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Unraveling ABP1-mediated auxin signaling

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Frequently used abbreviations

ABP1: AUXIN BINDING PROTEIN1

AFB: AUXIN-RELATED F-Box protein

ARF: AUXIN RESPONSE FACTOR

Aux/IAA: auxin/indole-3-acetic acid

AUX1: AUXIN RESISTANT1

AuxRE: auxin responsive element

AXR: AUXIN RESISTANT

BFA: brefeldin A

CUL1: cullin 1

ER: Endoplasmic reticulum

GA: Golgi aparatus

GFP: Green Fluorescent Protein

IAA: indole-3-acetic acid

LAX: LIKE-AUX1

MT: microtubuls

NAA: naphthalene-1-acetic acid

PGP: P-glycoproteins of the ATP-binding cassette transporter family

PILS: Pin-likes

PIN: Pin-formed

PM: plasma membrane

Q-RT-PCR: semi-quantitative reverse transcriptase polymerase chain reaction

RFP: red fluorescent protein

RIC: ROP-interactive CRIB motif-containing protein

ROP: Rho of plants

ROP-GDP: Rho of plants – guanosine diphosphate

ROP-GTP: Rho of plants – guanosine triphosphate

SCF^{TIR}: S-phase kinase-associated protein1-cullin1-transport inhibitor response1

SKP2: S-phase kinase associated protein 2

SKS: SKU5 similar homologues

SKU5: skewed 5

TGN/EE: *trans*-Golgi network/early endosomes

TIR1: TRANSPORT INHIBITOR RESPONSE1

TMK: transmembrane kinase receptor–like kinases

U: ubiquitin

WT: wild type

Scope

Auxin, one of the plant hormones, is a key regulator of plant growth and development. At the cellular level, it controls different processes, such as cell expansion, division, and differentiation, that play a role in a plethora of developmental mechanisms, such as root meristem formation (Blilou et al., 2005; Friml et al., 2002), tropic responses (Abas et al., 2006; Baster et al., 2013; Ding et al., 2011; Rakusová et al., 2011), lateral organ development (Benkova et al., 2003; Reinhard et al., 2003) or embryo development (Friml et al., 2003; Weijers et al., 2005). An important feature of the auxin action is its differential distribution within tissues that is mediated by the polar auxin transport machinery, which can be dynamically regulated in response to internal and external stimuli. Receptors at the cell surface or cell interior are needed to sense and interpret fluctuations in the auxin distribution. Until now, three proteins or protein complexes that can bind auxin have been identified of which two are localized in the nucleus, the SKP-Cullin-F box-Transport Inhibitor Response 1/Auxin-related F-Box ($SCF^{TIR1/AFB}$) and the S-Phase Kinase-Associated Protein 2A (SKP2A), and one, the Auxin-Binding Protein 1 (ABP1), occurs predominantly at the endoplasmic reticulum (ER) and cell surface. Early studies have demonstrated that APB1 is involved in the rapid regulation of the membrane potential and ion fluxes at the plasma membrane and that it mediates the auxin-induced cell swelling, cell elongation, and cell division (Steffens et al., 2001; Yamagami et al., 2004, Braun et al., 2008, Tromas et al., 2009). Recently identified roles include expression of auxin-responsive genes (Tromas et al., 2013; Paque et al., 2014), cytoskeleton rearrangement (Chen et al., 2014), cell morphogenesis and shape of leaf epidermal pavement cells (Xu et al., 2010; Nagawa et al., 2012), and clathrin-mediated endocytosis (Robert et al., 2010).

In this PhD thesis, I focus on the investigation of potentially novel functions of ABP1 that are necessary for proper plant development, on unraveling new APB1 interactors and downstream players in the ABP1 signaling pathway, and on the characterization of impact of phosphorylation on PIN-FORMED3 (PIN3)-mediated biological processes.

The structure of ABP1 has been determined more than a decade ago (Woo et al., 2002), but until now its binding pocket has not been evaluated appropriately. A weak ABP1 allele, *abp1-5* (Xu et al., 2010) has been used as a template to design mutations in the binding pocket of ABP1 to prepare an ABP1 molecule unable to bind auxin. I wanted to assess for which already identified ABP1 roles the auxin binding is necessary and also to identify some still omitted novel roles of ABP1 essential for proper plant development. A number of mutant

variants have been prepared with amino acid substitution in the binding pocket and their influence on the auxin-binding ability has been examined. The *abp1-M2X* mutant variant (H94A/H96A) completely fails to bind auxin, thus, activating downstream components of the ABP1 signaling pathway, such as the ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN 4 (RIC4), or to inhibit internalization of PIN proteins from the PM. I revealed that this mutant variant is unable to support the auxin-dependent association of clathrin with the PM, to mediate PIN polarization after long auxin treatments, or facilitate the auxin-dependent secretion of ABP1 from the ER to the cell surface. These findings show the significance of auxin binding for the correct ABP1 function during different auxin-mediated cellular processes that are important for proper plant growth and development.

In the second part of my PhD thesis, I focus on the discovery of proteins that may interact with ABP1. Although the C-terminal peptide-binding protein 1 (CBP1) from maize (*Zea mays*) had been found to interact with ABP1 (Shimomura, 2006), the interaction was studied only *in vitro*. We identified a homolog of CBP1 in the glycosylphosphatidylinositol (GPI)-anchored protein SKEWED5 (SKU5) of *Arabidopsis thaliana* and confirmed its ability to bind ABP1 *in vivo*. The SKU5 localization pattern is similar to that of ABP1, namely at the ER and at the cell surface. Therefore, it may play a role in several important processes during ABP1 signaling, such as assistance during signal transmission through the PM or during ABP1 secretion to the cell surface. The phenotypes of the SKU5/SKU5-similar homologues (SKSs) deficiency largely overlap with defects in the ABP1 inactivation lines. We demonstrate that the secretion mechanism of SKU5 to the cell surface is triggered by the ABP1-dependent auxin signaling; meanwhile, the ABP1 secretion to the cell surface is promoted by the SKU5/SKSs-involved auxin signaling.

The results presented in the last part of my PhD validate the importance of phosphorylation and dephosphorylation of the PIN3 auxin efflux carrier during maintenance of the PIN polarity and in response to internal and external stimuli. Furthermore, the ABP1 signaling plays an important role during the auxin-mediated PIN3 relocation at the PM (Rakusová, unpublished data). To characterize the potential connection between these two mechanisms, we evaluated the importance of two phosphorylation sites of the PIN3 hydrophilic loop and their impact on the PIN3-mediated biological processes, such as gravitropic and phototropic responses. Mutations in these sites mimic either the phosphorylated or dephosphorylated status of the protein. We demonstrate the influence of these mutations on PIN3 trafficking and their function during auxin maximum formation, gravitropism, and meristematic activity in roots.

All together, the data presented in this PhD thesis provide new insights into the ABP1 signaling pathway thanks to the characterization of the structure of ABP1 binding pocket, revelation of its new interactors, and identification of new roles for ABP1 during plant development and environmental responses.

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Chapter 1.

Introduction

Adapted from review

Peter Grones and Jiří Friml*, Auxin transporters and binding proteins at a glance. (provisionally accepted in Journal of Cell Science).*

PG and JF wrote the manuscript.

Auxin-controlled plant growth and development

Each organism responds to environmental stimuli via signal transduction mechanisms that are harmonized by developmental answers. Signal perception by one or more receptors initiates nontranscriptional and/or transcriptional processes that may result in changes at the cellular, tissue, or even organismal level. Signals can be divided into two major groups: physical (such as light, gravity, temperature, and mechanical stress) or molecular (such as ions, nutrients, toxins, and hormones); generally, they are transduced by second messengers or by modifications in the phosphorylation status of certain proteins. In plants, phytohormones, a subgroup of signaling molecules, play a prominent role in all developmental processes during the whole plant's life cycle. Until now, several phytohormones have been identified, such as auxin, cytokinin, brassinosteroid, strigolactone, salicylic acid, gibberellin, ethylene, jasmonate, and abscisic acid, and their influence on plant development and physiology is still being characterized.

The existence of growth regulators had already been proposed by Charles Darwin at the end of 19th century. Light-induced differential cell elongation in grass coleoptiles had been suggested to be facilitated by the basipetal transport of signaling molecules (Darwin and Darwin, 1880), of which the uneven distribution regulates the bending of the plant toward the light source (Went, 1926; Cholodny, 1927). This growth hormone was first isolated from media (Salkowski, 1885) and characterized as indole-3-acetic acid (IAA) a few decades later (Kögl et al., 1934).

IAA was designated 'auxin' from the Greek verb '*auxein*', which means to enlarge or to grow, because of its importance during stem and coleoptile elongation, but also of its undeniable role in root formation (Went, 1934). Ever since, the presence of auxin has been found crucial for the regulation of various developmental processes, such as control of leaf abscission (Rubinstein, 1963), senescence (Ellis et al., 2005), fruit formation (De Jong et al., 2009), response to pathogens (Kazan and Manners, 2009; Fu and Wang, 2011), abiotic stress (Wang et al., 2010), establishment and maintenance of tropic responses toward light and gravity, and establishment and maintenance of polarity or apical dominance (Woodward and Bartel, 2005; Vanneste and Friml, 2009). Nevertheless, auxin is implicated in the pathways of other phytohormones and, therefore, via cross-talk influences almost all plant development processes (Vanstraelen and Benková, 2012).

Endogenous auxins, synthetic auxins, and auxin analogs

Endogenous auxin or IAA is abundantly present in the plant and can accomplish most of the auxin functions during plant growth and development (Zhao et al., 2010). However, besides IAA, three additional compounds with a similar structure and auxin activity occur naturally in plants. Indole-3-butyric acid (IBA) has been identified in potato (*Solanum tuberosum*) tubers (Blommaert, 1954), and occurs also in many other plant species (Ludwig-Müller, 2000). In *Arabidopsis thaliana*, the IBA levels are below detection limit (Novák et al., 2012), but are apparently more efficient in initiation of root formation than IAA (Zimmerman and Wilcoxon, 1935; Hartmann et al., 1990). Other developmental processes in which IBA is involved are leaf epinasty, cell division, stem bending (Zimmerman and Wilcoxon, 1935), root hair elongation (Strader and Bartel, 2009; Růžička et al., 2010), and cell expansion in cotyledons (Strader et al., 2010). Interestingly, IBA can be converted from IAA and *vice versa*, meaning that IBA can facilitate storage of active IAA (Bartel et al., 2001; Woodward and Bartel, 2005).

