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Chapter 1

General introduction

Fang Huang and Remko Offringa

1. Plant development regulated by plant hormones: auxin is key

Different from animals that can escape unfavorable conditions by moving, plants have established adaptability and flexibility in their development in response to environmental changes to compensate for their sessile lifestyle. In both plants and animals, the regulation and coordination of patterning, differentiation, morphogenesis and growth depend on signaling molecules, called hormones. The term hormone comes from the Greek *horman*, which means “to stimulate”. In the animal field hormones have been defined as molecules that are synthesized at one location and transported to act at another location in the body. This definition does not hold for plant hormones, as they can function both locally at the site of synthesis or after being transported to other parts of the plant. The five classical plant hormones are auxin, abscisic acid (ABA), cytokinins, gibberellins (GAs) and ethylene. Together with the more recently identified plant growth regulators salicylic acid (SA), brassinosteroids, jasmonic acid (JA) and some small peptides, these signaling molecules act both independently and in concert to regulate a broad spectrum of cellular and developmental processes.

The key regulator of plant developmental plasticity is the first discovered plant hormone auxin, or indole-3-acetic acid (IAA). Around 1880 studies by Darwin and his son on the bending of canary grass and oat coleoptiles towards unidirectional light suggested the existence of a transmittable substance that regulates this phototropic response. This substance was later identified as auxin, named after the Greek word for “to grow” (Went, 1937), and recently defined as the morphogenetic trigger in plant development (Benková et al., 2009). Apart from directing tropical growth responses, auxin also regulates many other developmental processes, such as apical dominance, phyllotaxis, and embryo-, root meristem-, and vascular patterning by controlling cell elongation, cell division and differentiation.

2. Auxin action: a result of differential distribution and cellular responses

How can a single molecule like auxin control so many different developmental processes? One critical characteristic of auxin is that it is actively transported from cell to cell in a polar manner, from its sites of synthesis in the young developing organs in the shoot toward the root apex through the central cylinder, and from the root apex toward the elongation zone of the root through the outer cell layers. This polar auxin transport (PAT) generates local auxin maxima and minima that drive the different developmental processes to shape the plant (Tanaka et al., 2006; Sorefan et al., 2009). The mechanism of PAT will be discussed in detail below. First we will present how auxin is perceived

by the target cells and translated into rapid cellular and gene expression responses, following its differential distribution.

The earliest auxin responses occur within 1-10 minutes, and include changes in membrane potential, elevation in cytosolic Ca^{2+} concentration and cytosol acidification. These primary cellular responses require a type of receptor, preferentially localized at the plasma membrane (PM), that is responsible for perception of auxin and the initiation of downstream signal transduction (Shishova and Lindberg, 2010). The AUXIN BINDING PROTEIN 1 (ABP1) was identified as an auxin-binding protein (Feldwisch et al., 1992), and is a best candidate for mediating early cellular auxin responses. The importance of ABP1 in plant development has been indicated by the embryo lethality of the *abp1* null mutant (Chen et al., 2001). ABP1 is an ER-localized and PM-associated protein, and it is suggested to act as a part of an auxin receptor complex at the PM to mediate auxin responses (Tromas et al., 2010). Recent detailed characterization of conditional ABP1 knock-down mutants has revealed that ABP1 is required for almost all auxin-mediated responses, including auxin-regulated gene expression (Braun et al., 2008).

The gene expression responses involve two families of proteins: the Auxin Response Factors (ARFs) and the Aux/IAA repressor proteins. ARF proteins act as transcription factors to activate or repress gene expression by binding to specific auxin responsive elements (AuxREs) present in promoters of auxin responsive genes through their N-terminal DNA binding domain (DBD) (Ulmasov et al., 1999). Whether ARFs activate or repress transcription is determined by the amino acid sequence of the central region adjacent to DBD (Mockaitis and Estelle, 2008). At the C-terminus two domains (III and IV) are present that are conserved in both ARF and Aux/IAA proteins, and mediate homo- or heterodimerization between these two families of proteins (Guilfoyle et al., 1998a; Guilfoyle et al., 1998b). Aux/IAA proteins function as short-lived transcriptional repressors, and the encoding genes have originally been identified as primary auxin responsive genes. Besides the C-terminal conserved dimerization domains (III and IV), Aux/IAAs have a central conserved domain (II) that is involved in the stability of Aux/IAA proteins (Worley et al., 2000; Ramos et al., 2001), and an N-terminal domain (I) that represses transcription (Tiwari et al., 2004). Recently, it was shown that the repressive activity of the Aux/IAA protein BODENLOS/IAA12 (BDL/IAA12) requires the interaction with the co-repressor TOPLESS (TPL) through its domain I (Szemenyei et al., 2008).

In response to auxin treatment, the Aux/IAA repressor proteins are degraded, which subsequently allows ARFs to initiate transcription as monomers or dimers. Auxin-induced Aux/IAA degradation requires the SCF^{TIR1} (Skp1/cullin/F-box protein) E3 ubiquitin ligase complex, with auxin acting as a molecular glue that stabilizes the interaction between domain II of Aux/IAAs and the F-box protein TRANSPORT INHIBITOR RECEPTOR 1 (TIR1) (Gray et al., 2001; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Mutations

in specific amino acids in domain II of Aux/IAA proteins disrupt the binding to TIR1, and result in their stabilization and auxin insensitivity-related gain-of-function mutant phenotypes (Liscum and Reed, 2002). Following their recruitment by the SCF^{TIR1} complex, the efficiency of which is dependent on both the auxin concentration and the TIR1 abundance, Aux/IAAs are ubiquitinated and degraded by the 26S proteasome (Maraschin, 2009).

