sensitive trafficking pathway led to the recruitment of PIN2-GFP proteins into a BFA-independent apical pathway or a BFA-insensitive pathway for degradation. We used this response to screen for mutants that we called *exocytosis* (*exo*) mutants. We found five *exo* mutants (Table 1, Figure 6), showing different resistant phenotypes based on the BFA bodies size, ranging from strong resistant mutants, such as *exo*-105AB1 (Figure 6B), *exo*-8B1 (Figure 6C) and *exo*-36B1 (Figure 6I) to partially resistant mutants, such as*exo*-14B3 (Figure 6D) and *exo*-6B2 (Figure 6E). The *exo*-36B1 mutant displayed clear BFA bodies after 24-h BFA treatment (compare Figures 6G and 6I) and even after germination on BFA (not shown).

Table 3. Genes within the mapping interval of *exo*-36B1.

Gene Locus	Description	Seq.%
AT5G23420	Encodes HMGB6, a protein belonging to the subgroup of HMGB (high mobility group B) proteins. Localized in the nucleus. Binds to supercoiled DNA in vitro. HMGB6 is phosphorylated by protein kinase CK2alpha within its acidic C-terminal domain.	–
AT5G23430	Transducin/WD40 repeat-like superfamily protein	–
AT5G23440	ferredoxin/thioredoxinreductase subunit A (variable subunit) 1 (FTRA1)	–
AT5G23450	Encodes a sphingosine kinase that specifically phosphorylates D-erythro-dihydrosphingosine (DHS), but not N-acetyl-DHS or D-threo-DHS. It also phosphorylates D-erythro-sphingosine, trans-4, trans-8-sphingadienine and phytosphingosine.	–
AT5G23460	unknown protein	N.M
AT5G23470	Haloaciddehalogenase-like hydrolase (HAD) superfamily protein	N.M
AT5G23480	SWIB/MDM2 domain;Plus-3;GYF	N.M
AT5G23490	unknown protein	N.M
AT5G23510	unknown protein	N.M
AT5G23520	smr (Small MutS Related) domain-containing protein	N.M
AT5G23530	carboxyesterase 18 (CXE18)	N.M
AT5G23535	KOW domain-containing protein; FUNCTIONS IN: structural constituent of ribosome	N.M
AT5G23540	Mov34/MPN/PAD-1 family protein; INVOLVED IN: response to salt stress, protein catabolic process, ubiquitin-dependent protein catabolic process	N.M.
AT5G23550	Got1/Sft2-like vescicle transport protein family	N.M.
AT5G23570	Required for posttranscriptional gene silencing and natural virus resistance.SGS3 is a member of an 'unknown' protein family. Members of this family have predicted coiled domains suggesting oligomerization and a potential zinc finger domain. Involved in the production of trans-acting siRNAs, through direct or indirect stabilization of cleavage fragments of the primary ta-siRNA transcript. Acts before RDR6 in this pathway.	N.M.
AT5G23575	Transmembrane CLPTM1 family protein	N.M.
AT5G23580	unique family of enzymes containing a single polypeptide chain with a kinase domain at the amino terminus and a putative calcium-binding EF hands structure at the carboxyl terminus; recombinant protein is fully active and induced by Ca2+	N.M.
AT5G23590	DNAJ heat shock N-terminal domain-containing protein	–.
AT5G23600	RNA 2'-phosphotransferase, Tpt1 / KptA family	N.M.
AT5G23610	unknown	N.M.
AT5G23630	A member of the eukaryotic type V subfamily (P5) of P-type ATPase cation pumps. Highly abundant in the endoplasmic reticulum and small vesicles of developing pollen grains and tapetum cells. MIA is also named PDR2 and was shown to be required for proper expression of SCARECROW (SCR), a key regulator of root patterning, and for stem-cell maintenance in Pi-deprived roots.	N.M.
AT5G23640	unknown protein	N.M.
AT5G23650	Homeodomain-like transcriptional regulator	N.M.
AT5G23660	Encodes a member of the SWEET sucrose efflux transporter family proteins.	N.M.
AT5G23665	pre-tRNA; tRNA-Gln (anticodon: TTG)	–
AT5G23670	Encodes the LCB2 subunit of serine palmitoyltransferase, an enzyme involved in sphingosine biosynthesis. The protein is localized to the endoplasmic reticulum.	–
AT5G23680	Sterile alpha motif (SAM) domain-containing protein	–
AT5G23690	Polynucleotide adenylyltransferase family protein; FUNCTIONS IN: RNA binding, nucleotidyltransferase activity	–
AT5G23700	unknown protein	–
AT5G23710	DNA binding; DNA-directed RNA polymerases	–
AT5G23720	Encodes a protein tyrosine phosphatase Propyzamide-Hypersensitive 1 (PHS1). One of the mutant alleles, phs1-1, is hypersensitive to the microtubule-destabilizing drug propyzamide, suggesting that PHS1 may be involved in phosphorylation cascades that control the dynamics of cortical microtubules in plant cells. .	–
AT5G23730	Encodes REPRESSOR OF UV-B PHOTOMORPHOGENESIS 2 (RUP2). Functions as a repressor of UV-B signaling.	–
AT5G23740	Encodes a putative ribosomal protein S11 (RPS11-beta).	–
AT5G23750	Remorin family protein	–

% : Genes were fully sequenced in the background of *endo*-19A5 and compared to wild type for finding mutations. N.M.: no mutations found

We found this mutant to have an enhanced FM4-64 uptake (Figures 6J and 6K). Moreover, the *exo*-36B1 mutant defect is not specific for apical PIN2, but also PIN1 shows such agglomerations (Figures 6L-6O). This mutant was partially mapped to the right arm of chromosome 5 (Figure 6P) to an interval containing a total of 34 genes, from which 19 were sequenced and showed no mutations (Table 3). Further work is needed to sequence the whole interval and select candidate genes to continue analysis.

Other mutants

During the screening we decided to pick up some other interesting mutants that may help to get new insights into PIN or AUX1 trafficking or polarity pathways. Our wild-type line for the genetic screen was in the *pin2aux1* mutant background and contained PIN2-GFP and AUX1-YFP constructs that were fully functional and rescued the agravitropic phenotype of the double mutant background. Hence, any defect in trafficking, polarity or transcription would have an impact on root gravitropism and would be visible when germinating seedlings on vertical plates. This was the case for a large proportion of mutants which displayed different degrees of root gravitropic defects in seedlings germinated in the absence of BFA treatment (Table 1). We checked all those mutants under the confocal microscope to observe any defect in AUX1-YFP, PIN2-GFP or both. To our surprise, most mutants tested showed defects for AUX1-YFP. For instance, the mutant 70A3 was agravitropic when germinated on vertical plates (Figures 7A and 7B) and it was impaired in *AUX1-YFP* expression (Figures 7C and 7D). But not only transcriptional defects were detected as mutations generating agravitropic roots. For instance, the mutant 56 looked under the stereomicroscope like having larger BFA bodies compared to the clear round-shaped bodies that appeared in wild type (Figure 7E). When we had a closer look under the confocal microscope we realized that mutant 56 had accumulated AUX1-YFP, and not PIN2-GFP, in a reticulate pattern that resembles the ER

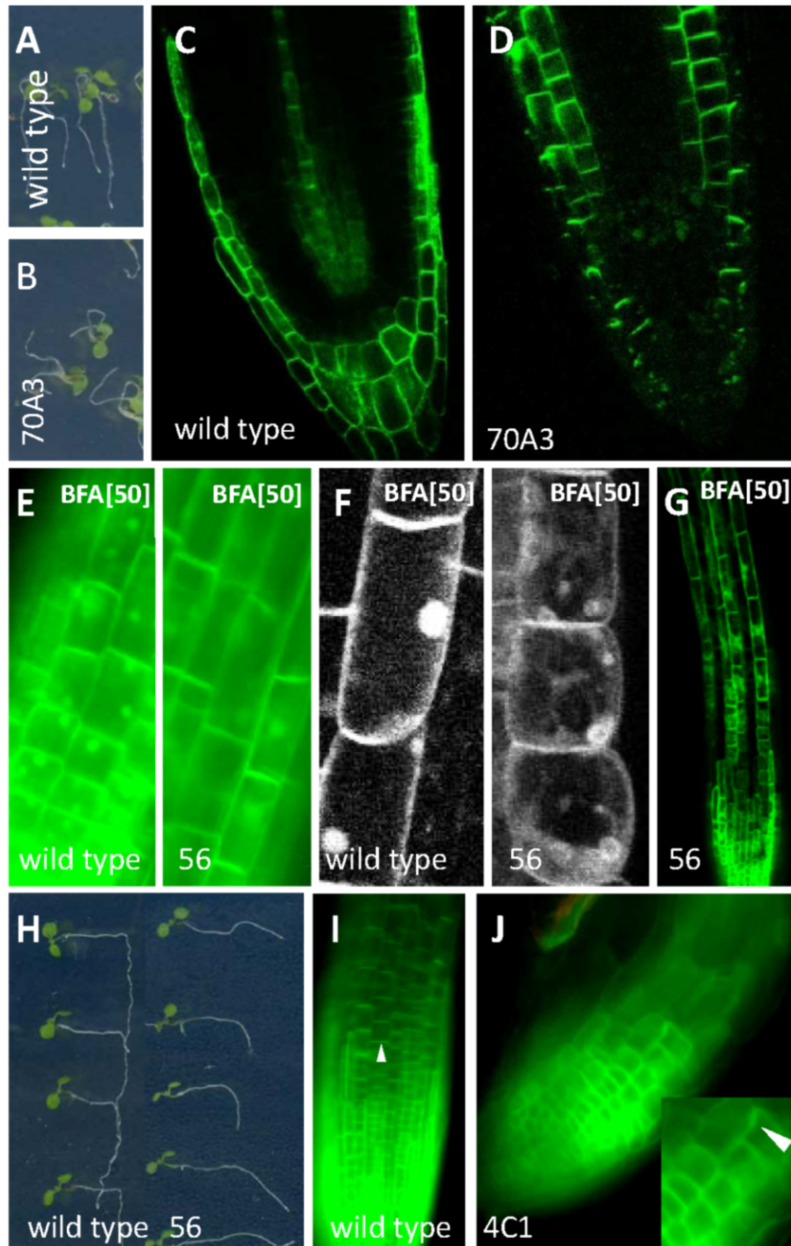

Figure 7. Other kind of mutants identified during the screening.
A-D. Several different mutant lines were agravitropic exemplified here by the line 70A3 (more details in Table 1). Vertically grown wild-type seedlings (A) compared to seedlings of the 70A3 mutant (B), which is clearly agravitropic. This defect was correlated with an abnormal AUX1-YFP distribution in the root tip (compare wild type in C with the 70A3 mutant in D).
E-H. Epifluorescence (E) and confocal (F, G) microscope pictures of mutant 56. This mutant was identified as having bigger agglomerations compared to wild type after 60 min treatment with BFA (E, F), that resemble ER retention of AUX1-YFP (G). As a consequence, this mutant was unable to bend the root in response to a gravitropic stimulus (H).
I-J. Epifluorescence microscope pictures of mutant 4C1 displaying apolar PIN2 in the epidermis (arrowhead in J) compared to the apical PIN2 in wild type (I).

(Figures 7F and 7G). As a consequence, the mutant 56 root was unable to respond properly to a gravitropic stimulus (Figure 7H). We also found mutants having defects in

PIN2-polarity and root patterning (Figures 7I and 7J), but all were lethal at later stages in development. This extremely interesting class of mutants requires working with segregating populations for their mapping and characterization.