First discovered in pea (*Pisum sativum*) seeds (Gandar and Nitsch, 1967; Marumo et al., 1968), 4-chloroindole-3-acetic acid (4-Cl-IAA) has been detected in the meantime in many different plant species, especially in members of the Fabaceae family (Engvild, 1975; Engvild, 1980; Engvild et al., 1978; Engvild et al., 1980; Hofinger and Böttger, 1979; Katamayama et al., 1987), but, until now, not in *Arabidopsis*. Therefore, the amount of information about its function is limited. Nevertheless, in pea it stimulates pericarp growth (Reinecke et al., 1995) and in maize (*Zea mays*) it is responsible for coleoptile elongation and protoplast swelling (Rescher et al., 1996; Steffens and Lüthens, 2000). As its stability is higher than that of IAA, it is active at lower concentrations than those of IAA (Marumo et al., 1973).

The third naturally occurring auxin-like compound is phenyl acetic acid (PAA). Thus far, it is the only identified IAA phenyl derivative, its working concentrations are much higher than those of IAA. Until now, PAA has been found in many plant species and it might play a role in the protection of roots against soil microorganisms (Morris and Johnson, 1984; Slininger et al., 2004; Somers et al., 2005).

Synthetic compounds have often a slightly different structure, but their biological activities are comparable with those of endogenous hormones. Structure–activity relationship analysis helped us to better understand the importance of functional groups of the auxin molecules. The indole group of IAA that has not been shown to be essential for the auxin activity can be substituted by any aromatic ring of comparable size. A group of known synthetic auxin

molecules consists of 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 3,6-dichloro-2-methoxybenzoic acid (dicamba), 4-amino-3,5,6-trichloropicolinic acid (picloram), and many others. All these compounds are more stable than IAA, possibly due to a reduced metabolic turnover (Dunlap et al., 1986). Similarly to IAA, these synthetic molecules can also be inactivated by conjugation with glucose (Barendse et al., 1987; Klems et al., 1998). Because of their toxicity at high concentrations for monocotyledonous and dicotyledonous plants, they are often used as herbicides (Grossmann, 2010), whereas at standard concentrations they are often used as initiators of adventitious root formation or for synchronization of flowering or fruit development.

Another group of synthetic auxins is formed by anti-auxins, molecules with structures similar to those of endogenous IAA, but with a different activity. Usually, they can bind to auxin receptors (TRANSPORT INHIBITOR RESPONSE1 [TIR1] (Ruegger et al., 1998; Kepinski and Lyser, 2005) or AUXIN-BINDING PROTEIN1 [ABP1] (Rück et al., 1993; Steffens et al., 2001); see below), but they cannot facilitate the subsequent auxin response. Different anti-auxins, such as tert-butoxycarbonylaminohexyl-IAA (BH-IAA), α-[phenylethyl-2-oxo]-IAA (PEO-IAA), or α-[2,4-dimethylphenylethyl-2-oxo]-IAA (auxinol), can bind TIR1/AUXIN SIGNALING F-BOX (AFB) proteins in the same manner as endogenous IAA, but they cannot promote the interaction with the domain II of (AUX)/IAA proteins and their consequent ubiquitination and degradation. Thus, they compete effectively with endogenous IAA and inactivate the TIR1/AFB signaling pathway. Nevertheless, they can still bind to ABP1 and inhibit endocytosis of PIN-FORMED (PIN) proteins at the plasma membrane (PM). By contrast, 5-floro-IAA (5-F-IAA) binds TIR1 and promotes degradation of AUX/IAA proteins, but cannot inhibit endocytosis of PIN proteins via ABP1 (Hayashi et al., 2008, Hayashi et al., 2012).

Auxin biosynthesis

Auxin, as a crucial player in almost every aspect of plant development and growth, is predominantly synthesized in shoot apical meristems, developing fruits, young leaves, and seeds (Ljung et al., 2005), but also in the quiescent center, provascular tissues of meristematic zones, and vascular tissues of hypocotyls and apical hooks (Stepanova et al., 2008). However, complete understanding of its biosynthetic machinery in plants still remains unclear. Auxin can be stored inside cells for sudden needs in the form of inactive auxin conjugates, such as IAA-sugars, IAA-esters, or IAA-amino acids, from which free and active IAA can be released

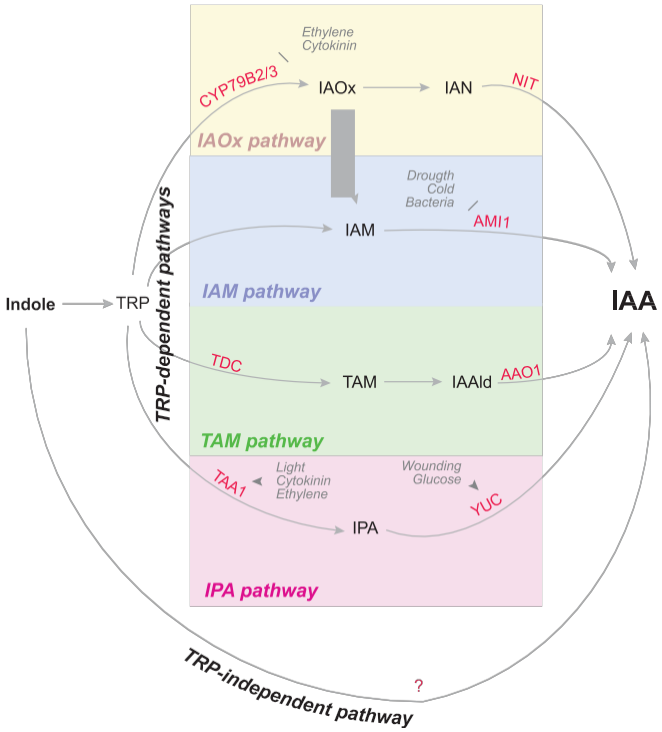

Figure 1. Auxin biosynthesis, tryptophan (TRP)-dependent and -independent auxin biosynthesis pathways. The TRP-derived pathways are highlighted in yellow (IAOx), blue (IAM), green (TAM), and red (IPA). The functional interdependence or redundancy of the proposed auxin biosynthesis routes is still a matter of debate. The pathways proposed here are largely based on the updated depiction by Mashiguchi et al. (2011). Auxin biosynthetic intermediates are shown in black, auxin biosynthesis enzymes in red, and internal and external triggers that regulate enzyme expression in gray. The identity of the enzymes catalyzing some of the suggested reactions remains elusive. AAO, ACETALDEHYDE OXIDASE; AMI1, AMIDASE 1; CYP79B2/3, CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2/3; IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; IPA, indole-3-ylpyruvic acid; NIT, NITRILASE; TAA1, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1; TAM, tryptamine; TDC, TRYPTOPHAN DECARBOXYLASE; TRP, tryptophan. Adapted from Rosquete et al. (2012)

by hydrolysis (Davies et al., 1999; Rampey et al., 2004; Ludwig-Muller, 2011; Korasick et al., 2013).

De novo synthesis of auxin usually starts from tryptophan as shown by the production of labeled IAA when plants were treated with labeled tryptophan (Wright et al., 1991; Normanly et al., 1993). Until now, several pathways that convert tryptophan into IAA have been characterized (Fig. 1), but the most prominent is the two-step conversion with IPA as an intermediate metabolite. Two major gene families involved in auxin biosynthesis, *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS* (*TAA*) and flavin monooxygenase-like protein-encoding *YUCCA* (*YUC*), were identified independently some time ago (Zhao et al., 2001; Cheng et al., 2006; Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009), but only recent studies confirmed that they participate in the same pathway (Mashiguchi et al., 2011; Stepanova et al., 2011, Won et al., 2011). *TAA* genes

catalyze transamination of tryptophan to IPA and *YUC* genes catalyze the oxidative decarboxylation of IPA into IAA. The auxin concentration of *taa* mutants is lower than that of wild-type plants, but overexpressed *TAA* does not exhibit any obvious developmental phenotypes, indicating that transamination is most probably not a rate-limiting step in the auxin biosynthesis process (Stepanova et al., 2008, Tao et al., 2008). Inactivation of a single *YUC* gene does not exhibit any developmental defects, most possibly because of the genetic redundancy among the 11 genes in the family. Only multiple *yuc* mutants, such as *yuc1/yuc4* or *yuc1/yuc2/yuc6*, display defective embryogenesis, growth, vascular formation, and flower development (Cheng et al., 2006, Cheng et al., 2007) and overexpression of *YUC* genes leads to auxin overproduction and consequent phenotypes (Chen et al., 2014).

The other tryptophan-dependent IAA-biosynthetic pathways are designated based on the intermediate products directly after tryptophan, such as indole-3-acetaldoxime (IAOx) that is produced by the cytochrome P450 enzymes CYP79B2 and CYP79B3 (Mikkelsen et al., 2004, Sugawara et al., 2009); indole-3-acetamide (IAM) that is catalyzed by the tryptophan-2-monooxygenase enzyme IAAM (Lehmann et al., 2010); and tryptamine (TAM) that is an IAA intermediate, but of which the true contribution to the auxin biosynthesis machinery is questionable (Tivendale et al., 2010). Additionally, the tryptophan-independent auxin biosynthesis pathway uses either indole or indol-3-glycerol phosphate as precursor, but the exact mechanism is still unclear (Normanly et al., 1993; Ouyang et al., 2000; Zhang et al., 2008). Therefore, the pathway with IPA as intermediate is thus far the best characterized and might be the most important of the auxin biosynthesis pathways.