The *Arabidopsis* genome encodes 23 ARFs and 29 Aux/IAA proteins, and the cell-type specific interactions between these two groups of proteins in response to auxin coordinately regulate gene expression and plant development. For example, BDL/IAA12-regulated MONOPTEROS (MP/ARF5) transcription factor activity is essential for root pole establishment during embryogenesis, as the *bdl* gain-of-function mutant, in which the IAA12/BDL protein is stabilized as a result from specific amino acid substitutions in domain II, produces seedlings lacking a root (Hamann et al., 2002). Different from Aux/IAA gain-of-function mutants, *aux/iaa* loss-of-function mutant plants display only very subtle defects due to functional redundancy between different *Aux/IAA* genes. In contrast, loss-of-function mutations in *ARF* genes interfere with tissue patterning, organ development and growth responses similar to Aux/IAA gain-of-function mutants (Liscum and Briggs, 1995; Sessions et al., 1997; Hardtke and Berleth, 1998; Liscum and Reed, 2002).

3. Auxin transport: a “unidirectional” way to shape the plant

In the 1970's, the chemiosmotic model was proposed to explain the molecular mechanism of PAT (Figure 1). This model postulates that auxin in the apoplast is protonated (IAAH) due to the acidic environment (pH 5.5), and can enter the cell by diffusion through the PM or by import carriers. In the more basic (pH 7.0) cytoplasm, auxin becomes deprotonated, and the resulting IAA⁻ anions can not pass the PM freely and thus become trapped. Auxin can only be exported from the cell by efflux carriers. By the asymmetric placement of these auxin carriers at the PM the direction of PAT is determined (Rubery and Sheldrake, 1973; Raven, 1975). Molecular genetics research in the model plant *Arabidopsis* has provided support for this model, through the identification of the AUX1 and LIKE-AUX1 (LAX) transporters as auxin influx carriers (Parry et al., 2001b), and the PIN-FORMED (PIN) proteins (Paponov et al., 2005) and the P-GLYCOPROTEIN ABC type transporters (ABCBs) (Geisler and Murphy, 2006) as auxin efflux carriers (Figure 1). Based on our current knowledge on the action of the three classes of auxin transporters, the PIN proteins are the rate limiting components in auxin efflux that determine the direction of PAT through their asymmetric localization (Petrásek et al.,

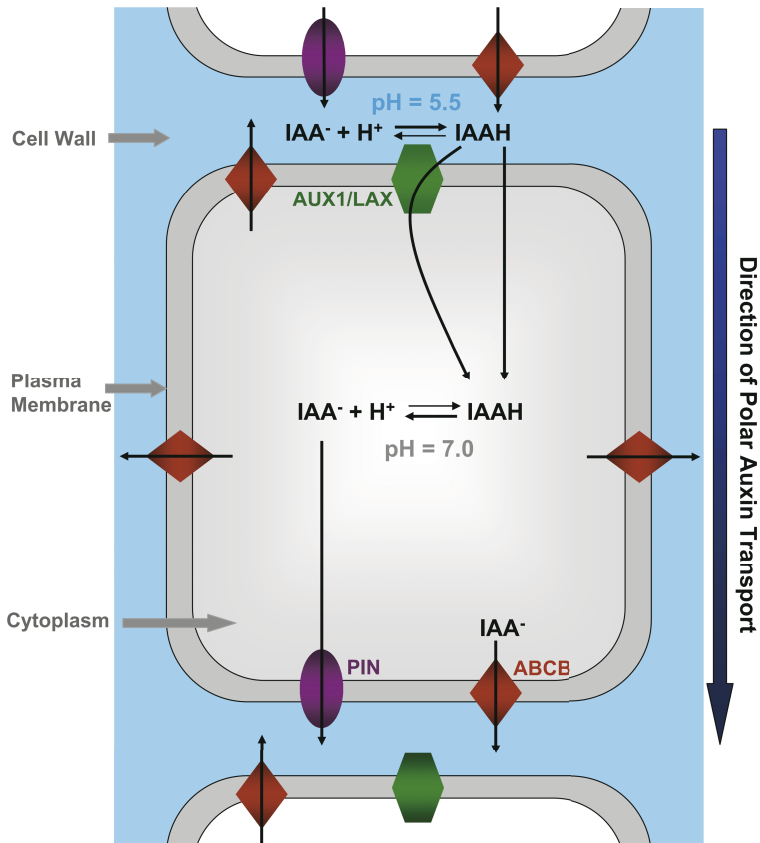


Figure 1. Mechanistic model for polar auxin transport based on the chemiosmotic hypothesis

Due to the lower pH in the plant cell wall the extracellular auxin, IAA exists predominantly in its protonated form (IAAH) which can pass through the PM to the cytosol by diffusion, or can be taken up by auxin influx carriers (AUX1/LAX). The higher pH in the cytosol induces IAAH deprotonation and the resulting anion can not freely pass the PM, and requires PM efflux carriers (PIN and ABCB) to transport out of the cell. The asymmetric placement of the PIN auxin efflux carriers at the PM determines the direction of auxin transport. The AUX1/LAX auxin influx carriers and the ABCB auxin transporters are modulators that determine the amount of auxin available for PIN-directed polar auxin transport.

2006; Wiśniewska et al., 2006), whereas the AUX/LAX and ABCB transporters rather modulate the strength of auxin flow, by controlling the auxin levels available for PIN-driven vectorial auxin transport (Kramer, 2004; Mravec et al., 2008). Below, these three classes of auxin transporters are discussed in more detail.