Taking all data together, the screening showed to be a powerful way to find PIN2 or AUX1 regulators not only at trafficking steps but also at transcriptional and post-transcriptional levels and it proves also to be a good screening for regulators of cell polarity.

CONCLUSION

Arabidopsis thaliana is a simple plant model system that has been exploited as a tool for forward genetic screens. In the last decade, several fluorescently tagged reporters have been used for diverse screenings aiming to map intracellular trafficking, compartments integrity and protein function that enhanced our knowledge in plant cell biology (Zwiewka and Friml, 2012). Here, we presented a novel fluorescent-based screen to identify new regulators of BFA-sensitive apical pathways that exploits BFA sensitivity as a tool and uses subcellular phenotyping as *modus operandi*. Using this strategy we were able to find mutants impaired in endocytic and exocytic pathways, protein stability and polar localization, proving to be an excellent method to find new regulators of subcellular trafficking pathways.

MATERIAL AND METHODS

Plant materials and growth conditions

Transgenic line PIN2:PIN2-GFP AUX1:AUX1-YFP *pin2 aux1* was generated by crossing two previously published lines (Abas et al., 2006; Swarup et al., 2004). For seedling phenotypic analysis, seeds were sterilized using chlorine gas for 4-8 hours, sown on Arabidopsis Medium (AM; 0,5x Murashige and Skoog (MS) salts, 1% sucrose, 0.8% agar, pH 5.9) and grown for 4 to 7 days at 18°C in a 16h:8h light:dark regime.

Genetic screen

For EMS mutagenesis, transgenic seeds of the PIN2:PIN2-GFP AUX1:AUX1-YFP *pin2 aux1* line were soaked in a solution containing 0.2% EMS for 8 hours, and washed extensively with water for at least 1 hour. These M1 seeds were germinated on soil and harvested in pools of 5 M1 plants. We screened 100 to 120 M2 seedlings coming from approximately 1,500 M1 plants (300 pools) as follows. Seedlings were sown on solid AM and 4 DAG random seedlings were manually transferred to solid AM containing 50 µM BFA (Molecular Probes) and each seedling was screened twice after 90 min and 15 hours of BFA treatment under a fluorescence stereomicroscope (Leica). Mutants showing less internalization after 90 min or displaying BFA internalization after long BFA treatments were further grown to harvest M3 seeds. A secondary screen was performed under Epifluorescence and confocal microscopes and immunolocalizations using anti-PIN1 and anti-PIN2 antibodies to confirm mutant phenotypes. Table 1 describes the amount of mutants found after primary screening and shows the phenotype confirmation by secondary screens.

Genetic mapping

endo and *exo* mutants were outcrossed to *Landsberg erecta* (L*er*) ecotype and F1 plants were allowed to self pollinate. The resulting F2 population was treated for 90 min with BFA in the case of *endo*-19A5 or 15 to 24 hours in the case of *exo*-36B1 and seedlings showing the respective mutant phenotypes were selected using an epifluorescence microscope. Genomic DNA was isolated from each individual plant. PCR-based linkage analysis was performed as previously described (Tanaka et al., 2009; Feraru et al., 2010) using single sequence length polymorphisms (SSLPs) and cleaved amplified polymorphic sequence (CAPS) molecular markers.

Immunolocalization and live cell imaging

Whole-mount root immunolocalization was performed using an automated system (Intavis in situ pro) as described previously (Sauer et al., 2006b) using anti-PIN1 and anti-PIN2 antibodies diluted 1 to 1000 and CY3-conjugated anti-rabbit antibody (Sigma) diluted 1 to 600. FM4-64 (Molecular Probes) was diluted one thousand times from 2 mM aqueous solution. FM4-64 labeling was done using a protocol previously described (Tanaka et al., 2009). BFA treatment was done for 30 min before FM4-64 pulse labeling for Figure 3. For live cell imaging, seedlings 4-7 DAG were mounted in a drop of liquid AM and immediately imaged.Confocal images were taken by Olympus FV1000 ASW or Carl Zeiss LSM710. Epifluorescence pictures were taken using a Zeiss Axioimager system.

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Chapter 6

Conclusions and Perspectives

Ricardo Tejos

Phospholipids and auxin are two main signaling molecules in plants. Auxin is a major determinant of tissue patterning from early on in plant life and it also regulates highly directional tropic responses. On the other hand, phosphoinositides regulate quick responses to the environment and also modulate cell polarity, thereby influencing developmental processes. In spite of their central role as plants regulators, auxin-phosphoinositide signaling interplay is not fully established. The evidences presented in this thesis provide further insights into the crosstalk between auxin and phosphoinositide signaling and their influence on plant development. In the following section I will attempt to give an integrative view on the results described in the previous sections and, furthermore, I will also present future perspectives that emerged from this research based on preliminary observations.

Plants have an outstanding developmental plasticity that has allowed them to successfully colonize every corner of the planet. Moreover, due to their sessile life style, plants have evolved special ways to cope with changes in environmental conditions such as nutrient and water availability and variations in light intensities. Such varieties of responses are in part accomplished by polarly localized proteins that are involved in embryonic and post-embryonic development, nutrient uptake and tropic responses (Reviewed in Chapter 1, Part I). The plant hormone auxin is a plant-specific determinant of cell and tissue polarity. The more prominent auxin, indole-3-acetic acid, is an intercellular signal that mediates directional responses in plants such as gravitropism and phototropism and it is behind the asymmetry generation during plant development (Grunewald and Friml, 2010). Auxin is transported by a network of polarly localized auxin exporters and importers (Blakeslee et al., 2005), among which the PIN family of auxin efflux carriers determines the directionality of transport (Wiśniewska et al., 2006). Many external and internal cues are integrated into changes in auxin transport, hence, auxin levels, by modulating PIN protein trafficking and polar localization (Boutté et al., 2007). Auxin itself is able to influence PIN endocytic trafficking, transcription and polarity and this feedback loop on its own transport has been proposed to enable dynamic PIN polar relocalization and changes in auxin transport directions (Berleth and Sachs 2001; Krupinski et al. 2010; Wabnik et al., 2011; Jönsson et al., 2006; Smith et al., 2006). Studying the mechanisms that control, maintain and determine such PIN polarity is a central question in biology as it is the foundation of what characterizes and defines a plant.

The current view on the auxin effect on PIN polarity is that it depends on the transcription of unkown regulators of PIN trafficking or localization (Sauer et al., 2006), although based on the available information we can not rule out the independence of other auxin-singaling mechanism such as the ABP1-dependent regulation of clathrin-mediated

endocytosis (Robert et al., 2010). Basal PIN proteins in root endodermis and cortex cells switch to a baso-lateral localization in response to external auxin treatments. Although this situation is far from being something that could be found in real circumstances, it may reflect a natural response to changes in auxin fluxes. Such dynamic PIN relocalizations have been described during organogenesis, embryo development and tropic responses concomitant with auxin flux or auxin maxima changes (Friml et al., 2003; Reinhardt et al., 2003; Benková et al., 2003; Blilou et al., 2005; Sauer et al., 2006; Kleine-Vehn et al., 2010; Ding et al., 2011; Rakusová et al., 2011). Therefore, we designed a microarray approach to fish out such regulators downstream of the auxin transcriptional response (Chapter 2, Table S1). From the candidate genes we extracted those that could be linked to changes in cell polarity and performed an initial screen using insertional mutants (Sauer and Friml, 2007). However, this approach did not give any conclusive evidence, so then we supposed a redundancy in function and we had to go for a gene family approach.

Phosphoinositide signaling for auxin-mediated development

As a prominent functional group of candidate genes appeared a phosphoinositide-related group (Sauer and Friml, 2007). Phosphoinositides are a group of phosphorylated phosphatidylinositol (PtdIns) lipids that are involved in several aspects of plant development and stress responses (Reviewed in Chapter 1, Part II). One gene candidate is the phosphatidylinositol 4-phosphate 5-kinase 1 (PIP5K1). PIP5K phosphorylates PtdIns 4-P to produce the important lipid messenger PtdIns(4,5)P_2. This phosphoinositide is necessary for clathrin-mediated endocytosis, exocytosis and polar cell growth (Reviewed in Chapter 1, Part II), besides being a substrate for generating second messengers important for stress responses (Munnik and Vermeer 2010). Therefore, we further characterized the PIP5K function in plants as it may be a good candidate for regulating

PIN trafficking and polarity. We demonstrated that auxin is able to induce a change in the global PtdIns(4,5)P_2 levels and particularly at the plasma membrane. We show that PIP5K1 and PIP5K2 are polarly localized in root cells where they colocalize with PINs. Furthermore, knockout double *pip5k1pip5k2* mutants display several developmental defects that we could link to a defective auxin response and PIN polar localization. Therefore, we propose that auxin, by transcriptionally regulating PIP5K, is able to modulate phosphoinositide contents, hence, polar PIN localization and auxin-dependent development (Chapter 2).

However, there is still room for speculations as phosphoinositide signaling complexity is enormous. For instance, at which level PtdIns(4,5)P_2 metabolism affects PIN polarity is not clear yet. Some evidence link PtdIns(4,5)P_2 to cell trafficking modulation (Mei et al., 2012), however, these evidences do not clarify the actual mechanism. We propose at least two parallel ways that could lead to a modulation of PIN polarity (Figure 1A). One is related to the function of PtdIns(4,5)P_2 as a scaffold molecule working in clathrin-mediated endocytosis (CME) and other trafficking processes. The other is related to the function of PtdIns(4,5)P_2 as precursor of diacylglycerol (DAG) and inositol (1,4,5)triphosphate (IP_3) and the connection with calcium signaling and PINOID. Both pathways, PINOID and CME, have been shown to play roles in PIN polar establishment and auxin-dependent development (Kitakura et al., 2011; Zhang et al., 2011). Some preliminary results reveal that probably there is a mixed contribution from both pathways (Figure 1B), however more work is needed to dissect the interplay between phosphoinositide and polar auxin transport and PIN polarity. For instance, the PID transcription is positively regulated by auxin, hence the observed down regulation could be a secondary effect of the reduced auxin contents in *pip5k1pip5k2* root tips (data not shown). Therefore, if the external application of auxin could restore PID levels and then

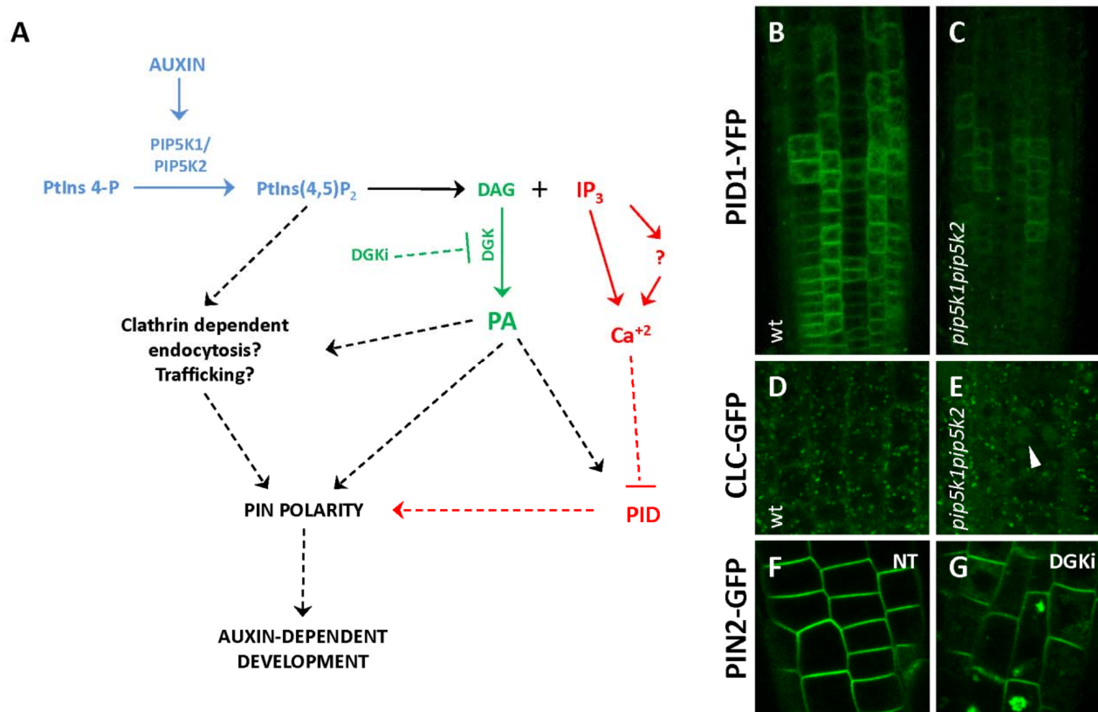

Figure 1. Phosphoinositide signaling modulation of PIN polarity.