Auxin transport at the cellular level

The plant auxin IAA controls plant growth and development by modulating fundamental cellular processes, such as cell division, expansion, and differentiation (Mockaitis and Estelle, 2008). Cellular auxin responses typically depend on auxin concentrations that result from intercellular auxin transport and auxin metabolism. Over the past years, auxin transport proteins and their regulators were characterized in detail, broadening our knowledge on the polar auxin transport mechanism and, hence, the auxin gradient formation, differential growth, and organogenesis (Grunewald and Friml 2010; Ganguly et al., 2012). Transport of auxin is usually accomplished via two combined pathways: a long-distance pathway, in which auxins are transported from their synthesis area in the shoot apical meristem toward the root, through phloem tissues (Swarup et al., 2001; Ljung et al., 2005) and a short-distance pathway, in which the cell-to-cell auxin transport is mediated by specific auxin influx and efflux

carriers (Fig. 2) (Vieten et al., 2007; Vanneste and Friml, 2009). Approximately 15% of the apoplastic auxin is protonated (IAAH), which allows its passage through the membrane by diffusion. The major part of auxin is transported actively via four different transporters: PIN proteins, AUX1/LIKE AUX1 (LAX), P-glycoproteins (PGPs) of the ATP-binding cassette (ABC) transporter family, and PIN-LIKES (PILS).

PIN proteins, also known as auxin efflux carriers, are localized, with some exceptions, at the PM and participate in the directional auxin transport (Petrášek et al., 2006) that is required for the maintenance of auxin maxima during various plant development processes, such as root meristem formation (Friml et al., 2002a; Blilou et al., 2005), tropic responses (Friml et al., 2002b; Abas et al., 2006), lateral organ development (Benková et al., 2003; Reinhard et al., 2003), and embryo development (Friml et al., 2003; Sauer and Friml., 2004; Weijers et al., 2005). Eight different genes belong to the PIN gene family and are expressed in an organ- or tissue-specific manner (Vieten et al., 2007; Žažimalová et al., 2007), of which five (PIN1, PIN2, PIN3, PIN4, and PIN7) localize at the PM and possess long hydrophilic loops (Viaene et al., 2013) that are the crucial components of the polar auxin transport. Among the *pin* loss-of-function mutants, *pin1* exhibits strong defects in development, resulting in the absence of lateral organs (Gälweiler et al., 1998), whereas *pin2* and *pin3* show a reduced ability to respond to gravity and light stimuli (Luschnig et al., 1998; Friml et al., 2002b). PIN5 and PIN8 localize subcellularly at the endoplasmic reticulum (ER), where they presumably facilitate the auxin homeostasis regulation by pumping auxin to and from the ER lumen (Mravec et al., 2009; Gaungly et al., 2010; Ding et al., 2012, Dal Bosco et al., 2012) and PIN6 has most probably a dual localization at the PM and at the ER (Cazzonelli et al., 2013).

The *AUX1/LAX* gene family consists of four auxin influx carriers that are responsible for the auxin transport from the apoplast into the cytoplasm (Bennett et al., 1996; Swarup et al., 2001; Swarup et al., 2008). Mutations in these genes exhibit severe phenotypes, such as agravitropic roots and reduced numbers of lateral roots in the *aux1* mutant, or delayed lateral root emergence in the *lax3* mutant. The exact roles of LAX1 and LAX2 are not completely clear, but might well be participation in the auxin transport (Swarup et al., 2008).

Members of the *Arabidopsi*s ABCB subfamily of the ABC superfamily have been identified as auxin transporters because loss-of-function mutants exhibited reduced auxin transport and, consequently, developmental defects (Noh et al., 2001; Geisler et al., 2005). The whole family contains 21 members from which ABCB1, ABCB4, ABCB14, ABCB15, and ABCB19 have been well characterized as auxin transporters (Kaneda et al., 2011; Titapiwatanakun et al., 2009). Because of functional redundancy, the single mutants exhibit only mild phenotypes

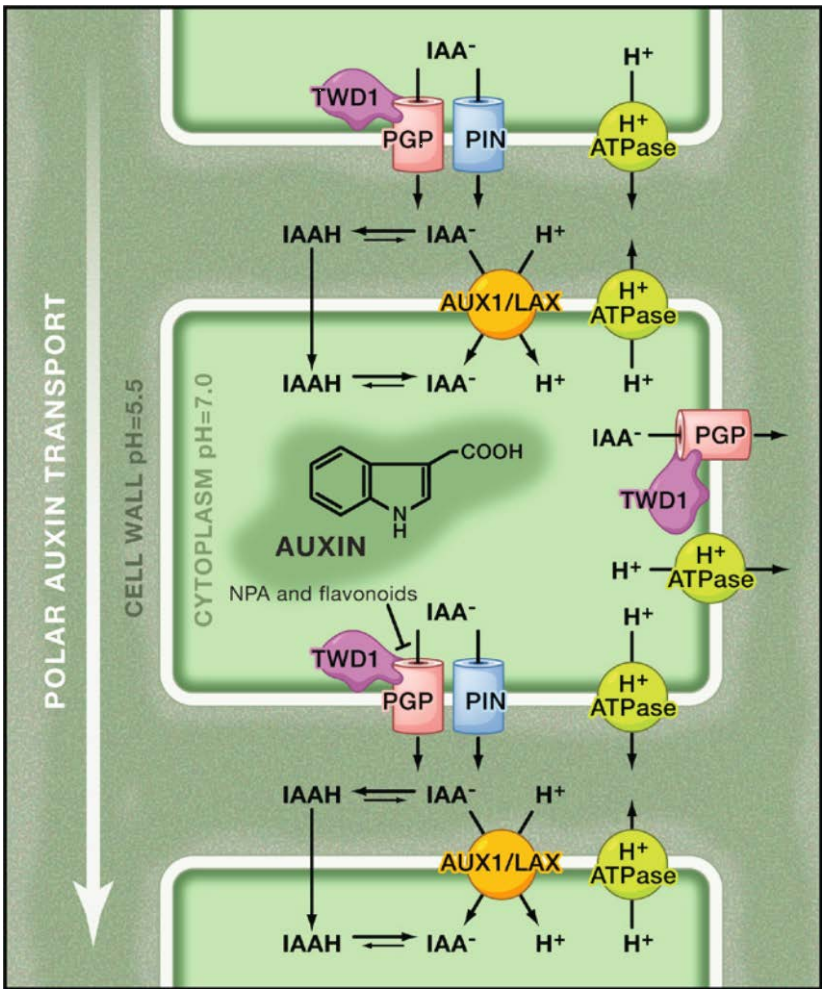

Figure 2. Chemiosmotic hypothesis for the polar auxin transport. The low pH in the apoplast (cell wall) is maintained throughout the activity of the plasma membrane H+ ATPases. In the relatively acidic environment, a fraction of the weak acid, indole-3-acetic acid (IAA), the major form of auxin, becomes protonated. The protonated (IAAH) form is more lipophilic and can diffuse freely through the plasma membrane into the cell. Besides passive diffusion, auxin is also actively taken up from the apoplast by the H+/IAA− symport mediated by the AUX1/LAX influx carriers. Once inside the neutral cytosol, auxin is deprotonated and becomes trapped inside the cell. Auxin can leave the cell by auxin efflux carriers, such as PIN-FORMED (PIN) proteins and P-glycoproteins (PGPs) of the ATP-Binding Cassette family B (ABCB) transporter family. ABCB activity can be modulated by 1-naphthylphthalamic acid (NPA) and flavonoids that interfere with the interaction of ABCB and a protein that regulates it, TWISTED DWARF 1 (TWD1). The polar subcellular localization of PIN proteins determines the direction of the auxin flow out of the cell and, thus, the unidirectional auxin flow within tissues. Adapted from Vanneste and Friml (2009).

and only multiple mutants show auxin-related phenotypes; for instance, the *abcb1/abcb19* double mutant reduces the basipetal auxin transport more than does the *pin1* mutant (Blakeslee et al., 2007).

The ABCB proteins are probably the only ones among the auxin transporters that work both as influx carriers and possibly mediate the basal intercellular auxin transport (Geisler et al., 2003; Cho et al., 2012; Cho and Cho, 2013).

Based on the PIN5 structural homology, the PILS protein family has been identified that localizes to the ER and also participates in auxin accumulation in the ER (Barbez et al., 2012).

There is some evidence that PILS proteins are evolutionarily older than PIN proteins, because they are found in unicellular algae and PIN proteins are not (Feraru et al., 2012).
Recently, the WALLS ARE THIN1 (WAT1) protein has been identified as a tonoplast-localized auxin transporter responsible for maintaining the intracellular auxin homeostasis (Ranocha et al., 2013). Active auxin transport preserves the optimal cellular auxin concentration required for proper plant growth and development.

Cellular trafficking of PIN proteins

In all multicellular organisms, the transmembrane (TM) protein sorting to the specific endomembrane structures or PM has to be tightly regulated in developmental and physiological processes. Most of the TM proteins are synthesized at the rough ER and translocated into its lipid bilayer. Proper folding and glycosylation of these proteins are checked by the ER quality control machinery, to which proteins belong, such as the Luminal-Binding Protein (BIP) and Calreticulin (CRT) (Fontes et al., 1991; Denecke et al., 1995; Gupta and Tuteja, 2011). Not properly folded proteins become substrates for the ER-associated protein degradation (ERAD) unit that will translocate them into the cytosol for degradation by the proteasome (Meusser et al., 2005; Müller et al., 2005). Properly folded proteins are sorted to the Golgi apparatus (GA) through an anterograde endomembrane vesicle trafficking that is operated by the coatomer protein complex COP-II (Fig. 3) (Sieben et al., 2008; Zelazny et al., 2009; Sorieul et al., 2011). Retrograde transport of the proteins with the ER retention signal from the GA to the ER is mediated via the COP-I coatomer complex-coupled endomembrane vesicles (Donohoe et al., 2007). For proper vesicle formation, the presence of the ADP-ribosylation factor (ARF) GTPases and the ARF GTPase guanine-nucleotide exchange factor (ARF-GEF) is essential, such as GNOM-LIKE1 (GNL1) in *Arabidopsis* (Richter et al., 2007; Teh and Moore, 2007; Du et al., 2013). In the GA, proteins go through several post-translational modifications steps, such as glycosylation, galactose α2,6-sialyation, tyrosine sulfation, and proteolytic cleavage of dibasic residues on prohormones (Duncan and Kornfeld, 1988; Baeuerle and Huttner, 1987; Sossin et al., 1990). The subsequent transport of the vesicle compartments from the GA through the *trans*-Golgi network/early endosomes (TGN/EEs) to the PM is operated by other ARFs and by the *Ras* genes from the Rat Brain (RAB) proteins (Batoko et al., 2000; Pinheiro et al., 2009). Several proteins from the RAB family actively participate in post-Golgi trafficking to the PM, such as RAB-E1^{d} or RABA1b/BEX5 (Zheng et al., 2005; Feraru et al., 2012). Another important ARF-GEF that acts on the ARF-type GTPases via its control of the directional auxin transport