3.1 The AUX1/LAX auxin influx carriers

The *AUX1* gene has been isolated from the *Arabidopsis aux1* mutant (Bennett et al.,

1996), which was originally obtained from a screen for mutants with auxin-resistant root growth (Pickett et al., 1990). *AUX1* encodes a transmembrane protein with homology to the bacterial amino acid/auxin permease (AAP) family of proton-driven transporters (Bennett et al., 1996; Swarup et al., 2004). The *aux1* agravitropic root phenotype can be rescued by the membrane permeable auxin 1-naphthaleneacetic acid (1-NAA), but not by auxins that require influx carrier activity for efficient uptake, such as IAA and 2,4-dichlorophenoxyacetic acid (2,4-D) (Marchant et al., 1999), suggesting that AUX1 is an auxin influx carrier. This is later supported by the fact that treatment of wild type *Arabidopsis* seedlings with the auxin influx carrier inhibitors 1-naphthoxyacetic acid (1-NOA) and 3-chloro-4-hydroxyphenylacetic acid (CHPAA) phenocopies the *aux1* mutant root phenotype (Parry et al., 2001a). The ability of AUX1 to mediate auxin influx has now been demonstrated in plants and heterologous cell systems, such as *Xenopus oocytes* (Yang et al., 2006).

Consistent with its function in root gravitropism, *AUX1* is most abundantly expressed in root tips (Bennett et al., 1996). At the cellular level, AUX1 is asymmetrically localized to the apical (shootward) membrane of protophloem cells, where it probably is involved in loading auxin into root tips (Swarup et al., 2001), and is localized to the basal (rootward) membrane of lateral root cap (LRC) and root epidermal cells, participating in auxin redistribution from the root tip to the elongation zone required for the root gravitropic response (Kleine-Vehn et al., 2006).

The *Arabidopsis* genome encodes three AUX1 related LAX proteins (LAX1 to 3) (Parry et al., 2001b). They share considerable sequence similarity, but are functionally distinct in a tissue-specific manner. LAX3 is expressed in the cortex and epidermis of the root where it promotes lateral root emergence by facilitating auxin-mediated cell separation (Swarup et al., 2008). Quadruple *aux1/lax* mutants exhibit defects in positioning of leaf primordia in the shoot meristem, indicating the involvement of the AUX1/LAX proteins in phyllotaxis (Bainbridge et al., 2008). As an essential component in auxin transport, AUX1 has been shown to mediate auxin-ethylene cross talk during apical hook development together with LAX3 (Vandenbussche et al., 2010), and auxin-ABA cross-talk during early seedling growth (Belin et al., 2009).

3.2 The PIN auxin efflux carriers

The PIN family proteins were named after the *PIN1* gene, which was initially identified in the *Arabidopsis pin-formed/pin1* mutants, that develop needle-shaped inflorescences, a phenotype that can be mimicked by growing plants on PAT inhibitors (Gälweiler et al., 1998). The *Arabidopsis* genome encodes eight PIN proteins, designated PIN1 to PIN8, six of which have been functionally characterized. Based on their characterization, PIN proteins can be divided into two groups, the PIN1-type proteins comprising PIN1, PIN2,

PIN3, PIN4 and PIN7, and the PIN5-type proteins comprising PIN5, PIN6 and PIN8 (Mravec et al., 2009). The predicted protein structure of the PIN1-type proteins consists of two sets of five transmembrane domains that are linked by a large central hydrophilic loop (HL), whereas the PIN5-type proteins have a smaller central HL (Mravec et al., 2009). The PIN1-type proteins are PM-localized and direct cell-to-cell auxin transport through their asymmetric distribution at the PM (Wiśniewska et al., 2006). They have redundant functions, and a loss-of-function mutation in one *PIN1-type* gene is often compensated for by the ectopic expression of other PINs (Blilou et al., 2005; Vieten et al., 2005). As a result, only mutants in multiple *PIN* genes show more pronounced phenotypes in embryogenesis, root patterning and lateral root initiation (Benková et al., 2003; Friml et al., 2003b; Blilou et al., 2005). The PIN5-type proteins localize to the endoplasmatic reticulum (ER), where they are thought to regulate intracellular auxin homeostasis and metabolism (Mravec et al., 2009).

The asymmetric distribution of the PIN1-type proteins at the PM is determined by cell type-specific signals. PIN1 is basally localized in vascular tissues in embryos, leaves and roots, where it contributes to PAT from the shoot to the root tip; and apically localized in the epidermis of embryos and the shoot apex where it generates auxin maxima that are initiation points of organogenesis (Gälweiler et al., 1998; Benková et al., 2003; Friml et al., 2003b; Reinhardt et al., 2003). PIN2 shows apical localization in epidermal cells and basal localization in cortical cells of the root, mediating auxin flow from the root tip to the elongation zone that regulates (tropic) root growth, and back to the organizing auxin maximum in the root tip (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998). PIN3 shows lateral localization at the inner surface of hypocotyl cells, and apolar distribution in root collumella cells, where it becomes polarized a few minutes after gravistimulation (Friml et al., 2002b). This, together with the observation that *pin3* loss-of-function mutants show delayed growth responses of the hypocotyl or root to unidirectional light or gravistimulation indicates that PIN3 mediates tropic growth responses. The recent observation that a shift from apical to apolar localization of PIN3 in the valve margin of the gynoecium is necessary to generate an auxin minimum that specifies the dehiscence zone, indicates that PIN3 also plays a role in tissue patterning (Sorefan et al., 2009). PIN4 shows apolar localization in root collumella cells, and is localized at the basal side of cells in the proximal root meristem cells, where it functions in confining the auxin maximum that is required for root meristem maintenance (Friml et al., 2002a). PIN7 has been reported to play a role during early embryogenesis. Immediately after division of the zygote, apical PIN7 localization in the basal cell is required to establish the auxin maximum in the apical cell that defines the embryo proper. At globular stage, PIN7 polarity is reversed to the basal membrane of the basal cell and the suspensor cells, mediating auxin efflux from the embryo (Friml et al., 2003b). Interestingly, when PIN1 is