A. Possible scenarios for PIN polarity modulation by phosphoinositide signaling. We propose an input of auxin on the transcription of PIP5Ks, that lead to increased levels of PtdIns(4,5)P_2 (blue pathway). This phosphoinositide can work as a scaffold molecule to recruit components of clathrin-mediated endocytosis, hence, modulating trafficking and PIN polarity. On the other hand, elevated levels of PtdIns(4,5)P_2 could have as a consequence increased amounts of diacylglycerol (DAG), inositol(1,4,5)-triphosphate (IP_3) and phosphatidic acid (PA). IP_3/Ca^{+2} signaling (red pathway) has some influence on PINOID (PID) activity, thereby affecting PIN polarity (suggested by Zhang et al., 2011). As an open possibility is the effect that PA could exert indirectly trough PID on PIN polarity or affecting other aspects of trafficking (green pathway).

B-G. Preliminary evidence supporting a broad impact of PIP5K activity on several phosphoinositide pathways. PID-YFP levels are down regulated in *pip5k1pip5k2* (C) compared to wild type (B); CLC-GFP is mistargeted to the vacuole in *pip5k1pip5k2* (arrowhead in E) compared to the exclusive plasma membrane and TGN localization seen in wild type (D); the DAG kinase inhibitor (DGKi) impairs PIN2-GFP trafficking (compare the not treated condition (F) with the one treated with 50 µM DGKi for 60 min (G). Live cell imaging under confocal microscope.

rescue *pip5k1pip5k2* mutant phenotypes then this would be a clear indication that PID pathway is the responsible for the observed defects seen in *pip5k1pip5k2* seedlings. Some preliminary observations indicated that auxin is able to increase PID-YFP in the double mutant but auxin was not able to restore the short RAM in the double mutant (data not shown).

So then most probably the PINOID pathway is not the main contributor to the auxin-phophoinositide modulation of PIN and cell polarity. Therefore the clathrin-mediated endocytic trafficking appears as a plausible candidate to the defects observed in *pip5k1pip5k2* mutants. Future experiments could clarify this link, although previous work have shown that clathrin and PtdIns(4,5)P_2 colocalize in endocytic vesicles in plants exposed to salt stress (see below), however it is not clear if this trafficking pathway is constitutivly present in plant cells.

Extra evidence for a critical role of PIP5K in plant development was presented in Chapter 3, where we described a male gametophytic defect in the double *pip5k1pip5k2* mutant. Lethality appeared quite early during pollen formation and as described for the polarity-related phenotypes (Chapter 2, Figure 2), there was some degree of functional redundancy also for the pollen development, although in this case, single mutants showed the phenotype as well (Chapter 3, Figures 3 and 4). The causes behind this defect are not totally clear. However, we propose a function for PIP5K in vacuole morphogenesis that could also be applied to explain other mutant phenotypes. Pollen derived from double *pip5k1pip5k2* mutants showed defects in vacuole formation at late stages of development (Chapter 3, Figure 5) and in root apical meristem (Chapter 3, Figure S3). Yet, it will be neecesary to extend the description of the phenotypes in early pollen development and to see if the described vacuolar phenotypes are also present in early stages. Furthermore, It would be necessary to clarify if the vacular defects can be linked to the pollen abortion. In spite of that, the only indication of phosphoinositide control of vacuole morphogenesis was linking PtdIns3-P/PtdIns(3,5)P_2 levels to vacuole trafficking and membrane homeostasis, therefore the results presented here are the first indications of PtdIns(4,5)P_2 control of vacuole morphology.

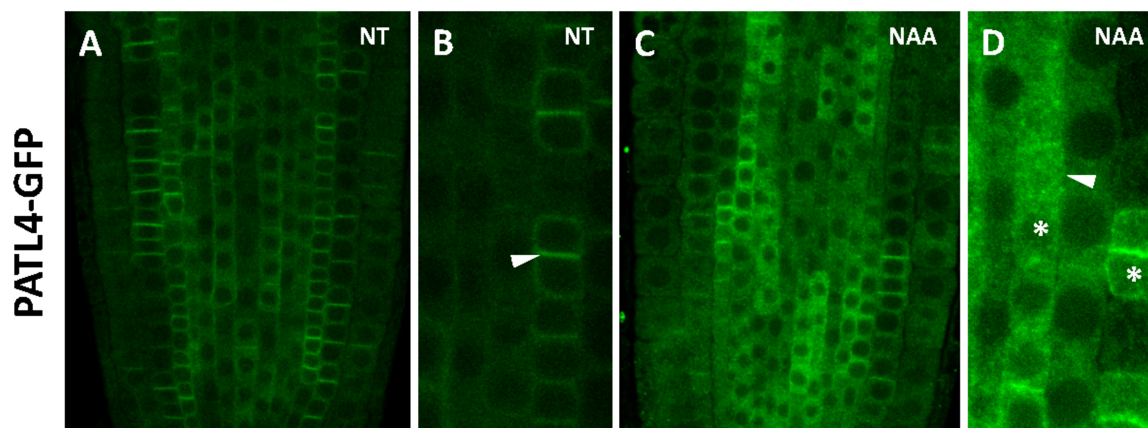

Figure 2. Auxin affects PATL4 localization.
A-D. PATL4-GFP is asymmetrically localized to the plasma membrane in root cells (A, arrowhead in B). After NAA treatment (C, D) part of the plasma membrane localization is lost (arrowhead in D) and it gets accumulated in intracellular vesicles (asterisks in D).
The PATL4-GFP line was treated for 4 hours with 10 µM NAA and fixed in PFA 4% for 1 hour under vacuum. After a few washes, seedlings were mounted and imaged in a confocal microscope.

Further evidence of the importance of phosphoinositide signaling for plant patterning and development came from the analysis of the phosphatidylinositol transfer proteins (PITPs) PATELLIN (PATL, Chapter 4). Although their precise molecular function is not clear, *in vitro* assays already suggest PITP involvement in phosphoinositide signaling (Reviewed in Chapter 1, Part I). PATELLINs are a small subgroup of the sec14-like proteins in Arabidopsis. We showed here that PATLs are plasma membrane-localized proteins that present some enrichment at apical-basal cell sides (Chapter 4, Figures 6 and 7). Multiple knockout mutants display embryo patterning defects and overexpression lines show strong seedling phenotypes and wavy roots. Although the exact role of PATLs was not elucidated in the present thesis, we propose a redundant and crucial role in plant development. Further experiments could clarify this. Some indications already showed a link for other Sec14-like proteins in regulating PtdIns4-P/PtdIns(4,5)P_2 homeostasis (Vincent et al., 2005), which could lead us back to the previous section and give us an indication for future directions. Furthermore, preliminary observations imply a role for auxin in PATL4 localization (Figure 2) on top of the effect on PATL transcription,

suggested by the microarray results (Chapter 4, Figure S1) that could open an interesting regulatory point for auxin in phosphoinositide signaling.

PIP5K trafficking to the nuclei, a module for stress-induced signaling?

Environmental stresses induce an array of cellular adjustments that facilitate plant survival. The plasma membrane is the primary sensor for heat and osmotic stresses (Kinnunen, 2000). The lipid bilayer, the structural component of all biological membranes, provides a place to sense external conditions and transmit them into the appropriate regulatory mechanisms. Beyond the spontaneous fluidity of lipids in membranes, they tend to self-organize into microdomains, which are believed to work as structural components as well as signaling hubs (Cacas et al., 2012; Kierszniowska et al., 2009). A large fraction of proteins with signaling functions were identified as variable members of lipid detergent-insoluble microdomains, while other cell wall related proteins are part of a core of proteins associated to such domains (Kierszniowska et al., 2009).

Heat, cold and osmotic stresses all induce phosphoinositol-dependent signaling cascades. Within minutes of stress application, phosphatidic acid (PA), PtdIns(4,5)P_2 and IP_3 accumulate (Pical et al., 1999; van Leeuwen et al., 2007; Minami et al., 2009; Mishkind et al., 2009; Munnik and Vermeer, 2010) and these changes correlate with rapid increases of the intracellular calcium concentration (DeWald et al., 2001). In the case of heat shock, cellular increases in PA occur through an activation of phospholipase D (PLD), a phosphoinositide-independent metabolic pathway, while the primary source of increased levels of IP_3, and hence calcium mobilization, is the PIP5K-synthesized PtdIns(4,5)P_2 (Mishkind et al., 2009). Plant cells deploy PtdIns(4,5)P_2 to different subcellular compartments: heat induces its accumulation to the plasma membrane, cytoplasmic structures and the nucleus. So far, the mechanisms for this diverse subcellular response and its consequences are not clear.

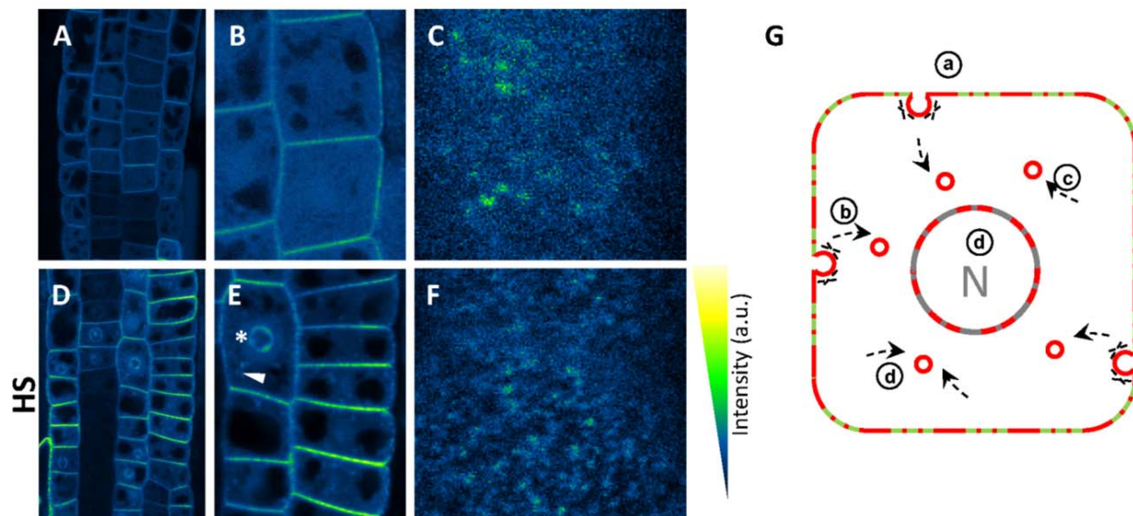

Figure 3. Heat shock induces PIP5K translocation to nuclei.
A-F. PIP5K1-YFP is localized to the plasma membrane and in some cytoplasmic pool (A, B). At the plasma membrane it is accumulated in clusters (arrowhead in C). After heat shock (HS; D, E), plasma membrane PIP5K1-YFP amounts increase and some endosome-like structures (arrowhead in E) and nuclear localization appear (asterisk in E). The number of plasma membrane PIP5K1 clusters increases after HS (F). Middle section (A, B, D, E) and surface view (C, F) of live root epidermal cells imaged on a confocal microscope after 1-h HS at 37°C (D, E, F) or in the control situation (A, B, C).
G. Future perspectives of research. PIP5K1-YFP translocation to the nuclei after HS opens some interesting questions for future research: signals and mechanism for plasma membrane stress perception (a); mechanism and routes for plasma membrane-nucleus trafficking (b) or cytosolic PIP5K recruitment to endosomes (c); and nuclear PIP5K-dependent signaling for stress responses (d).