and of the hormone gradient formation is the well-known GNOM, a brefeldin A (BFA)-sensitive key regulator of auxin-dependent developmental processes (Liu et al., 1993; Mayer et al., 1993; Geldner et al., 2004; Moriwaki et al., 2011; Wolters et al., 2011; Okumura et al., 2013). BFA is a fungal toxin that interferes with the exocytosis protein sorting machinery by inhibiting the ARF-GEFs, resulting in intracellular PIN accumulation in so-called "BFA bodies" (Geldner et al., 2001; Kleine-Vehn et al., 2008a). However, even in *gnom* full knock-out mutants, a small fraction of the PIN proteins still gets correctly sorted to the PM (Steinmann et al., 1999). Identification of the ARF-GTPase-activating protein (GAP) Vascular Network 3 (VAN3) clarified the regulation of the BFA-responsive PIN sorting machinery (Koizumi et al., 2005; Sieburth et al., 2006). VAN3 together with GNOM are responsible for control of the endocytic sorting of cargos and subsequent transcytosis (Naramoto et al., 2010). Another identified ARF-GEF is BFA-VISUALIZED ENDOCYTIC TRAFFICKING DEFECTIVE1. (BEN1/MIN7) that localizes to the early endocytic vesicles (Tanaka et al., 2009). Recently, VAN4 has been found to act as GEF regulator of RAB- and ARF-GTPases involved in post-Golgi protein sorting (Naramoto et al., 2014). In addition, another component that accounts for the PIN protein delivery at the PM is the protein complex called exocyst. Mutants of its subunits EXO70A1 and SEC8 did not differ in their PIN protein distribution rates at the PM, but they exhibited a decrease in the PIN protein release from the BFA bodies after removal of the drug (wash-out), hinting at a certain role of the exocyst complex in PIN recycling between PM and endocytic compartments (Fendrych et al., 2013).

Recycling of PIN proteins at the plasma membrane

Proper localization and maintenance of PIN proteins at the PM are a dynamic process that is required for preserve cell polarity, differential auxin distribution, and tissue patterning. Once the PIN proteins are endocytosed, they can undergo two different fates: either be recycled back to the PM or be targeted for vacuolar degradation. PIN proteins are internalized constitutively via the clathrin-coated vesicle (CCV) machinery (Kleine-Vehn and Friml, 2008; Dhonukshe et al., 2007), a process that is regulated by various ARF-GEF proteins. PM clathrin coat complexes consist of several proteins: clathrin light chain (CLC), clathrin heavy chain (CHC), heterotetrameric adaptor protein 2 (AP2) complex, and various accessory factors, such as AP180 or Epsin 1 (Traub, 2009). The two clathrin chains are crucial for proper functioning of the major part of the endocytosis, with particular focus on PIN proteins (Dhonukshe et al., 2007; Kitakura et al., 2011; Di Rubbo et al., 2013). *De novo* synthesized

PIN1 and PIN2 polarization requires a functional clathrin-mediated endocytosis machinery (Kleine-Vehn et al., 2011). Recently, the TPLATE adaptor complex has been identified as an essential component during the early stages of CCV formation through association with other essential components, such as CLC, CHC, and AP2 (Gadeyne et al., 2014). After the vesicles are fully formed at the PM, the scission process is controlled by dynamin-related proteins (DRP1 and DRP2).

Mutants in these genes show auxin-related phenotypes, such as agravitropism or altered polarity establishment (Collings et al., 2008; Bednarek and Backues, 2010; Fujimoto et al.,

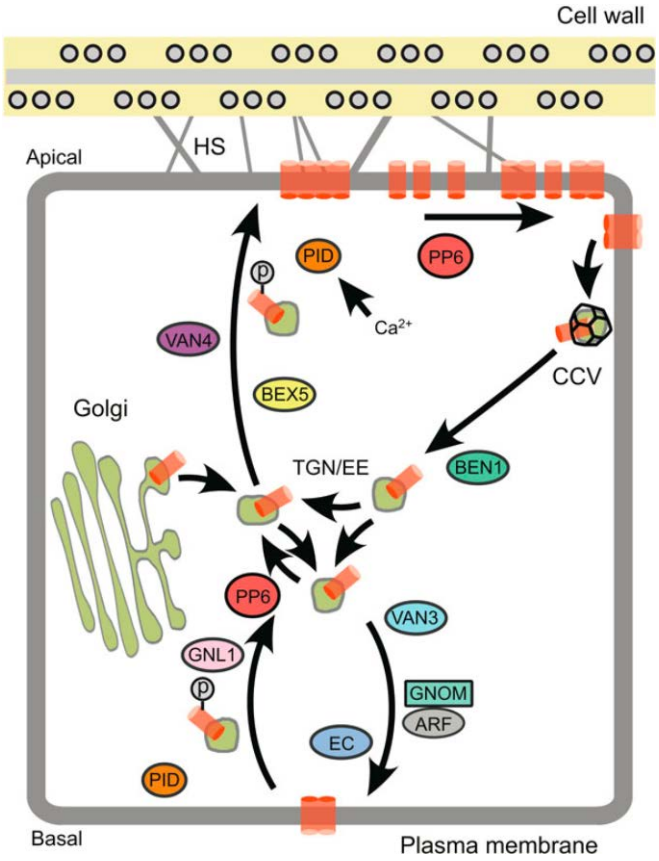

Figure 3. Regulation of plasma membrane protein recycling and polar localization. The ADP-ribosylation factor (ARF) GTPase guanine-nucleotide exchange factor (GEF) GNOM plays a key role in the recycling of PIN proteins (orange cylinders) between TGN/EE compartments and basal plasma membrane domains. In addition, regulators, such as the Rab-type GTPase BEX5 and the ARF-GEF proteins BEN1 and GNL1, are implicated in PIN trafficking. This process also depends on the activity of the exocyst complex (EC), which might modulate the exocytic sorting of PIN proteins. PIN targeting to the apical plasma membrane domain has been linked to PIN phosphorylation (p), presumably involving the activity of the AGC3-type protein kinase PINOID (PID) that, in turn, appears to be under the control of Ca2+ and phosphoinositide signaling. The activity of the PP6 phosphatase antagonizes PID, promoting dephosphorylation and PIN sorting to basal domains. PIN targeting to the plasma membrane has been suggested to involve 'super-polar' exocytosis, as reflected by the accumulation of PIN protein clusters at polar plasma membrane domains. Lateral diffusion of such protein clusters seems to be slow and to depend on a still unidentified crosstalk with cell wall components. For example, Hechtian strands (HS) that bridge the space between cell and plasma membrane have been suggested to act in this process. Adjacent to polar plasma membrane domains, clathrin-mediated endocytosis enforces internalization of PIN proteins in clathrin-coated vesicles (CCVs), preventing their further diffusion within the plasma membrane. Adapted from Luschnig and Vert (2014).

2010; Mravec et al., 2011). As soon as the PM proteins are internalized into endosomal compartments, they are further processed and sorted by the proteins SORTING NEXIN1 (SNX1) and VACUOLAR PROTEIN SORTING29 (VPS29) (Jaillais et al., 2006; Jaillais et al., 2007). The SNX1 endosomes are recruited to the microtubules with the help of the cytoplasmic linker-associated protein (CLASP) and are transported back to the PM (Ambrose et al., 2013). Different subunits of the retromer complex, such as VPS29 or VPS35, also participate in the trafficking process from the endosomes to the PM, thus preventing cargo degradation (Jaillais et al., 2007; Nodzyński et al., 2013).

Although the exact vacuolar targeting mechanism is not completely unraveled, ubiquitination has been suggested to play a crucial role. Before the cargo is targeted to the vacuole, it needs to be sorted into intraluminal vesicles of multivesicular bodies that then fuse with the vacuole. This intracellular targeting to the vacuole depends on lysine 63 (K63)-linked polyubiquitylation. PIN proteins are also modified by K63 polyubiquitin chains that rely on the E3 ubiquitin ligase RING-domain ligase (RGLG) (Yin et al., 2007; Leitner et al., 2012b). Consequently, these chains are recognized by the protein machinery, the endosomal sorting complex required for transport (ESCRT), that targets the cargos to the vacuole (Mukhopadhyay and Riezman, 2007; Lauwers et al., 2010). Mutations in ESCRT subunits block vacuolar sorting of ubiquitinated cargos (Herberth et al., 2012; Scheuring et al., 2012). Other components of the vacuolar sorting machinery, such as CHROMATIN MODIFYING PROTEIN1 (CHMP1) and ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM3 (AMSH3)-type deubiquitinase (DUB), have been shown to regulate the ESCRT-III subunit and to actively participate in vacuolar sorting of PM proteins, including PIN proteins (Spitzer et al., 2009; Isono et al., 2010; Katsiarimpa et al., 2011). Recently, also the component for initial recognition of ubiquitinated cargos has been identified as TARGET of Myb (TOM) (Korbei et al., 2013).