expressed in root epidermal cells where PIN2 shows apical localization, it localizes to the basal side of the cell, indicating that besides the cell-type specific determinants PIN polarity is also determined by PIN-specific sequences (Wiśniewska et al., 2006).

3.3 The ABCB auxin transporters

The third class of auxin transporters belongs to the multidrug resistant (MDR)/P-glycoproteins (PGPs) subfamily of ATP binding cassette (ABC) protein superfamily, that has now been renamed to the ABCB subfamily (Verrier et al., 2008). A role for ABCB proteins in auxin transport was first suggested by the finding that certain members bind to the PAT inhibitor NPA (1-naphthylphthalamic acid), and this was later supported by the fact that several ABCB proteins function in cellular efflux of natural and synthetic auxins by studies in whole plants, *Arabidopsis* protoplasts and heterologous systems (Geisler et al., 2005; Terasaka et al., 2005; Cho et al., 2007; Lewis et al., 2007). The best characterized members of the *Arabidopsis* ABCB proteins are ABCB1, ABCB4 and ABCB19. Microarray data show that the corresponding genes are expressed in both distinct and overlapping tissues, suggesting their functional redundancy and divergence (Titapiwatanakun and Murphy, 2009). Mutations in two of these genes (*ABCB1* and *ABCB19*) result in impaired PAT in seedling hypocotyls (Noh et al., 2001), and faster and greater phototropism and enhanced gravitropism (Noh et al., 2003). ABCB4 is involved in auxin transport processes controlling lateral root and root hair development (Santelia et al., 2005). Loss-of-function *abcb4* mutants show reduced basipetal auxin transport, resulting in reduced root gravitropic bending and elongation in line with its expression in root cap and epidermal cells (Terasaka et al., 2005).

In general, ABCB proteins do not show polar subcellular localization, and therefore they are not considered as the key drivers of PAT in the chemiosmotic model. Instead they seem to regulate PAT by modulating intracellular auxin levels (Mravec et al., 2008). In addition, ABCBs partially colocalize with PINs in a tissue-specific manner (Blakeslee et al., 2007), and ABCB19 has been found to stabilize PIN1 at the PM by physical interaction, and in this way to enhance auxin transport activity (Blakeslee et al., 2007; Titapiwatanakun et al., 2009). Despite the partially overlapping localization at the PM, trafficking of ABCB19 is not sensitive to BFA, and does not coincide with the dynamic cycling of PIN1 or PIN2 (Titapiwatanakun et al., 2009), whereas it is sensitive to the treatment of gravacin, which induces ABCB19 intracellular aggregation (Rojas-Pierce et al., 2007). In contrast, ABCB1 and ABCB4 are found to accumulate in endosomal compartments after BFA treatment (Terasaka et al., 2005; Blakeslee et al., 2007; Titapiwatanakun et al., 2009), suggesting distinct trafficking pathways involved in the regulation of different ABCB proteins.

4. Regulation of plant growth and development through PIN polarity manipulation

Our current understanding of the PAT mechanism indicates a key role of PIN proteins and their asymmetric distribution over the PM. In this thesis, we therefore focus our analysis on the regulation of polar localization of PIN transporters. PIN polarity is dynamic, and determined by trafficking pathways that have been extensively investigated (Geldner et al., 2001; Geldner et al., 2003; Dhonukshe et al., 2007; Dhonukshe et al., 2008; Kleine-Vehn et al., 2008a) (Figure 2), and that are modulated by developmental signals (Benková et al., 2003; Friml et al., 2003b), environmental cues (Friml et al., 2002b; Abas et al., 2006; Harrison and Masson, 2008) and auxin levels (Paciorek et al., 2005; Sauer et al., 2006). As we will discuss later, post-translational modifications of PIN proteins, such as phosphorylation (Michniewicz et al., 2007) and ubiquitination (Abas et al., 2006), are also involved in the manipulation of PIN polarity.

4.1 PIN polar localization regulated by trafficking mechanisms

The establishment and maintenance of cell polarity is critical for the development of multicellular organisms, because the individual cell polarity can be translated into tissue and organ polarities, which ultimately determine the body shape. An important factor in cell polarity establishment is the asymmetric distribution of components at the PM, which is the result of three consecutive steps: (1) Newly synthesized proteins are inserted into the rough ER and transported to the trans-Golgi network (TGN), where they are sorted and delivered to the cell surface. (2) Some proteins are retained at the PM and some are endocytosed into early endosomes (EE). (3) Endocytosed proteins are either recycled back to the original cell surface via recycling endosomes (RE), transported to vacuoles/lysosomes for degradation via late endosomes (LE) (also called multi-vesicular bodies [MVBs] or prevacuolar compartments [PVCs]), or transcytosed to the opposite cell surface (Rodriguez-Boulán et al., 2005).