We observe that heat shock induces a rapid subcellular relocalization of PIP5K in root cells. PIP5Ks are localized to the plasma membrane in normal conditions (Figures 3A and 3B). Upon heat treatments we observed two kind of responses. First, PIP5K localized to cytoplasmic vesicles and nuclei (Figures 3D and 3E), which might explain the patterns of PtdIns(4,5)P_2 previously observed under heat stress (Mishkind et al., 2009). Moreover, we also showed that the number of agglomerations that PIP5K normally forms at the plasma membrane increased after heat shock (Figures 3C and 3F, see Chapter 2). It was already proposed that PIP5K drives the formation of PtdIns(4,5)P_2, and thus IP_3 in response to heat stress, but the subcellular localization of PIP5K in response to stress is so far not known. Moreover, PIP5K clustering at the plasma membrane could be the first observation of protein localization at a microdomain that could work as a transducer from

plasma membrane perception to nuclear signaling. Even more, nuclear PIP5K-dependent phosphoinositide signaling is also not described previously.

Future work could help to link missing data to a more detailed view of stress perception, transduction and response in plants. For instance, a detailed study of PIP5K response to environmental stress should address few basic questions (Figure 3G): (i) which is the mechanism for stress perception at the plasma membrane? And what are the changes in PIP5K distribution at PM? (ii) is there a PIP5K translocation from plasma membrane to the nuclei? or is PIP5K being recruited from a cytosolic fraction to a unkown endosomal compartment and then translocated to the nuclei or also recruited to the nuclei? (iii) What are the roles of the PIP5K-mediated signaling inside the nuclei? The first two questions involve a detailed description of PIP5K localization and dynamics in response to stress using high resolution microscopy. Previous indications suggest that PtdIns(4,5)P_2 and clathrin colocalize in endosomes upon salt stress, so a detailed description of CME and the colocalization of PIP5K and clathrin it would give further insights of the mechanism of perception and transduction. Morever, colocalization studies with markers for intracellular compartments would shade light into the nature of the stress-induced PIP5K-endosomes. A crucial question is the identification of the mechanism of stress perception. A suitable approach would involve genetic screens, either chemical genetic or traditional forward genetic screens, although for both cases it is necessary for making the screens feasible the identification of a phenotype that is easily observable that would allow such high throuput screens.

Finally, the characterization of the intranuclear PIP5K–mediated signaling is a major chalange. Nuclear phosphoinositide signaling has been implicated in many essential processes such as DNA replication, recombinetion, RNA processing, mRNA export and cell cycle progression (Dieck et al., 2012). An initial step should be the characterization

of PIP5K downstream response and physiological output using knockouts and overexpressing lines: is PIP5K-signaling making the plant more resistant to stress? An easily observable phenotype that could appear comparing mutants and overexpressing lines could be the starting poing for the characterization of nuclear signaling as well as a good phenotype for a genetic screen. The study of PIP5K regarding stress responses could open several opportunities for improved resistance of crop to stressful environmental conditions.

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Summary

Polarity is an intrinsic characteristic of every biological system; it is at the foundations of how an organism responds to the physical environment, how it interacts with other organisms and how it is generated and maintained as an entity. Polarity can be expressed as asymmetry at structural levels (e.g. differential accumulation of proteins or lipids, or head-to-tail assembly) or as a differential response (e.g. plant bending toward the light, or directional swimming of a bacterium). Most of the time what we see as a directional response is the result of intrinsic structural asymmetries. That is why the mechanisms that determine, generate and maintain cell and tissue polarity are intense topics of research.

The plant hormone auxin is a simple yet powerful molecule. It is actively transported between cells by an array of transmembrane proteins, among which PINFORMED (PIN) proteins are polarly localized. The polar localization of PINs determines the local auxin gradients, hence influencing multiple developmental processes and differential growth responses. External and internal signals are integrated into polar auxin transport changes by modulating PIN trafficking and/or PIN polarity. Auxin feeds back on its own transport by affecting PIN transcription, PIN intracellular trafficking and PIN polarity by using different auxin receptors and downstream responses. In spite of the great importance of PIN intracellular trafficking regulation on plant's architecture and responses to the environment, there are still open questions.

In the present thesis we will present two approaches to characterize new regulators of PIN polarity and trafficking. We selected two gene families using a microarray based approach to find new regulators of auxin-mediated PIN and cell polarity and we concentrated on two phosphatydilinositol (PtdIns)-related proteins: phosphatydilinositol 4-phosphate 5- kinases (PIP5K) and PATELLIN (PATL). In Chapters 2 and 3 we functionally characterized PIP5K1 and PIP5K2 as important mediators of cell polarity.

PIP5Ks phosphorilate PtdIns4-P to produce the versatile messenger PtdIns(4,5)P_2. Auxin is able to transcriptionally regulate PIP5Ks, and as such controls overall PtdIns(4,5)P_2 levels and in particular those at the plasma membrane. Double knockout *pip5k1pip5k2* mutants display multiple auxin-related developmental defects (e.g. embryo patterning defects, reduced lateral root density and disconnected vascular bundles in leaves) that we could connect to aberrant auxin response patterning as a consequence of lost PIN polarity. Moreover, we demonstrated that PIP5Ks and markers for PtdIns4-P and PtdIns(4,5)P_2 are polarly localized in root cells where they colocalize with polar PIN1 and PIN2 (**Chapter 2**).

We further characterized the role of PIP5Ks in reproductive development. We showed that PIP5K1 and PIP5K2 are expressed early in pollen formation and their expression is induced by auxin. Single and double *pip5k* mutants display pollen lethality early during microgametogenesis. As a consequence *pip5k* mutants have reduced seed sets. Moreover, we observed that *pip5k1pip5k2* mutants display partial pollen lethality, and pollen grains that reached late development stages (i.e. Mature Pollen Grains) showed defects in vacuole formation and, as a consequence, pollen from *pip5k1pip5k2* exhibited reduced germination rates in *in vitro* conditions (**Chapter 3).**

We next examined the role of PATL proteins *in planta*. PATLs are putative phosphatidylinositol transfer proteins (PITP). PITPs work at the interphase between PtdIns metabolism and signaling having essential roles in trafficking processes. We used a genetic approach to dissect PATLs' role in plant development. We show that PATLs' overlapping expression patterns suggest a dual role during cell division and differentiation at different levels during plant development. We show that the multiple *patl23456* mutant displays embryo patterning defects, indicating a highly redundant function. All PATLs are membrane-associated proteins and show some degree of

asymmetric localization at the plasma membrane. Moreover, PATL4-GFP colocalizes with polar PINs in root cells, suggesting a role for PATLs in PIN polar establishment and plant development (**Chapter 4**).

In **Chapter 5** we present a forward genetic screen to identify new regulators of PIN2 intracellular trafficking. We used a subcellular screening to identify mutants that were responding differentially to short and long Brefeldin A treatment. We present the overall results of the screening and the initial characterization of the mutants found.

Curriculim vitae

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Tejos R, Sauer M, Vanneste S, Ischebeck T, Heilmann M, Schmid M, Heilmann I, Friml J. Auxin feed-back on cell and tissue polarity by modulation of phosphoinositide metabolism.

PRESENTATIONS AT SCIENTIFIC MEETINGS

Frontiers in plant biology: from discovery to applications. October 2012, Gent, Belgium (Poster Presentation)
"Auxin feed-back on cell and tissue polarity by modulation of phosphoinositide metabolism"

Annual ENPER meeting. August 2012, Madrid, Spain (Poster Presentation)
"PATELLIN family of Phosphatidylinositol transfer proteins regulates embryo patterning"

International Conference on Arabidopsis Research. July 2012, Vienna, Austria (Poster Presentation)
"PATELLIN family of Phosphatidylinositol transfer proteins regulates embryo patterning"

Annual ENPER meeting. September 2011, Assisi, Italy (Oral Presentation)
"Phosphatidylinositol 4-Phosphate 5-kinases mediate feed-back regulation of PIN polarity in Arabidopsis"

Membrane Dynamics of the Cell. September 2010, Dusseldorf, Germany (Poster Presentation)
"Fluorescence based screen to identify new components of the BFA-dependent PIN2 trafficking pathway"

Auxins and Cytokinins in Plant Development. July 2009, Prague, Czech Republic (Poster Presentation)
"Molecular mechanism of the auxin-dependent PIN lateralization"

Anual Meeting of the Cell Biology Society. October 2006, Pucón, Chile (Poster Presentation)
"Promoter swapping of the PIN family for reveal if cell- or protein-specific factors determine their polar localization"

Plant Biology Meeting. October 2006, Santiago, Chile (Poster Presentation)
"Promoter swapping between Pin1, Pin2 and Pin3, an experimental approach to understand factors that regulate polarly localized proteins in the plasma membrane"

Meeting of young scientist of the Millennium Scientific Initiative. September 2006, Punta de Tralca, Chile (Oral Presentation)
"Promoter swapping between Pin1, Pin2 and Pin3, an experimental approach to understand factors that regulate polarly localized proteins in the plasma membrane"

TEACHING EXPERIENCE

Supervision of Pavlina Koscova during her Master thesis. Research Project: ARCTICA proteins, new polarly localized proteins (2012)

Supervision of Maciej Adamowski during his Master thesis. Research Project: Patellin, a novel family of A. thaliana putative phosphatidylinositol transfer proteins (2011)

Supervised four students in their Bachelor Project (3rd Bachelor Biochemistry and Biotechnology, Gent University). Project: Molecular characterization of *patellin* mutants (2011)

Laboratory teacher of IV Advanced Course in Molecular Biotechnology oriented for school students. Organized by Millennium Nucleus in Plant Cell Biology and Biology Departament, UNAB (2007)

Laboratory teacher for the course Physiology, Plant Biology section, Faculty of Ecology and Natural Resources, UNAB (2007)

Laboratory teacher of III Advanced Course in Molecular Biotechnology oriented for school students. Organized by Millennium Nucleus in Plant Cell Biology and Biology Departament, UNAB (2006)

DNA`s Laboratory Teacher, Seed Project 2004, oriented for School teachers, Organized by Millennium Nucleus in Plant Cell Biology and the Faculty of Science, University of Chile (2006)

Acknowledgments

I will always remember the circumstances that surrounded my first trip outside my country. It was October 2007 and I was happily planning my master defense in Chile when the bad news reached me. Someone else had published a paper in Science telling more or less the same history I was writing in my thesis. At first sight this might sound like a debastating moment but… no, it wasn't− and I still don't know why. Anyway, there was an important consecuence arising from that event. When my former boss and me got the news we decided to contact the "competitors" and join them in an effort to save something of what I had been doing for the last two years. Not for nothing in Chile we say *"Si no puedes contra ellos, úneteles"* (something like "If you can't against them, join them"). So then, I contacted the famous (at least for me) Jiří Friml, thinking in going to Germany for three months, but here it happended the second of the afourtunate circumstances. Shortly after I decided to come to Europe Jiří also decided to move to Gent, which at the end was the city where I spent the last (almost) five years of my life doing my PhD. Everything that happened was not fully planned, but I am sure it couldn't have been better than it was. Gent is a perfect city to live: beautiful and small, where it's possible to go everywhere by bike, quiet but active during the day and crazy during the night… Also, the VIB is a great institute to work, full of enthusiastic people, where it was possible to find moral and technical support.