Phosphorylation of PIN proteins as a polarity regulator

PIN proteins are responsible, among others, for the intracellular auxin transport and for the formation and maintenance of auxin maxima (Tanaka et al., 2006). The polar localization of PIN proteins at the PM is highly dynamic and regulated by different developmental signals (Benková et al., 2003; Friml et al., 2003; Paciorek et al., 2005; Heisler et al., 2005; Sauer et al., 2006) and by various environmental stimuli (Friml et al., 2002; Harrison and Masson, 2008; Ding et al., 2011). One of the important regulation processes of the PIN polar targeting is protein phosphorylation. Identification of the serine/threonine protein kinase PINOID (PID)

revealed that PID controls the apical-to-basal PIN localization (Benjamins et al., 2001; Friml et al., 2004). *PID* overexpression mutants stimulate a basal-to-apical PIN translocation that causes a disruption of the auxin maxima and the collapse of the root meristem, leading to agravitropic root growth (Benjamins et al., 2001; Friml et al., 2004). In contrast, the *pid* mutant triggers a apical-to-basal PIN translocation and deprives auxin from meristems, resulting in *pin*-like inflorescences (Reinhardt et al., 2003; Friml et al., 2004). *In vitro* and *in vivo* assays confirmed that PID interacts with hydrophilic loops of PIN proteins and phosphorylates them most probably at the PM (Michniewicz et al., 2007). This regulation process is balanced by the PP6-type heterotrimeric phosphate holoenzyme, which consists of a PP2A regulatory A subunit (roots curl in NPA (RCN1)/PP2AA1, PP2AA2, or PP2AA3), a catalytic C subunit (PHYTOCHROME-ASSOCIATED SERINE/THREONINE PROTEIN PHOSPHATASE1 [FyPP1] or FyPP3), and a SIT4-ASSOCIATED PROTEINS (SAPS) domain-like regulatory B subunit (SAL1 to SAL4), and is responsible for dephosphorylation of PIN proteins (Michniewicz et al., 2007; Dai et al., 2012).

In the hydrophilic loop of the PIN1 protein, three evolutionarily conserved motifs have been identified (TPRXS(N/S)) that are the phosphorylation target of the PID kinase. Substitution of serines with alanines in these conserved motifs (dephosphorylation-mimicking mutations) resulted in a constitutive basal localization of the PIN1 protein in the root, even in the *PID* overexpression background, whereas substitution of serines with glutamic acid (phosphorylation-mimicking mutations) led to an apical localization of PIN1 (Huang et al., 2010). Both these versions did not complement the *pin* mutant, suggesting that reversible phosphorylation of the PIN1 hydrophilic loop by PID and the PP6 complex is essential for maintaining a dynamic PIN1 asymmetric PM localization and for formation of the necessary local auxin maxima that are crucial for proper organ initiation and development (Heisler et al., 2005; Huang et al., 2010). These three phosphorylation motifs are highly conserved among long *Arabidopsis* PIN proteins. Similar results have been obtained after mutations in the PIN2 hydrophilic loop. Dephosphorylation-mimicking, serine-to-alanine mutations exhibited predominantly a basal localization of the PIN2 protein and its insensitivity to *PID* overexpression (Dhonukshe et al., 2010).

Two other protein kinases, WAG1 and WAG2, that belong to the same protein family as PID, are functionally redundant with the PID kinase (Galvan-Ampudia and Offringa, 2007; Cheng et al., 2008; Dhonukshe et al., 2010). Both WAG1 and WAG2 phosphorylate the PIN2 hydrophilic loop at three conserved motifs and, similarly to the PID kinase, also overexpression of *WAG1* and *WAG2* initiates basal-to-apical PIN translocation (Dhonukshe et

al., 2010). Besides PID, WAG1, and WAG2, also the D6 protein kinase (D6PK) has been shown to phosphorylate PIN1 and PIN3 and to help in the maintenance of their proper polar localization (Zourelidou et al., 2009, Willige et al., 2013). Although D6PK phosphorylates PIN proteins in a manner similar to that of PID, it has different phospho site preferences (Zourelidou et al., 2014). The D6PK localization is regulated by auxin, because it is translocated from the PM to the intracellular endomembranes. Interestingly, auxin transport-dependent tropic responses are strictly controlled by this kinase (Barbosa et al., 2014). The subcellular distribution and trafficking of PIN proteins, closely ruled by several protein kinases and phosphatases, are modulated via reversible phosphorylation.

Auxin perception and signal transduction

Changes in cellular auxin concentration are chemically transduced by auxin signaling systems to mediate different cellular responses. Identification and characterization of auxin receptors over the past decades greatly helped us to understand the auxin signaling mechanism. Three auxin receptor/coreceptor systems have been revealed and their contributions to auxin signaling have been clarified in the past few years. The best characterized is the TIR1/AFB-AUX/IAA coreceptor system that regulates auxin-dependent transcription in the nucleus (Ruegger et al., 1998; Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). More recently, SKP2a has been found to be able to bind auxin and to be one of the key regulators of cell division (Jurado et al., 2010). Finally, ABP1 is a main player in fast nontranscriptional auxin responses (Fig. 4) (Rück et al., 1993; Steffens et al., 2001; David et al., 2007; Braun et al., 2008).

Auxin-mediated transcriptional regulation by TIR1

The TRANSPORT INHIBITOR RESPONSE1 (TIR1) had first been identified in a forward-genetic screen for mutants that are resistant to auxin transport inhibitors (Ruegger et al., 1997). TIR1 and the AUXIN SIGNALING F-BOX (AFB) proteins are F-box components of a nuclear S-PHASE KINASE-ASSOCIATED PROTEIN1–CULLIN1–F-BOX (SCF)-type E3 ubiquitin ligase (see below). In the presence of high auxin concentrations, TIR1 or AFB-containing SCF complex targets the transcriptional repressors AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE (AUX/IAA) (29 members in *Arabidopsis*) for degradation by the 26S proteasome via polyubiquitination (Fig. 5) (Gray et al., 2001; Dharmasiri et al., 2005b; Kepinski and Leyser 2005; Petroski and Deshaies, 2005; Tan et al., 2007; dos Santos Maraschin et al., 2009). AUX/IAA degradation releases the AUXIN RESPONSE FACTOR

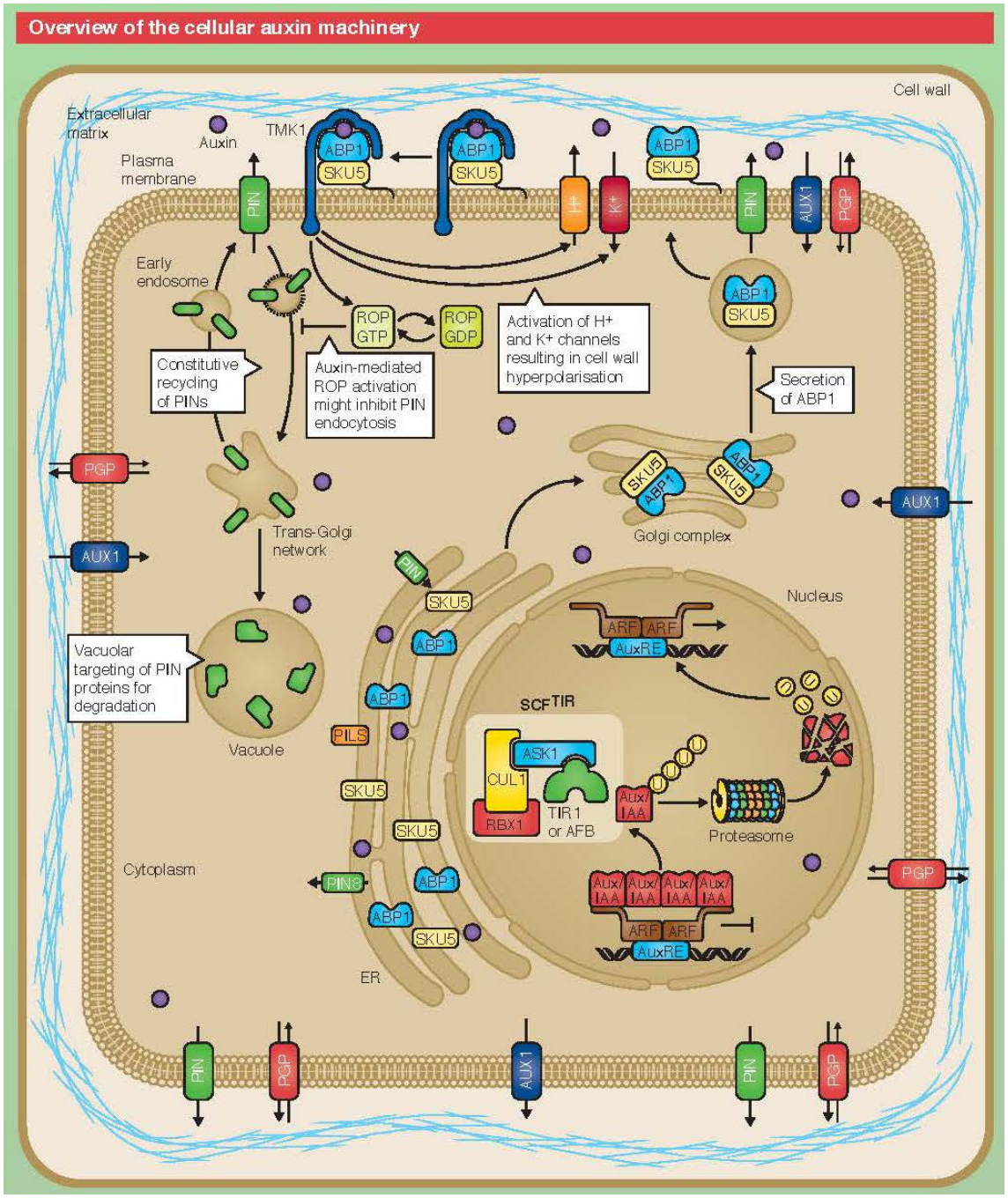

Figure 4. Model for auxin signaling in the cell. TIR1/AFB auxin receptor is an F-box protein that forms an SCF E3 ubiquitin ligase complex between SKP (ASK1) and cullin1 (CUL1). SCF^{TIR1}/AFB catalyzes the ubiquitination of auxin/IAA proteins (Aux/IAAs) in the presence of auxin. The activity of the auxin response factor (ARF) transcription factors is blocked by Aux/IAA. The auxin-induced degradation of Aux/IAA repressors recovers the ARF activity and activates the transcription of auxin-responsive genes. Auxin-binding protein 1 (ABP1) is an ER-localized protein, but small amounts of functional ABP1 protein act at the plasma membrane as an auxin receptor. At the cell surface, it binds the transmembrane receptor-like kinase1 (TMK1) that transmits the signal to the cytosol. Auxin blocks the clathrin-mediated endocytosis of PIN from the plasma membrane via the ABP1 signaling. The ABP1 signal activates K+ channels and H^+-ATPases to induce rapid auxin responses, such as turgor-induced growth. The ABP1 signal also activates Rho GTPases, which are ROP2- and ROP6-mediated processes that control the interdigitated growth of leaf epidermal pavement cells. Abbreviations: AUX, auxin resistant 1; AuxRE, auxin-responsive element; PGP, P-glycoproteins of the ATP-binding cassette transporter family; PILS, PIN-LIKES; PIN, PIN-FORMED; RBX1, RING-BOX 1; ROP-GDP, Rho of plants – guanosine diphosphate; ROP-GTP, Rho of plants – guanosine triphosphate; SKU5, SKEWED 5; U, ubiquitin. Adapted from Grones and Friml (2014).