In contrast to the intensive research on intracellular trafficking of PM proteins in animals and yeast, knowledge about recycling pathways of polar cargoes in plant cells has emerged only recently, with the polar distribution and intracellular trafficking of PIN proteins being most intensively studied. Newly synthesized PIN proteins arrive at the PM in a non-polar manner, and PIN polarity is established by clathrin-mediated endocytosis and subsequent recycling to the PM (Dhonukshe et al., 2007; Dhonukshe et al., 2008). The recycling pathway involves the function of GNOM, an ARF-GEF (guanine-nucleotide exchange factor for ADP ribosylation factor) involved in exocytosis of PINs (Geldner et al., 2003). ARFs are small GTP-binding proteins that act as molecular switches to regulate

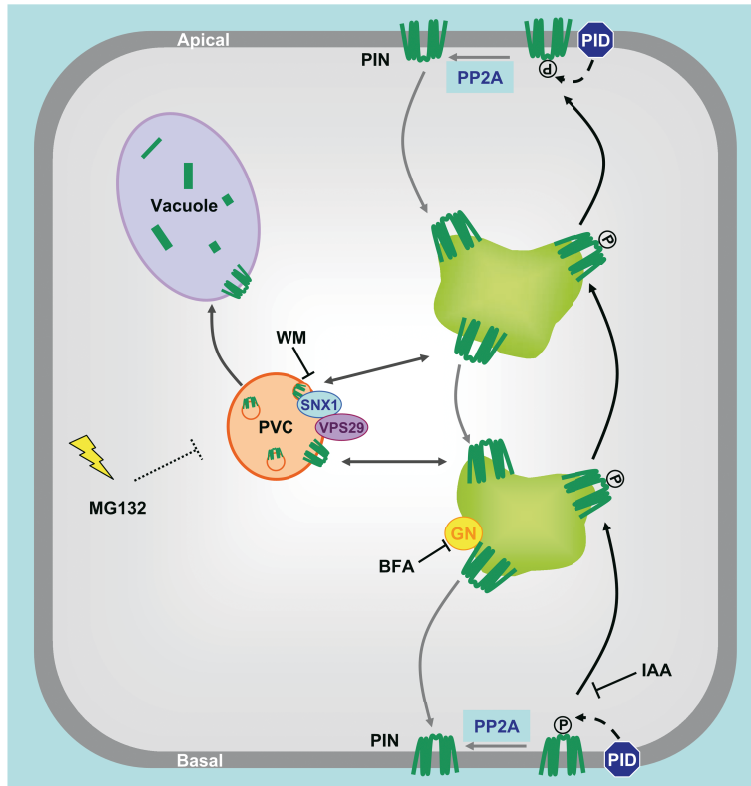


Figure 2. Model for the dynamic subcellular distribution of PIN auxin efflux carriers.

PIN proteins are PM localized auxin efflux transporters that direct PAT through their asymmetric distribution at the PM. Following their de novo biosynthesis, PINs are placed at the PM apolarly, and undergo constitutive endocytosis and recycling back to the PM to establish and maintain their asymmetric distribution. The endocytosis step is clathrin- and sterole-dependent, and can be inhibited by auxin as a feedback regulation of its own transport. The recycling pathway involves the function of ARF-GEF proteins. The BFA-sensitive ARF-GEF GNOM (GN) is required for the basal recycling, whereas, other BFA-resistant ARF-GEF proteins are involved in the apical recycling of PIN-coated vesicles. The basal (rootward) and apical (shootward) recycling pathways are interconnected, and PINs can be recruited from the basal to the apical domain of the PM, so called transcytosis. This process involves the functions of the PID kinase and the PP2A phosphatases, through antagonistic phosphorylation of the hydrophilic loop of PIN proteins. The internalized PINs can be sorted to the vacuole for degradation via the prevacuolar compartment (PVC), and the PVC-located components of the retromer complexes SNX1 (inhibited by Wortmannin [WM]) and VPS29 are likely involved in PIN trafficking from the PVC back to the endosomes. Light signaling and proteasome inhibitor MG132 reduce PIN vacuolar targeting indirectly.

vesicle trafficking, and the interconversion between the active form ARF-GTP and the inactive form ARF-GDP is regulated by two classes of proteins, ARF-GEFs as positive regulators, and ARF-GAPs (ARF GTPase activating proteins) as negative regulators.

GNOM is a target of the fungal toxin brefeldin A (BFA) (Geldner et al., 2003), and pharmacological interference of GNOM function by BFA treatment blocks PIN1 recycling to the basal PM, and induces PIN1 accumulation into so called BFA-compartments (Geldner et al., 2001). Consistently, genomic interference with GNOM function leads to depolarization of PIN1 during embryogenesis, which causes strong defects in embryo axis formation (Steinmann et al., 1999). Nevertheless, apically-localized PIN2 in the root epidermis is less sensitive to BFA treatment, suggesting that apical PINs are recycled in a GNOM-independent manner (Geldner et al., 2003; Kleine-Vehn et al., 2008a). The BFA effect is time- and dosage-dependent, and long-term BFA treatment induces transcytosis of PIN1 from the basal to the apical domain of the cell, indicating that the basal and apical recycling pathways are interconnected (Kleine-Vehn et al., 2008a). Mutant analysis has shown that the ARF-GAP SFC/VAN3 and three homologous are involved in auxin transport-mediated leaf vein pattern formation (Koizumi et al., 2005; Sieburth et al., 2006). PIN1 intracellular accumulation in response to BFA treatment shows different morphology in the *sfc/van3* mutant compared to wild type (Sieburth et al., 2006), suggesting that SFC/VAN3 is also a regulator of PIN trafficking, possibly acting in the opposite pathway to GNOM.