I would like to acknowledge everyone that contributed and participate in my formation as a scientist. I greatly appreciate the support during all these years from my boss, Jiří. I had learned a lot from you, it has been a pleasure to be part of your lab for all these years. Of course, I spent most of my life in Gent in the Auxin lab sharing good moments with all my past and present colleagues from and I was lucky to get to know different kind of people and diverse cultures. I thank you all! I would like to mention here the special participation of the postdocs from the lab that contributed to the writing of the present thesis: Dr Tomasz Nodzyński (dziękuję); Dr. Sibu Simon (doc, thanks!); Dr. Steffen Vanneste (Dank u!); Dr. Tom Viaene (Dank u!); and Dr. Helene Robert (Merci!).

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Finally, I would like to thank to my families (sorry, in Spanish). Quisiera dedicar esta tesis a mis dos familias. Mi familia de siempre, en la que uno crece y, por supuesto, no elige. En este sentido, me considero un afortunado por los padres y hermanos que tengo. Estan y estaran siempre conmigo y yo estare siempre con uds. Mama, la vida nos da cosas amargas y dulces al mismo tiempo, espero que esta parte de mi vida que plasmo en esta tesis le traiga un sabor dulce. Tenga claro que este logro es parte de la cosecha de lo que sembraron con mi papá. Mi otra familia es la que estoy formando ahora. La que uno elige. Claudia, esto es para ti, por tu paciencia en los primeros años de mi vida en Bélgica, por tu compañia en este ultimo tiempo, y por todo tu amor sincero y porfundo. Espero que esto lo sientas como un triunfo nuestro. Te amo.

Papá, más de alguna vez me recordaste cuanto te gusto el pequeño gesto que te hice cuando termine el colegio y la promesa que cumpli a tiempo cuatro años despues. Por supuesto, ahora no será posible hacer la segunda parte. Te extraño.

Annex 1

Candidate genes for auxin-regulated PIN polarity

Table S1. Related to Chapter 2. Candidates genes differentially regulated in *HS::axr3-2* compared to auxin-regulated genes in wild type. The gene model description is depicted as it appears in TAIR database. In bold are indicated the candidates that were tested for defects on PIN polarity.

Locus identifier	Primary Gene Symbol	Gene Model Description
AT1G02400	GIBBERELLIN 2-OXIDASE 6 (GA2OX6)	Encodes a gibberellin 2-oxidase that acts on C19 gibberellins but not C20 gibberellins.
AT1G02850	BETA GLUCOSIDASE 11 (BGLU11)	beta glucosidase 11 (BGLU11)
AT1G03820		unknown protein
AT1G03870	FASCICLIN-LIKE ARABINOOGALACTAN 9 (FLA9)	fasciclin-like arabinogalactan-protein 9 (Fla9)
AT1G04040		HAD superfamily, subfamily IIIB acid phosphatase
AT1G05530	UDP-GLUCOSYL TRANSFERASE 75B2 (UGT75B2)	Encodes a protein with glucosyltransferase activity with high sequence homology to UGT1 (AT1G05560). It belongs to an UGT subfamily that binds UDP-glucose but not UDP-glucuronate, UDP-galactose, or UDP-rhamnose as the glycosyl donor. UGT2 was shown to be able to use abscisic acid as glycosylation substrate in the presence of UDP-glucose.
AT1G08500	EARLY NODULIN-LIKE PROTEIN 18 (ENODL18)	early nodulin-like protein 18 (ENODL18); FUNCTIONS IN: electron carrier activity, copper ion binding; LOCATED IN: anchored to membrane;
AT1G14350	FOUR LIPS (FLP)	Encodes a putative MYB transcription factor involved in stomata development, loss of FLP activity results in a failure of guard mother cells (GMCs) to adopt the guard cell fate, thus they continue to divide resulting in abnormal stomata consisting of clusters of numerous guard cell-like cells. This phenotype is enhanced in double mutants with MYB88.
AT1G14540		Peroxidase superfamily protein
AT1G15210	ATP-BINDING CASSETTE G35 (ABCG35)	pleiotropic drug resistance 7 (PDR7)
AT1G15580	INDOLE-3-ACETIC ACID INDUCIBLE 5 (IAA5)	auxin induced protein
AT1G19220	AUXIN RESPONSE FACTOR 19 (ARF19)	Encodes an auxin response factor that contains the conserved VP1-B3 DNA-binding domain at its N-terminus and the Aux/IAA-like domains III and IV present in most ARFs at its C-terminus. The protein interacts with IAA1 (yeast two hybrid) and other auxin response elements such as ER7 and ER9 (yeast one hybrid). ARF19 protein can complement many aspects of the arf7 mutant phenotype and , together with ARF7, is involved in the response to ethylene. In the arf7 arf19 double mutant, several auxin-responsive genes (e.g. IAA5, LBD16, LBD29 and LBD33) are no longer upregulated by auxin.
AT1G21980	**PHOSPHATIDYLINOSITOL-4-PHOSPHATE 5-KINASE 1 (PIP5K1)**	**Type I phosphatidylinositol-4-phosphate 5-kinase. Preferentially phosphorylates PtdIns4P. Induced by water stress and abscisic acid in Arabidopsis thaliana. Expressed in procambial cells of leaves, flowers and roots. A N-terminal Membrane Occupation and Recognition Nexus (MORN) affects enzyme activity and distribution.**
AT1G22330		RNA-binding (RRM/RBD/RNP motifs) family protein
AT1G22530	**PATELLIN 2 (PATL2)**	**PATELLIN 2 (PATL2**

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT1G23080	PIN-FORMED 7 (PIN7)	Encodes a novel component of auxin efflux that is located apically in the basal cell and is involved during embryogenesis in setting up the apical-basal axis in the embryo. It is also involved in pattern specification during root development. In roots, it is expressed at lateral and basal membranes of provascular cells in the meristem and elongation zone, whereas in the columella cells it coincides with the PIN3 domain. Plasma membrane-localized PIN proteins mediate a saturable efflux of auxin. PINs mediate auxin efflux from mammalian and yeast cells without needing additional plant-specific factors. The action of PINs in auxin efflux is distinct from PGPs, rate-limiting, specific to auxins and sensitive to auxin transport inhibitors. PINs are directly involved of in catalyzing cellular auxin efflux.
AT1G25230		Calcineurin-like metallo-phosphoesterase superfamily protein;
AT1G25450	3-KETOACYL-COA SYNTHASE 5 (KCS5)	Encodes KCS5, a member of the 3-ketoacyl-CoA synthase family involved in the biosynthesis of VLCFA (very long chain fatty acids).
AT1G28370	ERF DOMAIN PROTEIN 11 (ERF11)	encodes a member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 15 members in this subfamily including ATERF-3, ATERF-4, ATERF-7, and leafy petiole.
AT1G28380	NECROTIC SPOTTED LESIONS 1 (NSL1)	This gene is predicted to encode a protein involved in negatively regulating salicylic acid-related defense responses and cell death programs.
AT1G28680		HXXXD-type acyl-transferase family protein
AT1G29500		SAUR-like auxin-responsive protein family
AT1G29510	SMALL AUXIN UPREGULATED 68 (SAUR68)	SMALL AUXIN UPREGULATED 68 (SAUR68);
AT1G33590		Leucine-rich repeat (LRR) family protein
AT1G33790		jacalin lectin family protein;
AT1G50660		unknown protein;
AT1G52050		Mannose-binding lectin superfamily protein;
AT1G52830	INDOLE-3-ACETIC ACID 6 (IAA6)	An extragenic dominant suppressor of the hy2 mutant phenotype. Also exhibits aspects of constitutive photomorphogenetic phenotype in the absence of hy2. Mutants have dominant leaf curling phenotype shortened hypocotyls and reduced apical hook. Induced by indole-3-acetic acid.
AT1G55330	ARABINOGALACTAN PROTEIN 21 (AGP21)	Encodes a putative arabinogalactan-protein (AGP21).

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT1G55740	SEED IMBIBITION 1 (SIP1)	seed imbibition 1 (SIP1
AT1G56020		unknown protein
AT1G60000		RNA-binding (RRM/RBD/RNP motifs) family protein
AT1G60010		unknown protein
AT1G62770		Plant invertase/pectin methylesterase inhibitor superfamily protein
AT1G63830		PLAC8 family protein;
AT1G64390	GLYCOSYL HYDROLASE 9C2 (GH9C2)	glycosyl hydrolase 9C2 (GH9C2)
AT1G64405		unknown protein
AT1G69530	EXPANSIN A1 (EXPA1)	Member of Alpha-Expansin Gene Family. Naming convention from the Expansin Working Group (Kende et al, Plant Mol Bio). Involved in the formation of nematode-induced syncytia in roots of Arabidopsis thaliana.
AT1G70230	TRICHOME BIREFRINGENCE-LIKE 27 (TBL27)	Encodes a member of the TBL (TRICHOME BIREFRINGENCE-LIKE) gene family containing a plant-specific DUF231 (domain of unknown function) domain. TBL gene family has 46 members, two of which (TBR/AT5G06700 and TBL3/AT5G01360) have been shown to be involved in the synthesis and deposition of secondary wall cellulose, presumably by influencing the esterification state of pectic polymers. A nomenclature for this gene family has been proposed (Volker Bischoff & Wolf Scheible, 2010, personal communication).
AT1G70560	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1)	TAA1 is involved in the shade-induced production of indole-3-pyruvate (IPA), a precursor to IAA, a biologically active auxin. It is also involved in regulating many aspects of plant growth and development from embryogenesis to flower formation and plays a role in ethylene-mediated signaling. This enzyme can catalyze the formation of IPA from L-tryptophan. Though L-Trp is expected to be the preferred substrate in vivo, TAA1 also acts as an aminotransferase using L-Phe, L-Tyr, L-Leu, L-Ala, L-Met, and L-Gln.
AT1G70940	PIN-FORMED 3 (PIN3)	A regulator of auxin efflux and involved in differential growth. PIN3 is expressed in gravity-sensing tissues, with PIN3 protein accumulating predominantly at the lateral cell surface. PIN3 localizes to the plasma membrane and to vesicles. In roots, PIN3 is expressed without pronounced polarity in tiers two and three of the columella cells, at the basal side of vascular cells, and to the lateral side of pericycle cells of the elongation zone. PIN3 overexpression inhibits root cell growth. Protein phosphorylation plays a role in PIN3 trafficking to the plasma membrane.
AT1G72900		Toll-Interleukin-Resistance (TIR) domain-containing protein;