(ARF) from the AUX/IAA-ARF heteromer, resulting in ARF-ARF dimerization and induction of auxin target gene expression (Guilfoyle and Hagen, 2007; Korasick et al., 2014, Nanao et al., 2014).

Auxin-dependent coimmunoprecipitation and yeast two-hybrid (Y2H) assays revealed that TIR1 and ARF have a different affinity toward various AUX/IAA family members, depending on the particular pair (Calderon Villalobos et al., 2012; Gray et al., 2001; Greenham et al., 2011; Shimizu-Mitao and Kakimoto, 2014; Yu et al., 2013).

Besides a conserved F-box domain, TIR1 also contains a leucine-rich repeat (LRR) domain that is essential for its binding to AUX/IAA proteins (Gray et al., 1999; Tan et al., 2007). This observation was strengthened by the identification of two point mutations in this TIR domain (D170E and M473L) that enhance the interaction of TIR with AUX/IAA proteins, resulting in their faster degradation and, thus, in enhanced transcription of auxin-responsive genes in the plant (Yu et al., 2013). The TIR1 and AFB protein family consists of six genes, all of which are involved in auxin sensing to some extent (Dharmasiri et al., 2005b; Greenham et al., 2011). TIR1 and AFB2 are positive regulators of auxin signaling (Parry et al., 2009; Havens et al., 2012). There is evidence that AFB4 and AFB5 function as auxin receptors, but their exact role still needs to be clarified as well as the role of AFB1 and AFB3 in this process (Calderon-Villalobos et al., 2010; Greenham et al., 2011; Parry et al., 2009; Walsh et al., 2006).

The AUX/IAA proteins are encoded by early auxin-responsive genes and act as transcriptional repressors in auxin responses (Mockaitis and Estelle, 2008). Most AUX/IAA proteins consist of four domains (Tiwari et al. 2001; Tiwari et al. 2004). Domain II was shown to be important for recognition by TIR1 during auxin-induced destabilization of AUX/IAA proteins (Shimizu-Mitao and Kakimoto, 2014). Domains III and IV share certain structural similarities with ARFs and contribute to homo- or heterodimerization within and between these two protein families (Guilfoyle and Hagen, 2012).

ARF transcription factors (a family of 23 members in *Arabidopsis*) contain a DNA-binding domain (DBD) that recognizes the auxin response *cis* element (AuxRE) located in the promoter of auxin-responsive genes (Guilfoyle and Hagen, 2007; Ulmasov et al., 1997). Recently, a dimerization domain has been identified in the DBD that is necessary for dimerization of ARFs; it cooperates with DNA binding and is essential for proper ARF function *in vivo* (Boer et al., 2014). At low auxin levels, AUX/IAA proteins are bound to ARFs and undergo heterotypic ARF-AUX/IAA interactions that are stronger and, thus, more favorable than homotypic ARF-ARF interactions (Fig. 5). Multiple AUX/IAA proteins seem

to be required to efficiently repress the formation of an ARF-ARF dimer (Korasick et al., 2014, Nanao et al., 2014). The recruitment of the corepressor TOPLESS and its associated

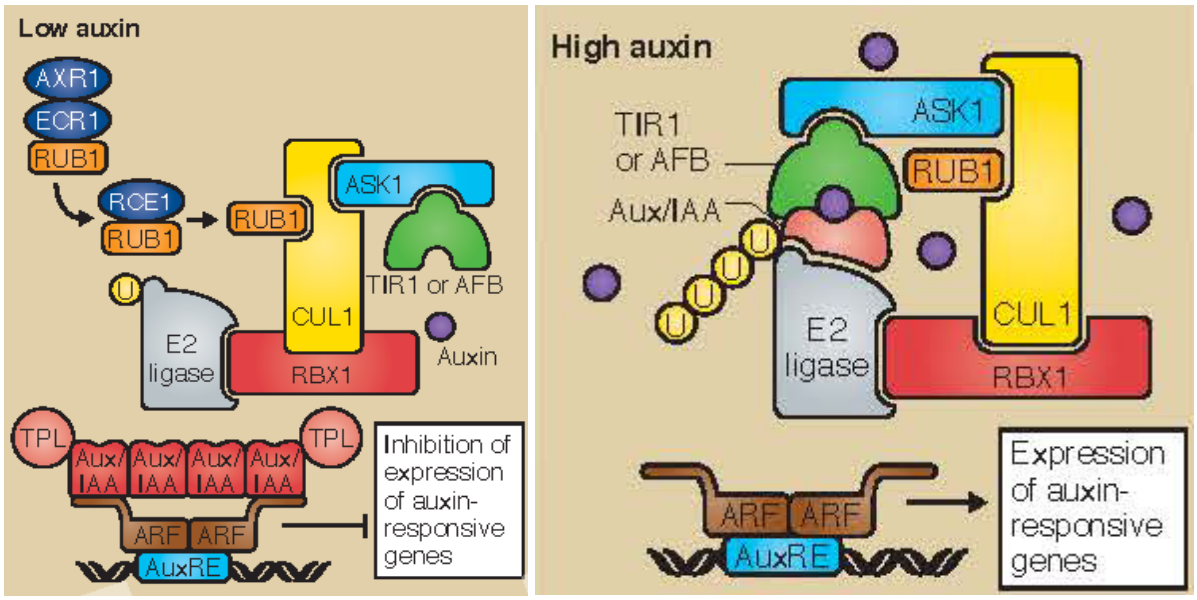

Figure 5. **The SCF^{TIR1}-Aux/IAA-ARF and SKP2 auxin response pathways**. The TIR1/AFB interaction with the Aux/IAA coreceptor/transcriptional repressors increases at high auxin concentrations, promoting Aux/IAA ubiquitination and degradation. The transcriptional activity of the activator ARFs is modulated by the levels of Aux/IAAs and repressor ARFs. Abbreviations: ARF, auxin response factor; ASK1, apoptosis signal-regulating kinase 1; AUX/IAA, auxin/indole-3-acetic acid inducible; AuxRE, auxin-responsive element; AXR1, auxin-resistance 1; CUL1, cullin 1; ECR1, E1 C-terminal related 1; RBX1, ring-box 1; RCE1, RUB 1-conjugating enzyme 1; RUB1, related to ubiquitin 1; TPL, TOPLESS; U, ubiquitin. Adapted from Grones and Friml (2014).

chromatin-modifying machinery to this complex results in inhibition of transcription (Szemenyei et al., 2008; Tiwari et al., 2001).

Four different proteins participate in the formation of the SCF^{TIR} complex: TIR1, RING-BOX1 (RBX1), CULLIN1 (CUL1), and *Arabidopsis* SKP1-LIKE1 (ASK1). The activity of the SCF^{TIR1}complex is regulated by covalent coupling of RELATED TO UBIQUITIN1 (RUB1), a protein similar to ubiquitin, to the CUL1 subunit of SCF (del Pozo and Estelle, 1999). This process is coordinated by several other enzymes, of which the most important is AUXIN-RESISTANCE1 (AXR1). Modification of CUL1 by RUB1 appears to be highly important for SCF^{TIR1} activity and normal auxin responses (del Pozo et al., 2002). Mutations in the *AXR1* gene often result in an auxin-insensitive phenotype, such as reduced apical dominance, fewer lateral roots, or reduced gravitropic response due to a decrease in the number of RUB1-CUL1 complexes (Lincoln et al., 1990; Leyser et al., 1993). Deconjugation of RUB1 from CUL1 also contributes to SCF^{TIR1} regulation that is mediated by another enzymatic multiprotein complex, the COP9 signalosome (CSN) (Serino and Pick, 2013). Mutations in most of the CSN components confer auxin-resistant phenotypes to plants and

result in defects in auxin-related developmental processes (del Pozo et al., 2002; Dharmasiri et al., 2003).

Cell type-specific auxin responses may also be influenced by additional regulators of the SCF^{TIR1}-AUX/IAA-ARF pathway. For instance, TIR1 activity has been shown to be modulated by nitric oxide-mediated S-nitrosylation that leads to an increased TIR1–AUX/IAA interaction (Terrile et al., 2012). Recent reports show an involvement of the SCF^{TIR1}-AFB pathway in degradation of PIN proteins and their vacuolar targeting (Baster et al., 2013), as well as links between the auxin perception at the cell surface and the auxin signaling machinery in the nucleus (Tromas et al., 2013).