Apart from its importance in polarity establishment, endocytosis is recognized in plants as an important step to regulate PM protein abundance, by subsequent targeting of internalized proteins to the vacuole for degradation. Several PIN proteins have been shown to undergo vacuolar targeting in the dark, including PIN1 and PIN2 (Laxmi et al., 2008; Shirakawa et al., 2009), finally leading to the degradation of the proteins (Kleine-Vehn et al., 2008b). The vacuolar targeting process requires the transport of the internalized PINs from EE to the PVC, and finally to the vacuole. The trafficking between EE and PVC is reversible, and the PVC-localized protein SORTING NEXIN1 (SNX1) (Jaillais et al., 2008) is indicated in the recruitment of transporting PIN cargoes from PVC to the EE (Kleine-Vehn et al., 2008b). SNX1 is sensitive to the phosphatidylinositol-3-OH kinase (PI-3K) inhibitor wortmannin, and treatment with wortmannin induces PIN2 accumulation in SNX1-endosomes, different from GNOM-endosomes (Jaillais et al., 2006). SNX1 is a subunit of the retromer complex that in yeast and mammalian systems is involved in the recycling of proteins from MVBs to the TGN (Bonifacio and Rojas, 2006). Another subunit of the retromer complex Vacuolar Protein Sorting 29 (VPS29) colocalizes with SNX1 (Jaillais et al., 2008). *Arabidopsis* mutants lacking VPS29 show altered PIN1 localization and altered morphology of SNX1-containing endosomes (Jaillais et al., 2007), suggesting that the retromer complex also functions in the endosomal recycling of PINs.

4.2 Internal signals regulating PIN polarity dynamics

Many developmental programs in plants are inextricably linked to the polar localization of PIN proteins. For example, during early embryogenesis from the one-cell to the 16-cell stage, PIN1 is localized at the PM without any detectable polarity. From the 32-cell stage on, however, PIN1 becomes polarly localized to the basal side of provascular cells facing the hypophysis, generating the auxin maximum that defines the QC and columella precursors, or to the apical membrane of epidermal cells to drive the initiation of cotyledon primordia (Friml et al., 2003b). Changes in PIN1 polar localization have also been observed during lateral root development. At the earliest stage, PIN1 is found at the transverse side of the initial founder cells, and later, its polarity changes to the lateral side pointing to the primordium tip. After emergence of the lateral root, PIN1 is predominantly targeted to the cell membrane facing the apex of the young lateral root, resembling its polar localization in the primary root (Benková et al., 2003). These rearrangements of PIN polarity, regulated by developmental cues, allow gradual establishment of auxin gradients that are important for the subsequent programs of plant development.

Almost three decades ago, Sachs formulated the canalization hypothesis to explain auxin transport-driven tissue polarization and patterning. The model proposed that auxin feeds back on its own transport by reorienting transport components (Sachs, 1981). Several recent observations are in line with this hypothesis. Firstly, auxin was found to promote its own efflux from cells by inhibiting their endocytosis and increasing the abundance of PINs at the PM (Paciorek et al., 2005). In addition, it was shown that auxin orients its own flux by Aux/IAA-ARF dependent regulation of PIN polar targeting, and that this intracellular feed back loop steers processes such as regeneration and vascular differentiation (Sauer et al., 2006).

In animals and yeast, polar targeting and endocytic recycling of PM proteins rely on membrane sterols. In plants several lines of evidence suggest a role for sterols in PIN polarity establishment. Firstly, following BFA treatment internalized sterols co-accumulated with PIN2 in BFA-compartments in an actin-dependent manner, implying a link between endocytic sterol and PIN trafficking (Grebe et al., 2003). Secondly, two mutants defective in membrane sterol biosynthesis, *sterol methyltransferase 1 (stm1)* and *cyclopropylsterol isomerase1-1 (cpi1-1)*, showed defects in auxin distribution and regulated plant development, due to disturbed polar localization of PIN1 and PIN3 for *stm1* (Willemsen et al., 2003), and of PIN2 for *cpi1-1* (Men et al., 2008). The *cpi1-1* mutant was found to display altered PIN2 endocytosis (Men et al., 2008). As endocytosis is essential for the generation of PIN polar localization (Dhonukshe et al., 2008), PIN2 polarity in the *cpi1-1* mutant is lost, leading to root agravitropism (Men et al., 2008). Similar observations are obtained by either chemically or genetically inhibiting the sterol biosynthetic enzyme FACKEL (FK) or in the sterol biosynthetic mutant *cvp1-3/stm2*. In the absence of sterol biosynthesis, the auxin inhibitory effect on PIN2 endocytosis

is reversed, confirming the importance of sterols in PIN2 endocytosis and polarity establishment (Pan et al., 2009).

4.3 PIN polarity dynamics induced by external signals

Plants can sense and adapt to environmental changes by multiple ways, and because of the central role of auxin in plant development, the modulation of PIN polar localization by external signals, such as gravity and light forms an important mechanism in plant adaptability. In response to changes in the gravity vector or in the direction of the light source, plants optimize the orientation or position of their organs by means of differential growth, a process that is called gravitropism or phototropism, respectively. During root gravitropism, cell expansion is reduced on the lower side relative to the upper side of the root in the elongation zone, causing the root tip bending toward the gravity vector (Mullen et al., 1998). In shoot phototropism, cell elongation on the dark side is stronger relative to cells at the light side, and this causes the shoot tip bending toward the light source. Almost a century ago, Cholodny and Went proposed that tropic growth responses are directed by differential auxin distribution, and that due to differential sensitivities, elevated auxin levels in the root elongation zone inhibit cell growth, whereas they promote cell growth in the shoot (Went, 1937).