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT1G73590	PIN-FORMED 1 (PIN1)	Encodes an auxin efflux carrier involved in shoot and root development. It is involved in the maintenance of embryonic auxin gradients. Loss of function severely affects organ initiation, pin1 mutants are characterised by an inflorescence meristem that does not initiate any flowers, resulting in the formation of a naked inflorescence stem. PIN1 is involved in the determination of leaf shape by actively promoting development of leaf margin serrations. In roots, the protein mainly resides at the basal end of the vascular cells, but weak signals can be detected in the epidermis and the cortex. Expression levels and polarity of this auxin efflux carrier change during primordium development suggesting that cycles of auxin build-up and depletion accompany, and may direct, different stages of primordium development. PIN1 action on plant development does not strictly require function of PGP1 and PGP19 proteins.
AT1G73620		Pathogenesis-related thaumatin superfamily protein
AT1G73780		Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein;
AT1G74660	MINI ZINC FINGER 1 (MIF1)	Encodes MINI ZINC FINGER 1 (MIF1) which has a zinc finger domain but lacks other protein motifs normally present in transcription factors. MIF1 physically interact with a group of zinc finger-homeodomain (ZHD) transcription factors, such as ZHD5 (AT1G75240), that regulate floral architecture and leaf development. Gel mobility shift assays revealed that MIF1 blocks the DNA binding activity of ZHD5 homodimers by competitively forming MIF1-ZHD5 heterodimers. Constitutive overexpression of MIF1 caused dramatic developmental defects, seedlings were non-responsive to gibberellin (GA) for cell elongation, hypersensitive to the GA synthesis inhibitor paclobutrazol (PAC) and abscisic acid (ABA), and hyposensitive to auxin, brassinosteroid and cytokinin, but normally responsive to ethylene.
AT1G74790		catalytics
AT1G75500	WALLS ARE THIN 1 (WAT1)	An Arabidopsis thaliana homolog of Medicago truncatula NODULIN21 (MtN21). The gene encodes a plant-specific, predicted integral membrane protein and is a member of the Plant-Drug/Metabolite Exporter (P-DME) family (Transporter Classification number: TC 2.A.7.3).
AT1G77280		Protein kinase protein with adenine nucleotide alpha hydrolases-like domain
AT1G78100		F-box family protein;
AT1G78420		RING/U-box superfamily protein
AT2G01430	HOMEOBOX-LEUCINE ZIPPER PROTEIN 17 (HB17)	homeobox-leucine zipper protein 17 (HB17);
AT2G01910	(ATMAP65-6)	Binds microtubules. Induces a crisscross mesh of microtubules, not bundles. Not involved in microtubule polymerization nor nucleation. Localizes to mitochondria.
AT2G02620		Cysteine/Histidine-rich C1 domain family protein

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT2G03730	ACT DOMAIN REPEAT 5 (ACR5)	Member of a small family of ACT domain containing proteins. ACT domains are thought to be involved in amino acid binding.
AT2G03830	ROOT MERISTEM GROWTH FACTOR 8 (RGF8)	Encodes a root meristem growth factor (RGF). Belongs to a family of functionally redundant homologous peptides that are secreted, tyrosine-sulfated, and expressed mainly in the stem cell area and the innermost layer of central columella cells. RGFs are required for maintenance of the root stem cell niche and transit amplifying cell proliferation. Members of this family include: At5g60810 (RGF1), At1g13620 (RGF2), At2g04025 (RGF3), At3g30350 (RGF4), At5g51451 (RGF5), At4g16515 (RGF6), At3g02240 (RGF7), At2g03830 (RGF8) and At5g64770 (RGF9).
AT2G05940		**Protein kinase superfamily protein;**
AT2G14960	(GH3.1)	encodes a protein similar to IAA-amido synthases. Lines carrying an insertion in this gene are hypersensitive to auxin.
AT2G18690		unknown protein;
AT2G18800	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 21 (XTH21)	xyloglucan endotransglucosylase/hydrolase 21 (XTH21);
AT2G18980		Peroxidase superfamily protein
AT2G22500	UNCOUPLING PROTEIN 5 (UCP5)	Encodes one of the mitochondrial dicarboxylate carriers (DIC): DIC1 (AT2G22500), DIC2 (AT4G24570), DIC3 (AT5G09470).
AT2G23170	(GH3.3)	encodes an IAA-amido synthase that conjugates Asp and other amino acids to auxin in vitro.
AT2G25790		**Leucine-rich receptor-like protein kinase family protein;**
AT2G28400		Protein of unknown function, DUF584;
AT2G29460	GLUTATHIONE S-TRANSFERASE TAU 4 (GSTU4)	Encodes glutathione transferase belonging to the tau class of GSTs. Naming convention according to Wagner et al. (2002).
AT2G30040	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 14 (MAPKKK14)	member of MEKK subfamily
AT2G30140		UDP-Glycosyltransferase superfamily protein
AT2G30930		unknown protein
AT2G33310	AUXIN-INDUCED PROTEIN 13 (IAA13)	Auxin induced gene, IAA13 (IAA13).

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT2G34650	**PINOID (PID)**	**Encodes a protein serine/threonine kinase that may act as a positive regulator of cellular auxin efflux, as a a binary switch for PIN polarity, and as a negative regulator of auxin signaling. Recessive mutants exhibit similar phenotypes as pin-formed mutants in flowers and inflorescence but distinct phenotypes in cotyledons and leaves. Expressed in the vascular tissue proximal to root and shoot meristems, shoot apex, and embryos. Expression is induced by auxin. Overexpression of the gene results in phenotypes in the root and shoot similar to those found in auxin-insensitive mutants. The protein physically interacts with TCH3 (TOUCH3) and PID-BINDING PROTEIN 1 (PBP1), a previously uncharacterized protein containing putative EF-hand calcium-binding motifs. Acts together with ENP (ENHANCER OF PINOID) to instruct precursor cells to elaborate cotyledons in the transition stage embryo. Interacts with PDK1. PID autophosphorylation is required for the ability of PID to phosphorylate an exogenous substrate. PID activation loop is required for PDK1-dependent PID phosphorylation and requires the PIF domain. Negative regulator of root hair growth. PID kinase activity is critical for the inhibition of root hair growth and for maintaining the proper subcellular localization of PID.**
AT2G35930	PLANT U-BOX 23 (PUB23)	Encodes a cytoplasmically localized U-box domain containing E3 ubiquitin ligase that is involved in the response to water stress and acts as a negative regulator of PAMP-triggered immunity.
AT2G35980	YELLOW-LEAF-SPECIFIC GENE 9 (YLS9)	Encodes a protein whose sequence is similar to tobacco hairpin-induced gene (HIN1) and Arabidopsis non-race specific disease resistance gene (NDR1). Expression of this gene is induced by cucumber mosaic virus, spermine and during senescence. The gene product is localized to the chloroplast.
AT2G36220		unknown protein
AT2G39350	ATP-BINDING CASSETTE G1 (ABCG1)	ABC-2 type transporter family protein
AT2G39370		unknown protein;
AT2G39700	EXPANSIN A4 (EXPA4)	putative expansin.
AT2G40540	POTASSIUM TRANSPORTER 2 (KT2)	putative potassium transporter AtKT2p (AtKT2) mRNA,
AT2G41100	**TOUCH 3 (TCH3)**	**encodes a calmodulin-like protein, with six potential calcium binding domains. Calcium binding shown by Ca(2+)-specific shift in electrophoretic mobility. Expression induced by touch and darkness. Expression may also be developmentally controlled. Expression in growing regions of roots, vascular tissue, root/shoot junctions, trichomes, branch points of the shoot, and regions of siliques and flowers.**
AT2G41380		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein;

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT2G41810		_ unknown
AT2G42430	LATERAL ORGAN BOUNDARIES-DOMAIN 16 (LBD16)	LOB-domain protein gene LBD16. This gene contains one auxin-responsive element (AuxRE).
AT2G42440		Lateral organ boundaries (LOB) domain family protein; CONTAINS InterPro DOMAIN/s: Lateral organ boundaries, LOB (InterPro:IPR004883)
AT2G42570	TRICHOME BIREFRINGENCE-LIKE 39 (TBL39)	Encodes a member of the TBL (TRICHOME BIREFRINGENCE-LIKE) gene family containing a plant-specific DUF231 (domain of unknown function) domain. TBL gene family has 46 members, two of which (TBR/AT5G06700 and TBL3/AT5G01360) have been shown to be involved in the synthesis and deposition of secondary wall cellulose, presumably by influencing the esterification state of pectic polymers. A nomenclature for this gene family has been proposed (Volker Bischoff & Wolf Scheible, 2010, personal communication).
AT2G42870	PHY RAPIDLY REGULATED 1 (PAR1)	Encodes PHYTOCHROME RAPIDLY REGULATED1 (PAR1), an atypical basic helix-loop-helix (bHLP) protein. Closely related to PAR2 (At3g58850). Up regulated after simulated shade perception. Acts in the nucleus to control plant development and as a negative regulator of shade avoidance response. Functions as transcriptional repressor of auxin-responsive genes SAUR15 (AT4G38850) and SAUR68 (AT1G29510).
AT2G43290	**MULTICOPY SUPPRESSORS OF SNF4 DEFICIENCY IN YEAST 3 (MSS3)**	**Encodes calmodulin-like MSS3.**
AT2G43590		Chitinase family protein
AT2G43880		Pectin lyase-like superfamily protein;
AT2G45400	(BEN1)	involved in the regulation of brassinosteroid metabolic pathway
AT2G45420	LOB DOMAIN-CONTAINING PROTEIN 18 (LBD18)	LOB domain-containing protein 18 (LBD18)
AT2G47130		NAD(P)-binding Rossmann-fold superfamily protein
AT2G47140		NAD(P)-binding Rossmann-fold superfamily protein
AT2G47260	WRKY DNA-BINDING PROTEIN 23 (WRKY23)	Encodes a member of WRKY Transcription Factor; Group I. Involved in nematode feeding site establishment.
AT2G47440		Tetratricopeptide repeat (TPR)-like superfamily protein Eukaryotes - 33 (source: NCBI BLink).
AT3G01190		Peroxidase superfamily protein