ABP1 is a crucial regulator of fast nontranscriptional responses

Since its original identification as a soluble 22-kDa large glycoprotein (Leblanc et al., 1997; Watanabe and Shimomura, 1998), ABP1 has been extensively studied for its role as an auxin receptor candidate. Its crystal structure has been elucidated some time ago and revealed that the ABP1 protein forms a β-barrel structure and a dimer upon crystallization (Woo et al, 2002). The binding pocket is rather small and buried deep inside of the molecule. It possesses a zinc ion (can be substituted with copper) that can be chelated by three histidine and one glutamate residues. Metal ion interaction with the carboxyl group of the auxin molecule and the aromatic ring is coordinated at the correct place by the seven hydrophobic residues. Binding of auxin did not exhibit excessive changes, but only a minor one at the C-terminal end of the ABP1 molecule that can form active dimeric structures necessary for signaling (Fig. 6). The presence of the KDEL retention motif at its C-terminus suggests that ABP1 is predominantly localized in the ER, but a small fraction can also be found in the apoplast, the extracellular space between the PM and the cell wall (Jones and Herman, 1993). Although most of ABP1 occurs in the ER, the pH of the ER is not considered to be favorable for auxin binding. The best environment for auxin binding, as experimentally proven *in vitro* on purified proteins, is at pH 5.5 (K_D of 10^{-7} M of NAA) of the apoplast, implying that ABP1 senses auxin at the cell surface (Hesse et al., 1989; Jones and Herman, 1993; Diekmann et al., 1995; Tian et a., 1995; Leblanc et al., 1999, Woo et al., 2002). Early studies demonstrated that ABP1 is involved in the rapid regulation of membrane potential and ion fluxes at the PM and that it can positively control the auxin-induced cell swelling of *Arabidopsis* protoplasts and pea hypocotyls (Steffens et al., 2001; Yamagami et al., 2004). In the presence of auxin, ABP1 activates the H+ pump ATPase that acidify the extracellular space, triggering expansins that are essential agents for cell wall loosening (Cosgrove, 2000). Stimulation of the proton pump

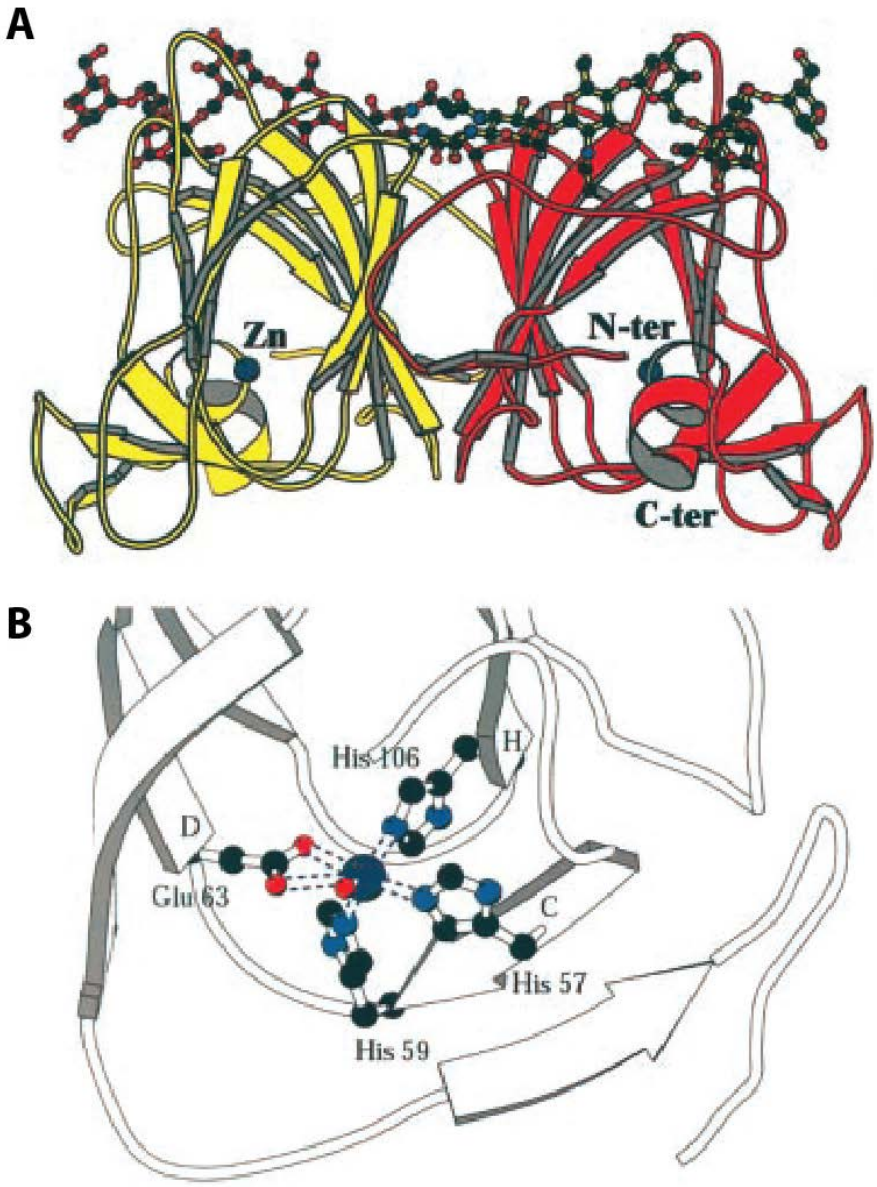

Figure 6. The overall structure of crystalized ABP1 protein from *Zea mays*. (A) Schematic representation of the overall fold of the ABP1 dimer with the b-strands drawn as arrows, the a-helices as helices, and with the bound zinc ions represented as dark blue spheres, one at the centre of each subunit. The orientation of the red subunit is similar to that shown in (A). The subunits comprising the dimer are related by a vertical 2-fold rotation axis. Asn95 and the observed N-linked sugar residues [Manp(a1,6)±(Manp(a1,3))±Manp(b1,4)±GlcpNAc(b1,4)±GlcpNAc(b1,N)-Asn] are also shown. (B) The zinc-binding site in detail. The protein ligands are His57 (strand C), His59 (at the end of strand C), Glu63 (at the beginning of strand D) and His106 (at the beginning of strand H). A single water molecule completes the octahedral coordination sphere. The zinc±nitrogen (His NE2) distances are between 2.2 and 2.3 AÊ and the zinc±oxygen distances are 2.4 AÊ to Glu63 OE1, 3.1 AÊ to Glu63 OE2 and 2.2 A Ê to the water molecule (shown as a small red sphere). Adapted from Woo et al., (2002)

also causes hyperpolarization of the membrane potential and activates K+ inward channels that are necessary for the for cell expansion crucial water uptake (Rück et al., 1993; Philippar et al, 1999, 2004). *ABP1* overexpression in tobacco (*Nicotiana tabacum*) plants showed only

mild phenotypes, namely epinastic leaf curvature (Jones et al., 1998), but the *abp1* knockout mutant has been reported to cause an embryo-lethal phenotype (Chen et al., 2001). Heterozygous *abp1*/*ABP1* knockout mutants also exhibit auxin-related defects, including root skewing, slightly elongated roots and hypocotyls, apical dominance reduction, as well as decreased basipetal auxin transport (Effendi et al., 2011).

Measurements of auxin transport implied that ABP1 has an important role in the polarized auxin transport mechanism. Auxin-regulated retention of PIN1 and other cargos at the PM was demonstrated to be mediated by ABP and to be independent of the SCF^{TIR1} machinery (Paciorek et al., 2005; Robert et al., 2010). At low levels of cellular auxin, ABP1 reduces the cellular efflux of auxin by promoting PIN endocytosis. By contrast, at high auxin levels, auxin binds to ABP1 and inhibits endocytosis to stimulate auxin export from the cell (Čovanová et al., 2013; Nagawa et al., 2012). ABP1 positively regulates the recruitment of clathrin to the PM, which can be inhibited by auxin binding, leading to the reduced internalization of PIN proteins and enhanced auxin efflux. Auxin differentially regulates the association of CHC with either the PM or the *trans*-Golgi Network (TGN), or early endosomes (EEs), in a manner dependent on ABP1, but independent of TIR1/AFB. In the presence of auxin, the association of CLC with the PM and TGN/EE is reduced dramatically, while, at the same time, CHC begins to associate with these compartments (Robert et al., 2010; Wang et al., 2013).

The question how ABP1 transmits the auxin signal from the cell surface to the cytosol to regulate different cellular processes has long been an enigma, but has recently been answered with the identification of an ABP1 transmembrane-interacting partner. The PM-localized transmembrane receptor-like kinase (TMK1) has been shown to interact with ABP1 in an auxin-dependent manner (Fig. 7) (Dai et al., 2013; Xu et al., 2014). ABP1 and TMK1 form a cell surface auxin-sensing complex that activates known downstream players of the signaling pathway, such as the small GTPases Rho of plants (ROPs) and their associated ROP-interactive CRIB motif-containing proteins (RICs) (Xu, et al, 2010; Chen et al., 2012).

Auxin can activate both ROP2 and ROP6 within 30 seconds and can promote interdigitated growth of the epidermal pavement cells in *Arabidopsis* leaves to produce a variety of cell shapes resembling a jig-saw puzzle (Fig. 7) (Xu et al., 2010). The ROP2-RIC4 and ROP6-RIC1 pairs act antagonistically on convex and concave sides of the lobes of pavement cells. ROP2-RIC4 stabilizes the actin cytoskeleton in the lobes (Fu et al., 2002), reducing PIN1 endocytosis and, thereby, promoting its localization to the PM in the lobes (Nagawa et al., 2012). By contrast, ROP6 loads RIC1 onto the microtubules, inhibiting exocytosis and, thus,