Sensing and growth responses in root gravitropism are accomplished by two spatially distinct root tissues: i) specialized statocytes in the collumella that sense changes in the gravity vector and trigger the onset of differential auxin distribution, and ii) the root elongation zone that responds to the differential auxin levels between the upper and the lower side of the root by differential growth (Chen et al., 1999). Many auxin transport components are functionally involved in root gravitropism, including the auxin influx carrier AUX1, as well as the efflux carriers PIN2 and PIN3, which are expressed in the gravity-responding and -sensing tissues. By a largely unknown signaling mechanism, the changes in the gravity vector are translated into rapid relocalization of PIN3 in root columella cells from apolar distribution to unilateral polarity (Friml et al., 2002b). PIN3 relocation triggers enhanced auxin flow to the lower side of the root, which enhances PIN2 abundance at the PM by inhibiting endocytosis (Paciorek et al., 2005). At the upper side of the reoriented root, PIN2 in the epidermal cells is internalized and subsequently degraded (Abas et al., 2006). The resulting unequal abundance of PIN2 at the upper and lower sides of the root leads to the asymmetric auxin distribution, that subsequently drives the gravitropic growth response.

Phototropism is mediated by both blue light and asymmetric auxin distribution. Pathways for the phototropic growth response from light perception by the phototropin photoreceptors PHOT1 and PHOT2 to the downstream signaling cascade are not clear yet. But evidence has been provided that the auxin efflux transporter PIN3 localized at

the inner membrane of the hypocotyl cells is involved in the asymmetric auxin distribution (Friml et al., 2002b). Until now, the relationship between differential auxin distribution and PIN3 localization during phototropic responses is still unclear. However, based on the mechanism underlying root gravitropism, PIN3 might undergo subcellular relocation to direct differential auxin distribution in the shoot.

4.4 PIN polarity regulated by phosphorylation

Reversible phosphorylation is one of the most important post-translational modifications to modulate protein function. Phosphorylation studies have been extensively performed in different systems, and cumulative evidence supports that phosphorylation can influence multiple characteristics of proteins, including biological activity (Ben-Levy et al., 1995), subcellular location (Hubbard and Cohen, 1993), ubiquitination and protein degradation (Karin, 1999), or protein-protein interactions (Anderson et al., 1990; Ramm et al., 2006). Especially, phosphorylation of cargo proteins is an important mechanism for the regulation of their polar delivery to the PM. For example, in mammalian epithelial cells, phosphorylation of the basally-localized immunoglobulin receptor at a single serine residue results in its accumulation at the apical cell membrane (Casanova et al., 1990).

Similar process has recently been found in plants, correlating PIN polarity alteration with phosphorylation by the PINOID (PID) serine/threonine protein kinase (Friml et al., 2004; Michniewicz et al., 2007). The *PID* gene was identified through the *Arabidopsis pid* loss-of-function mutants that phenocopy the inflorescence defects of the *pin1* mutants (Bennett et al., 1995). The *PID* loss- and gain-of-function mutant phenotypes indicated a role of PID as a positive regulator of PAT (Benjamins et al., 2001). More recently, it was found that PID regulates PAT by determining PIN polar localization. A high dose of PID activity (*PID* overexpression) in roots induces a basal-to-apical PIN polarity switch, leading to auxin-related agravitropic root growth and collapse of the primary root meristem; whereas, reduced PID activity (*pid* loss-of-function) causes an apical-to-basal PIN1 polarity shift, resulting in pin-like inflorescences and defects in auxin-regulated embryo development (Friml et al., 2004). *In vitro* and in protoplast studies have shown that PID phosphorylates the PINHL, and that the PP2A phosphatases act antagonistically with PID to determine the phosphorylation status of the PINs and their polar targeting (Michniewicz et al., 2007).

PID belongs to the plant AGC family of serine/threonine protein kinases, named after the yeast and animal orthologs cAMP-dependent protein kinase A (PKA), cGMP-dependent protein kinase G (PKG), and protein kinase C (PKC) (Bögge et al., 2003). The role for AGC kinases in the regulation of PM protein recycling has been widely reported in mammalian systems. Phosphorylation of serine 256 of aquaporin-2 (AQP2) by PKA is necessary for the exocytosis of AQP2 from intracellular vesicles to the apical

membrane in response to vasopressin-induced cAMP increase (Fushimi et al., 1997; van Balkom et al., 2002). PKC phosphorylation of the cytoplasmic domain of insulin-responsive aminopeptidase (IRAP), a component of the secretory complexes of the glucose transporter GLUT4, plays a key role in the recruitment of GLUT4 to the PM in response to insulin in adipocytes (Ryu et al., 2002). PKC phosphorylation can also down-regulate PM transporter localization. For example, the constitutive recycling of the dopamine transporter (DAT) is regulated by PKC phosphorylation, as PKC phosphorylation accelerates DAT endocytosis rate and reduces recycling, resulting in DAT sequestration from the PM to endosomal compartments (Loder and Melikian, 2003). Another important chemical messenger serotonin, belonging to the same group of compounds as dopamine, is transmitted by the serotonin transporter (SERT). PKC down-regulates SERT activity, probably by sequential phosphorylation, initially on serine residues independent of trafficking and subsequently on threonine residues to enhance endocytosis (Jayanthi et al., 2005). These data suggest that animal AGC kinases share common mechanisms in the regulation of cargo trafficking between PM and endosomes through phosphorylation of specific residues, and that these mechanisms may be conserved in plants as well.