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT3G02850	STELAR K+ OUTWARD RECTIFIER (SKOR)	Encodes SKOR, a member of Shaker family potassium ion (K+) channel. This family includes five groups based on phylogenetic analysis (FEBS Letters (2007) 581: 2357): I (inward rectifying channel): AKT1 (AT2G26650), AKT5 (AT4G32500) and SPIK (also known as AKT6, AT2G25600); II (inward rectifying channel): KAT1 (AT5G46240) and KAT2 (AT4G18290); III (weakly inward rectifying channel): AKT2 (AT4G22200); IV (regulatory subunit involved in inwardly rectifying conductance formation): KAT3 (also known as AtKC1, AT4G32650); V (outward rectifying channel): SKOR (AT3G02850) and GORK (AT5G37500). Mediates the delivery of K+ from stelar cells to the xylem in the roots towards the shoot. mRNA accumulation is modulated by abscisic acid. K+ gating activity is modulated by external and internal K+.
AT3G02885	GAST1 PROTEIN HOMOLOG 5 (GASA5)	GAST1 protein homolog 5 (GASA5); INVOLVED IN: response to gibberellin stimulus;
AT3G06460		GNS1/SUR4 membrane protein family;
AT3G07010		Pectin lyase-like superfamily protein
AT3G07390	AUXIN-INDUCED IN ROOT CULTURES 12 (AIR12)	isolated from differential screening of a cDNA library from auxin-treated root culture. sequence does not show homology to any known proteins and is predicted to be extracellular.
AT3G09280		unknown protein
AT3G12700		Eukaryotic aspartyl protease family protein;
AT3G13380	BRI1-LIKE 3 (BRL3)	Similar to BRI, brassinosteroid receptor protein.
AT3G14690	CYTOCHROME P450, FAMILY 72, SUBFAMILY A, POLYPEPTIDE 15 (CYP72A15)	putative cytochrome P450
AT3G15540	INDOLE-3-ACETIC ACID INDUCIBLE 19 (IAA19)	Primary auxin-responsive gene. Involved in the regulation stamen filaments development.
AT3G16180		Major facilitator superfamily protein
AT3G16570	RAPID ALKALINIZATION FACTOR 23 (RALF23)	Encodes RALF23, a member of a diversely expressed predicted peptide family showing sequence similarity to tobacco Rapid Alkalinization Factor (RALF), and is believed to play an essential role in the physiology of Arabidopsis. Consists of a single exon and is characterized by a conserved C-terminal motif and N-terminal signal peptide. RALF23 is significantly downregulated by brassinolide treatment of seedlings. Overexpression of AtRALF23 impairs brassinolide-induced hypocotyls elongation, and mature overexpressing plants are shorter and bushier. RALF23 overexpression produces slower growing seedlings with roots that have reduced capacity to acidify the rhizosphere.
AT3G18280		Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein;
AT3G18560		unknown protein;
AT3G19200		unknown protein;
AT3G19320		Leucine-rich repeat (LRR) family protein

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT3G20015		Eukaryotic aspartyl protease family protein
AT3G20830		**AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family protein;**
AT3G21250	ATP-BINDING CASSETTE C8 (ABCC8)	member of MRP subfamily
AT3G21700	(SGP2)	Monomeric G protein. Expressed in root epidermal cells that are destined to become atrichoblasts. Also expressed during pollen development and in the pollen tube tip.
AT3G23030	INDOLE-3-ACETIC ACID INDUCIBLE 2 (IAA2)	auxin inducible gene expressed in the nucleus
AT3G23730	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 16 (XTH16)	xyloglucan endotransglucosylase/hydrolase 16 (XTH16); FUNCTIONS IN: hydrolase activity, acting on glycosyl bonds, hydrolase activity, hydrolyzing O-glycosyl compounds, xyloglucan:xyloglucosyl transferase activity; INVOLVED IN: carbohydrate metabolic process, cellular glucan metabolic process
AT3G24750		unknown protein
AT3G26610		Pectin lyase-like superfamily protein
AT3G26760		NAD(P)-binding Rossmann-fold superfamily protein
AT3G28850		Glutaredoxin family protein
AT3G42800		unknown protein
AT3G43800	GLUTATHIONE S-TRANSFERASE TAU 27 (GSTU27)	Encodes glutathione transferase belonging to the tau class of GSTs. Naming convention according to Wagner et al. (2002).
AT3G44990	XYLOGLUCAN ENDO-TRANSGLYCOSYLASE-RELATED 8 (XTR8)	xyloglucan endo-transglycosylase
AT3G48520	CYTOCHROME P450, FAMILY 94, SUBFAMILY B, POLYPEPTIDE 3 (CYP94B3)	CYP94B3 is a jasmonoyl-isoleucine-12-hydroxylase that catalyzes the formation of 12-OH-JA-Ile from JA-Ile. By reducing the levels of this the biologically active phytohormone, CYP94B3 attenuates the jasmonic acid signaling cascade. CYP94B3 transcript levels rise in response to wounding.
AT3G49350		**Ypt/Rab-GAP domain of gyp1p superfamily protein**
AT3G49360	6-PHOSPHOGLUCONOLACTONASE 2 (PGL2)	6-phosphogluconolactonase 2 (PGL2)
AT3G49700	1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 9 (ACS9)	encodes a a member of the 1-aminocyclopropane-1-carboxylate (ACC) synthase (S-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14) gene family. Mutants produce elevated levels of ethylene as etiolated seedlings.

Table S1. continuation

Locus identifier	**Primary Gene Symbol**	**Gene Model Description**
AT3G50660	DWARF 4 (DWF4)	Encodes a 22α hydroxylase whose reaction is a rate-limiting step in brassinosteroid biosynthetic pathway. The protein is a member of CYP90B gene family. CLM is an epi-allele with small, compressed rosette, reduced internode length, and reduced fertility, appears in selfed ddm mutant plants possibly due to loss of cytosine methylation. Transcripts accumulate in actively growing tissues, and GUS expression is negatively regulated by brassinosteroids. Localized in the endoplasmic reticulum. The in vitro expressed protein can perform the C-22 hydroxylation of a variety of C27-, C28- and C29-sterols. Cholesterol was the best substrate, followed by campesterol. Sitosterol was a poor substrate.
AT3G51410		Arabidopsis protein of unknown function (DUF241)
AT3G51670	**PATL6**	**SEC14 cytosolic factor family protein / phosphoglyceride transfer family protein**
AT3G54000		Uncharacterised conserved protein UCP022260
AT3G54770		RNA-binding (RRM/RBD/RNP motifs) family protein
AT3G54950	PATATIN-RELATED PHOSPHOLIPASE IIIBETA (pPLAIIIbeta)	Encodes pPLAIIIbeta, a member of the Group 3 patatin-related phospholipases. pPLAIIIbeta hydrolyzes phospholipids and galactolipids and additionally has acyl-CoA thioesterase activity. Alterations of pPLAIIIβ result in changes in lipid levels and composition.
AT3G55690		unknown protein;
AT3G55720		Protein of unknown function (DUF620)
AT3G56230		BTB/POZ domain-containing protein
AT3G56880		VQ motif-containing protein
AT3G58190	LATERAL ORGAN BOUNDARIES-DOMAIN 29 (LBD29)	This gene contains two auxin-responsive element (AuxRE).
AT3G60550	CYCLIN P3;2 (CYCP3;2)	cyclin p3;2 (CYCP3;2);
AT3G60630	HAIRY MERISTEM 2 (HAM2)	Belongs to one of the LOM (LOST MERISTEMS) genes: AT2G45160 (LOM1), AT3G60630 (LOM2) and AT4G00150 (LOM3). LOM1 and LOM2 promote cell differentiation at the periphery of shoot meristems and help to maintain their polar organization.
AT3G60640	AUTOPHAGY 8G (ATG8G)	AUTOPHAGY 8G (ATG8G)
AT3G61490		Pectin lyase-like superfamily protein;
AT3G62100	INDOLE-3-ACETIC ACID INDUCIBLE 30 (IAA30)	Encodes a member of the Aux/IAA family of proteins implicated in auxin signaling. IAA30 lacks the conserved degron (domain II) found in many family members. IAA30 transcripts are induced by auxin treatment and accumulate preferentially in the quiescent center cells of the root meristem. Overexpression of IAA30 leads to defects in gravitropism, root development, root meristem maintenance, and cotyledon vascular development. Target of LEC2 and AGL15. Promotes somatyic embryogenesis.

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT3G63440	CYTOKININ OXIDASE/DEHYDROGENASE 6 (CKX6)	This gene used to be called AtCKX7. It encodes a protein whose sequence is similar to cytokinin oxidase/dehydrogenase, which catalyzes the degradation of cytokinins.
AT4G00080	UNFERTILIZED EMBRYO SAC 11 (UNE11)	unfertilized embryo sac 11 (UNE11)
AT4G01870		tolB protein-related
AT4G03820		Protein of unknown function (DUF3537)
AT4G08040	1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 11 (ACS11)	encodes an aminotransferase that belongs to ACC synthase gene family structurally
AT4G09570	CALCIUM-DEPENDENT PROTEIN KINASE 4 (CPK4)	Encodes a member of Calcium Dependent Protein Kinase (CDPK) gene family.Positive regulator of ABA signaling. Phosphorylates ABA responsive transcription factors ABF1 and ABF4.
AT4G11280	1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE 6 (ACS6)	encodes a a member of the 1-aminocyclopropane-1-carboxylate (ACC) synthase (S-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14) gene family
AT4G12110	STEROL-4ALPHA-METHYL OXIDASE 1-1 (SMO1-1)	Encodes a member of the SMO1 family of sterol 4alpha-methyl oxidases. More specifically functions as a 4,4-dimethyl-9beta,19-cyclopropylsterol-4alpha- methyl oxidase.
AT4G12410		SAUR-like auxin-responsive protein family ; CONTAINS InterPro DOMAIN/s: Auxin responsive SAUR protein (InterPro:IPR003676); BEST Arabidopsis thaliana protein match is: SAUR-like auxin-responsive protein family (TAIR:AT4G22620.1); Has 1137 Blast hits to 1128 proteins in 26 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 1136; Viruses - 0; Other Eukaryotes - 1 (source: NCBI BLink).
AT4G12720	(NUDT7)	Encodes a protein with ADP-ribose hydrolase activity. Negatively regulates EDS1-conditioned plant defense and programmed cell death.
AT4G12730	FASCICLIN-LIKE ARABINOGALACTAN 2 (FLA2)	AF333971 Arabidopsis thaliana fasciclin-like arabinogalactan-protein 2 (Fla2) mRNA, complete cds
AT4G12880	EARLY NODULIN-LIKE PROTEIN 19 (ENODL19)	early nodulin-like protein 19 (ENODL19)
AT4G13180		NAD(P)-binding Rossmann-fold superfamily protein).
AT4G13195	CLAVATA3/ESR-RELATED 44 (CLE44)	Belongs to a large gene family, called CLE for CLAVATA3/ESR-related, encoding small peptides with conserved carboxyl termini. The C-terminal 12 amino acid sequence of CLE44 is identical to that of a dodeca peptide (TDIF, tracheary element differentiation inhibitory factor) isolated from Arabidopsis and functions as a suppressor of plant stem cell differentiation. TDIF sequence is also identical to the C-terminal 12 amino acids of CLE41 (At3g24770).
AT4G14130	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 15 (XTH15)	xyloglucan endotransglycosylase-related protein (XTR7)

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT4G14560	INDOLE-3-ACETIC ACID INDUCIBLE (IAA1)	auxin (indole-3-acetic acid) induced gene (IAA1) encoding a short-lived nuclear-localized transcriptional regulator protein.
AT4G14750	IQ-DOMAIN 19 (IQD19)	IQ-domain 19 (IQD19); CONTAINS InterPro DOMAIN/s: IQ calmodulin-binding region (InterPro:IPR000048)
AT4G17350		DOMAIN/s: Pleckstrin-like, plant (InterPro:IPR013666), Protein of unknown function DUF828 (InterPro:IPR008546), Pleckstrin homology (InterPro:IPR001849
AT4G17490	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 6 (ERF6)	Encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ATERF-6). The protein contains one AP2 domain. There are 18 members in this subfamily including ATERF-1, ATERF-2, AND ATERF-5.
AT4G17870	PYRABACTIN RESISTANCE 1 (PYR1)	Encodes a member of the PYR (pyrabactin resistance)/PYL(PYR1-like)/RCAR (regulatory components of ABA receptor) family proteins with 14 members. PYR/PYL/RCAR family proteins function as abscisic acid sensors. Mediate ABA-dependent regulation of protein phosphatase 2Cs ABI1 and ABI2.
AT4G20460		NAD(P)-binding Rossmann-fold superfamily protein
AT4G21200	GIBBERELLIN 2-OXIDASE 8 (GA2OX8)	Encodes a protein with gibberellin 2-oxidase activity which acts specifically on C-20 gibberellins.
AT4G21850	METHIONINE SULFOXIDE REDUCTASE B9 (MSRB9)	methionine sulfoxide reductase B9 (MSRB9);
AT4G21870		HSP20-like chaperones superfamily protein
AT4G22530		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
AT4G22620		SAUR-like auxin-responsive protein family
AT4G22780	ACT DOMAIN REPEAT 7 (ACR7)	Member of a family of ACT domain containing proteins . ACT domains are involved in amino acid binding .
AT4G24160		Encodes a soluble lysophosphatidic acid acyltransferase with additional triacylglycerol lipase and phosphatidylcholine hydrolyzing enzymatic activities. Plays a pivotal role in maintaining the lipid homeostasis by regulating both phospholipid and neutral lipid levels.
AT4G25250		Plant invertase/pectin methylesterase inhibitor superfamily protein
AT4G26320	ARABINOGALACTAN PROTEIN 13 (AGP13)	arabinogalactan protein 13 (AGP13);
AT4G27260	(WES1)	encodes an IAA-amido synthase that conjugates Asp and other amino acids to auxin in vitro. Lines carrying insertions in this gene are hypersensitive to auxin.
AT4G27280		Calcium-binding EF-hand family protein;
AT4G27290		**S-locus lectin protein kinase family protein**
AT4G28640	INDOLE-3-ACETIC ACID INDUCIBLE 11 (IAA11)	Auxin induced gene, IAA11 (IAA11).

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT4G29900	AUTOINHIBITED CA(2+)-ATPASE 10 (ACA10)	one of the type IIB calcium pump isoforms. encodes an autoinhibited Ca(2+)-ATPase that contains an N-terminal calmodulin binding autoinhibitory domain.
AT4G30170		Peroxidase family protein
AT4G30420		nodulin MtN21 /EamA-like transporter family protein
AT4G30450		glycine-rich protein
AT4G31320		SAUR-like auxin-responsive protein family
AT4G31910		HXXXD-type acyl-transferase family protein
AT4G32280	INDOLE-3-ACETIC ACID INDUCIBLE 29 (IAA29)	Auxin inducible protein.
AT4G34150		Calcium-dependent lipid-binding (CaLB domain) family protein;
AT4G34710	ARGININE DECARBOXYLASE 2 (ADC2)	encodes a arginine decarboxylase (ADC), a rate-limiting enzyme that catalyzes the first step of polyamine (PA) biosynthesis via ADC pathway in Arabidopsis thaliana. Arabidopsis genome has two ADC paralogs, ADC1 and ADC2. ADC2 is stress-inducible (osmotic stress). Double mutant analysis showed that ADC genes are essential for the production of PA, and are required for normal seed development. Overexpression causes phenotypes similar to GA-deficient plants and these plants show reduced levels of GA due to lower expression levels of AtGA20ox1, AtGA3ox3 and AtGA3ox1.
AT4G35210		Arabidopsis protein of unknown function (DUF241);
AT4G35320		unknown protein
AT4G37290		unknown protein
AT4G37295		unknown protein
AT4G37590	NAKED PINS IN YUC MUTANTS 5 (NPY5)	A member of the NPY gene family (NPY1/AT4G31820, NPY2/AT2G14820, NPY3/AT5G67440, NPY4/AT2G23050, NPY5/AT4G37590). Involved in auxin-mediated organogenesis.
AT5G01750		Protein of unknown function (DUF567)
AT5G01840	OVATE FAMILY PROTEIN 1 (OFP1)	Encodes a member of the plant specific ovate protein family. Members of this family have been shown to bind to KNOX and BELL-like TALE class homeodomain proteins. This interaction may mediate relocalization of the TALE homeodomain from the nucleus to the cytoplasm. Functions as a transcriptional repressor that suppresses cell elongation.
AT5G02760		**Protein phosphatase 2C family protein**
AT5G03960	IQ-DOMAIN 12 (IQD12)	IQ-domain 12 (IQD12)
AT5G04980		DNAse I-like superfamily protein
AT5G05160	**REDUCED IN LATERAL GROWTH1 (RUL1)**	**Encodes a receptor-like kinase that activates secondary growth, the production of secondary vascular tissues.**
AT5G06080	LOB DOMAIN-CONTAINING PROTEIN 33 (LBD33)	LOB domain-containing protein 33 (LBD33)

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT5G10210		C2 calcium-dependent membrane targeting
AT5G10430	ARABINOGALACTAN PROTEIN 4 (AGP4)	Encodes arabinogalactan-protein (AGP4).
AT5G12050		unknown protein;
AT5G13910	LEAFY PETIOLE (LEP)	Encodes a member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family (LEAFY PETIOLE). The protein contains one AP2 domain. There are 15 members in this subfamily including ATERF-3, ATERF-4, ATERF-7, and LEAFY PETIOLE. Acts as a positive regulator of gibberellic acid-induced germination.
AT5G15890	TRICHOME BIREFRINGENCE-LIKE 21 (TBL21)	Encodes a member of the TBL (TRICHOME BIREFRINGENCE-LIKE) gene family containing a plant-specific DUF231 (domain of unknown function) domain. TBL gene family has 46 members, two of which (TBR/AT5G06700 and TBL3/AT5G01360) have been shown to be involved in the synthesis and deposition of secondary wall cellulose, presumably by influencing the esterification state of pectic polymers. A nomenclature for this gene family has been proposed (Volker Bischoff & Wolf Scheible, 2010, personal communication).
AT5G16110		unknown protein
AT5G16120		alpha/beta-Hydrolases superfamily protein
AT5G17340		Putative membrane lipoprotein
AT5G18560	(PUCHI)	Encodes PUCHI, a member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 15 members in this subfamily including ATERF-3, ATERF-4, ATERF-7, and leafy petiole. PUCHI is required for morphogenesis in the early lateral root primordium of Arabidopsis. Expressed in early floral meristem (stage 1 to 2). Required for early floral meristem growth and for bract suppression. Triple mutant with bop1 and bop2 displays a strong defect in the determination of floral meristem identity with reduced LFY expression and the lack of AP1 expression.
AT5G19530	ACAULIS 5 (ACL5)	Encodes a spermine synthase. Required for internode elongation and vascular development, specifically in the mechanism that defines the boundaries between veins and nonvein regions. This mechanism may be mediated by polar auxin transport. Though ACL5 has been shown to function as a spermine synthase in E. coli, an ACL5 knockout has no effect on the endogenous levels of free and conjugated polyamines in Arabidopsis, suggesting that ACL5 may have a very specific or altogether different in vivo function.
AT5G20230	BLUE-COPPER-BINDING PROTEIN (BCB)	Al-stress-induced gene
AT5G24100		**Leucine-rich repeat protein kinase family protein**
AT5G26930	GATA TRANSCRIPTION FACTOR 23 (GATA23)	Encodes a member of the GATA factor family of zinc finger transcription factors. Controls lateral root founder cell specification.
AT5G27000	KINESIN 4 (ATK4)	Encodes a kinesin-like protein that binds microtubules in an ATP-dependent manner.
AT5G39670		Calcium-binding EF-hand family protein;
AT5G40540		**Protein kinase superfamily protein**

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT5G42050		DCD (Development and Cell Death) domain protein
AT5G43700	AUXIN INDUCIBLE 2-11 (ATAUX2-11)	Auxin inducible protein similar to transcription factors.
AT5G47250		LRR and NB-ARC domains-containing disease resistance protein;
AT5G47370	(HAT2)	homeobox-leucine zipper genes induced by auxin, but not by other phytohormones. Plays opposite roles in the shoot and root tissues in regulating auxin-mediated morphogenesis.
AT5G47440		CONTAINS InterPro DOMAIN/s: Pleckstrin-like, plant (InterPro:IPR013666), Protein of unknown function DUF828 (InterPro:IPR008546), Pleckstrin homology (InterPro:IPR001849)
AT5G48150	PHYTOCHROME A SIGNAL TRANSDUCTION 1 (PAT1)	Member of GRAS gene family. Semi-dominant mutant has a reduced response to far-red light and appears to act early in the phytochrome A signaling pathway.
AT5G49360	BETA-XYLOSIDASE 1 (BXL1)	Encodes a bifunctional {beta}-D-xylosidase/{alpha}-L-arabinofuranosidase required for pectic arabinan modification. Located in the extracellular matrix. Gene is expressed specifically in tissues undergoing secondary wall thickening. This is a member of glycosyl hydrolase family 3 and has six other closely related members.
AT5G49450	BASIC LEUCINE-ZIPPER 1 (bZIP1)	basic leucine-zipper 1 (bZIP1)
AT5G51670		Protein of unknown function (DUF668)
AT5G52450		MATE efflux family protein
AT5G52900		unknown protein
AT5G53250	ARABINOGALACTAN PROTEIN 22 (AGP22)	arabinogalactan protein 22 (AGP22)
AT5G54130		Calcium-binding endonuclease/exonuclease/phosphatase family
<u>AT5G54380</u>	**THESEUS1 (THE1)**	**Encodes THESEUS1 (THE1), a receptor kinase regulated by Brassinosteroids and required for cell elongation during vegetative growth.**
<u>AT5G54490</u>	**PINOID-BINDING PROTEIN 1 (PBP1)**	**Encodes a PINOID (PID)-binding protein containing putative EF-hand calcium-binding motifs. The interaction is dependent on the presence of calcium. mRNA expression is up-regulated by auxin. Not a phosphorylation target of PID, likely acts upstream of PID to regulate the activity of this protein in response to changes in calcium levels.**
AT5G54500	FLAVODOXIN-LIKE QUINONE REDUCTASE 1 (FQR1)	Encodes a flavin mononucleotide-binding flavodoxin-like quinone reductase that is a primary auxin-response gene.
AT5G54510	DWARF IN LIGHT 1 (DFL1)	Encodes an IAA-amido synthase that conjugates Ala, Asp, Phe, and Trp to auxin. Lines overexpressing this gene accumulate IAA-ASP and are hypersensitive to several auxins. Identified as a dominant mutation that displays shorter hypocotyls in light grown plants when compared to wild type siblings. Protein is similar to auxin inducible gene from pea (GH3).
AT5G57100		Nucleotide/sugar transporter family protein

Table S1. continuation

Locus identifier	Primary Gene Symbol	Gene Model Description
AT5G57520	ZINC FINGER PROTEIN 2 (ZFP2)	Encodes a zinc finger protein containing only a single zinc finger.
AT5G60450	AUXIN RESPONSE FACTOR 4 (ARF4)	Encodes a member of the ARF family of transcription factors which mediate auxin responses. ARF4 appears to have redundant function with ETT(ARF3) in specifying abaxial cell identity.
AT5G60520		Late embryogenesis abundant (LEA) protein-related;
AT5G60660	PLASMA MEMBRANE INTRINSIC PROTEIN 2;4 (PIP2;4)	A member of the plasma membrane intrinsic protein subfamily PIP2.When expressed in yeast cells can conduct hydrogen peroxide into those cells. Mutants exhibit longer root hairs.
AT5G62280		Protein of unknown function (DUF1442)
AT5G64250		Aldolase-type TIM barrel family protein
AT5G65390	ARABINOGALACTAN PROTEIN 7 (AGP7)	arabinogalactan protein 7 (AGP7)
AT5G65670	INDOLE-3-ACETIC ACID INDUCIBLE 9 (IAA9)	auxin (indole-3-acetic acid) induced gene
AT5G67430		Acyl-CoA N-acyltransferases (NAT) superfamily protein