In *Arabidopsis*, PID and 22 other kinases form the plant-specific AGCIII kinase family where PID groups into the AGC3 subclade together with three other kinases WAG1, WAG2 and AGC3-4 (Galván-Ampudia and Offringa, 2007). The localization of all four kinases at the PM (Galván-Ampudia and Offringa, 2007), and their genetic interactions (Cheng et al., 2008), suggest functional redundancy among the AGC3 kinases. It will therefore be interesting to test whether the role of PID as PIN polarity determinant by phosphorylating the PINHL is conserved for the other three AGC3 kinases.

Protein subcellular relocation regulated by phosphorylation has been shown to be critical for the biological function of animal AGC kinases. For example, the conventional PKC can be phosphorylated by its upstream AGC kinase 3-phosphoinositide-dependent protein kinase1 (PDK1) (Mora et al., 2004), inducing its conformational change and subsequent autophosphorylation (Toker and Newton, 2000). Several plant AGC kinases including PID, have also been shown to be phosphorylated by the plant ortholog PDK1 (Zegzouti et al., 2006). PDK1 trans-phosphorylation enhances PID autophosphorylation, similar to the regulation in animals (Zegzouti et al., 2006). Interestingly, a mutated form of PID with a point mutation in the ATP binding domain displays cytoplasmic localization in root hair cells (Lee and Cho, 2006), suggesting that PID kinase activity is critical for its trafficking to the PM. The recent discovery that PDK1-dependent phosphorylation is required for PID relocation from PM to endomembrane structures and microtubules (Galván Ampudia, 2009), together with the previous reports, indicates that PDK1 phosphorylation-regulated AGC kinase relocation is an evolutionarily conserved

mechanism from animals to plants.

5. Thesis outline

The identification and characterization of PID as the molecular determinant in PIN polar localization by phosphorylating PINHL reveals the importance of kinase phosphorylation in PIN-mediated polar auxin transport. However, to date, the phosphorylation targets within PINHL are still unknown and evidence supporting the biological importance of PINHL phosphorylation is still lacking. Previously, two residues in the PIN1HL have been shown to be phosphorylated (Michniewicz et al., 2007) and to be functionally important in PIN polar targeting (Zhang et al., 2010). *In vitro* assays showed that they are not phosphorylated by PID (Zhang et al., 2010). The objective of the research described in this thesis is to identify the PID phosphorylation targets within the PINHL, to use this information to test the substrate specificity of other plant AGC kinases, and to prove their importance in PIN-mediated programmed and adaptive plant development.

Chapter 2 describes the identification of the central serine residues located in three evolutionary conserved TPRXS(N/S) motifs within PIN1HL to be phosphorylated by PID. Suggested by the conservation of the serine residues among several *Arabidopsis* PIN proteins, they are also demonstrated to be phosphorylated by PID in PIN2HL in **Chapter 3**. Interestingly, the three serines are not only phosphorylation targets for PID, but also for the other two closely related AGC3 kinases, WAG1 and WAG2, as serine to alanine substitutions completely abolished PIN2HL phosphorylation by those kinases. These analyses suggested that our identified phosphoserines might have similar functions in the regulatory mechanism by AGC3 kinases. Furthermore, the conservation of the motif sequence within PIN proteins from different plant species implies that this regulation may be operational in all land plants.

The subsequent genetic analyses and localization studies described in **Chapter 2** demonstrated the importance of the three serines for PIN1 polar localization and function in auxin-related plant development. Loss-of-phosphorylation PIN1:GFP proteins show an apical-to-basal polarity switch in epidermis cells of the shoot apical meristem, or mislocalization in embryos, giving rise to defects in organogenesis and embryogenesis. Conversely, phosphomimic PIN1:GFP proteins, in which the three serines have been replaced by glutamic acid, show apical localization in the shoot apex, but can not complement *pin1* inflorescence defects. In *Arabidopsis* roots, basally-localized non-phosphorylatable PIN1:GFP and apically-localized phosphomimic PIN1:GFP proteins are insensitive to *PID* overexpression, and exhibit antagonistic effects on the *35S::PID*-induced root collapse phenotype, consistent with an increased or decreased auxin

accumulation in root tips.

The importance of phosphorylation of the three serines in PIN2 was studied in **Chapter 3**. Replacement of the serines with alanines in a PIN2:VENUS construct resulted in defects in root growth and gravitropic response, correlating with an enhanced auxin accumulation in the columella and LRC cells of the root, and a symmetric auxin distribution in the LRC at the upper and lower side of the root upon gravity stimulation. Interestingly, the loss-of-phosphorylation PIN2:VENUS protein exhibited both PM and intracellular accumulation, and an apical-to-basal polarity change was detected in young epidermal cells in the root tip. These observations resemble those for PIN2 in the *pid wag1 wag2* triple mutant, corroborating that the three kinases act redundantly by phosphorylating the same three serines in the PINHL.

In **Chapter 4**, by using tobacco BY-2 suspension cells, *Arabidopsis* protoplasts, and *Arabidopsis* plant lines, we provide further evidence that phosphorylation of the three conserved serines by PID enhances PIN1 PM localization, and reduces targeting of PIN1 to vacuole for degradation in a dose-dependent manner. These results corroborate the previously proposed role of PID as a positive regulator of PAT (Benjamins et al., 2001; Lee and Cho, 2006).