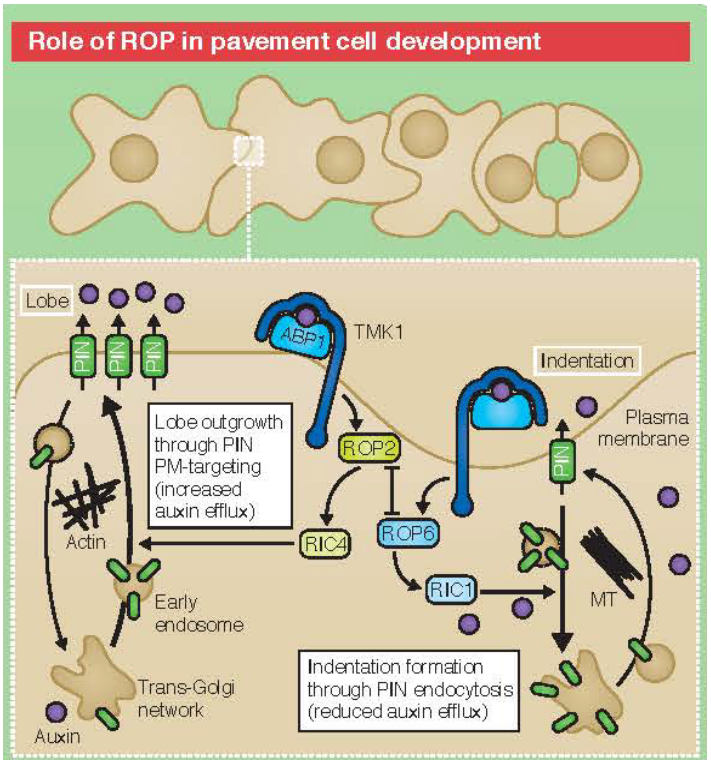

Figure 7. Model of ROP-regulated vesicle trafficking and auxin signaling in leaf pavement cells. Lobe and indention formations are controlled by ABP1-triggered Rho-GTPase pathways: ROP2-RIC4 signaling promotes lobe outgrowth (more auxin efflux) via actin assembly and ROP6-RIC1 signaling suppresses lobe outgrowth, but triggers indention formation (less auxin efflux) via microtubule organization. Only in lobes, exocytosis and, in turn, polarization of PIN1 are enhanced by ROP2-RIC4 signaling. Abbreviations: ABP1, auxin binding protein 1; PIN, PIN-FORMED; MT, microtubules; RIC1, ROP-interactive CRIB motif-containing protein 1; RIC4, ROP-interactive CRIB motif-containing protein 4; ROP2, rho of plants 2; ROP6, rho of plants 6; TMK, transmembrane kinase receptor–like kinases; U, ubiquitin. Adapted from Grones and Friml (2014).

generating indentations (Fu et al., 2005). Once PIN1 is stabilized in the lobes, ABP1 senses the exported auxin and acts through ROP6 in the opposite cell, where indentations are formed (Xu et al., 2010). In *Arabidopsis* roots, ABP1-activated ROP6-RIC1 recruits the microtubule-severing protein katanin (KTN1) during cortical microtubule rearrangements by inducing the detachment of branched microtubules (Lin et al., 2013). Through this mechanism, ROP6-RIC1 can regulate the association of clathrin with the PM for clathrin-mediated endocytosis (Chen et al., 2012).

Although the majority of the ABP1 effects mentioned above are related to nontranscriptional processes, there are some indications that ABP1 may also influence auxin-regulated transcription. A few years ago, several immunomodulation mutants of ABP1 had been produced. The SS12S6 and SS12K9 lines conditionally expressed single-chain fragment variables (scFv) that were derived from a characterized anti-ABP1 antibody previously shown to block the protein activity (Leblanc et al., 1999a; David et al., 2001, 2007). The SS12S6 line possesses a construct that allows the antibody to reach the apoplast where it can inhibit the

function of the ABP1 protein, whereas SS12K9 produces antibodies that cannot leave the ER and inhibit the function of ABP1 in this compartment (David et al., 2007). The conditional knockdown mutants of ABP1 revealed that regulation of cell elongation and cell division is impaired and that the mutants also display alterations in gene expression patterns in response to auxin (Braun et al., 2008; Tromas et al., 2009). The expression levels of some of the auxin-inducible genes, such as *AUX/IAA*, *SAUR*, and *GH3*, are reduced in the ABP1 knockdown mutant and in the *abp1*/*ABP1* heterozygous mutant, suggesting that ABP1 can also affect the expression of auxin-responsive genes (Braun et al., 2008; Effendi et al., 2011). Active ROP (GTP-ROP) from tobacco expressed in *Arabidopsis* enhances the transcription rate of auxin-responsive genes, whereas expression of an inactive ROP (GDP-ROP) reduces gene transcription. This observation provides evidence that ROP-mediated signaling might act through TIR1/AFB–AUX/IAA or SKP2a to affect transcription (Tao et al., 2002). There is also some support that ABP1 may influence components that are involved in the G1-to-S phase transition, either independently of or through SKP2a activity (Zažímalová et al., 2010). Interestingly, recent work indicates that ABP1 is also a negative regulator of the SCF^{TIR1}-AUX/IAA-ARF pathway (Tromas et al., 2013). ABP1 can counteract the phenotypes that are caused by the loss of the TIR1/AFB complex and, hence, is likely to act genetically upstream of these F-box proteins. Furthermore, knockdown of ABP1 also increased the degradation of AUX/IAA through the SCF^{TIR1} ubiquitin ligase pathway (Tromas et al., 2013). Moreover, an involvement of ABP1 in cell wall loosening and consequent cell expansion has already been described a decade ago (Steffens et al., 2001; Yamagami et al., 2004). Only recently, ABP1 has been found to control, through a $SCF^{TIR1/AFB}$-dependent pathway, the expression of a broad range of cell wall-related genes, especially those that mostly participate in cell wall remodeling. In particular, ABP1 plays a crucial role in regulating the expression of genes involved in remodeling of xyloglucan side chains that are essential for the spatial and temporal regulation of cell expansion (Paque et al., 2014). Recently, a connection between ABP1 and phytochromes (phyA and phyB) has also been proposed. ABP1 negatively regulates phyB-dependent signaling, and, thus, hypocotyl elongation during the shade avoidance syndrome (Effendi et al., 2013). Taken together, these results suggest that auxin sensing at the cell surface may influence the sensitivity of the nuclear auxin receptors and, therefore, that ABP1 mediates not only nontranscriptional, but also transcriptional auxin responses.

SKP2, an atypical auxin-binding protein

Both auxin receptor systems, ABP1 and TIR1/AFB–AUX/IAA, are involved in the processes of auxin-dependent cell expansion and cell division (Schneck et al., 2010; Chen et al., 2001). The direct effect of the cell surface-localized ABP1 on auxin-mediated cell cycle control implied the existence of a nuclear target (Braun et al., 2008). SKP2 has been proposed as the potential downstream component that regulates the proteolysis of cell cycle-related transcription factors. During the G1-to-S checkpoint in the cell cycle, some transcription factors and other proteins need to be degraded before the next phase can commence. SKP2, assumed to be a part of the SCF complex, participates in this process and so regulates positively cell cycle progression (Jurado et al., 2008). Of the two *SKP2* genes in *Arabidopsis*, *SKP2a* can bind to auxin in cell-free assays and pull-down experiments, whereas *SKP2b* cannot (Jurado et al., 2010; Manzano et al., 2012). Binding of SKP2a to auxin enhances the interaction between SKP2a and the cell division-related transcription factors EF2C and DIMERIZATION PARTNER OF E2FB (DPB), which are both subsequently degraded, thus allowing cell cycle progression (Fig. 8) (del Pozo et al., 2006; Jurado et al., 2008).

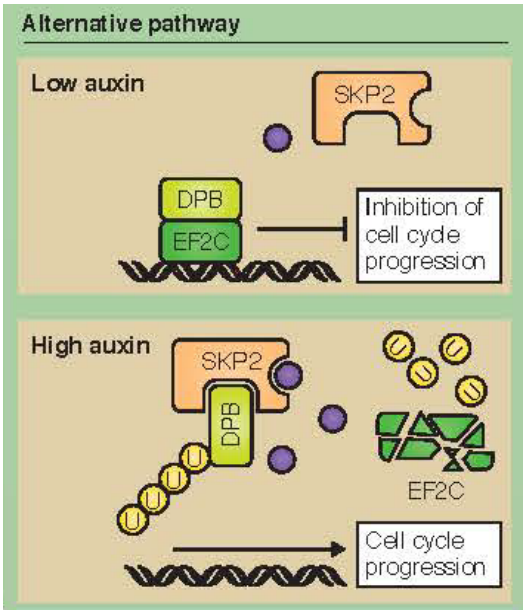

Figure 8. Model of SCF^{SKP2a}–auxin interactions. The DPb-E2Fc herterodimer represses transcription of a subset of E2F target genes. SKP2a binds auxin and degrades ubiquitinylated DPb and E2Fc. Auxin promotes degradation of Skp2a during a later phase of the cell cycle. Abbreviations: DPB, dimerization partner of E2FB; E2FC, transcription factor E2F; SKP2, S-phase kinase-associated protein 2; U, ubiquitination. Adapted from Grones and Friml (2014)

In the *skp2* mutant, the SKP2 interaction with transcription factors cannot occur and EF2C and DPB accumulate in the cell. Although E2FB, another cell division transcription factor, is degraded in an SKP2-independent manner, E2FB is stabilized by auxin by an unknown

mechanism (Jurado et al., 2010). Thus, besides the TIR1/AFB–dependent auxin perception mechanism, SKP2 might provide an alternative pathway that contributes to the final response to auxin in the nucleus.

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Chapter 2.

Auxin binding to ABP1 is crucial for its cellular functions and developmental roles

Adapted from

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Auxin binding to ABP1 is crucial for its cellular functions and developmental roles

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The plant hormone auxin is a key regulator of plant growth and development. Cellular auxin levels are sensed and interpreted by distinct receptor systems that activate a broad range of cellular responses. The Auxin-Binding Protein1 (ABP1) that has been identified based on its ability to bind auxin with high affinity is a prime candidate for the extracellular receptor responsible for mediating a range of auxin effects, in particular, the fast nontranscriptional ones. Genetic studies revealed the importance of ABP1 in many developmental processes, including embryogenesis, organogenesis, and tissue polarization. However, whether and how crucial the role of ABP1 is for its functions has not been addressed. Here, we show that the auxin-binding pocket of ABP1 is essential for its known cellular functions and developmental roles. In total, 16 different *abp1* mutants were prepared that possessed substitutions in the metal core or in the hydrophobic amino acids of the auxin-binding pocket as well as neutral mutations. Their analysis revealed that an intact auxin-binding pocket is a prerequisite for ABP1 to activate downstream components of the ABP1 signaling pathway, such as Rho of Plants (ROPs), to mediate the clathrin association with membranes for endocytosis regulation or to promote ABP1 secretion to the cell surface. *In planta* analyses demonstrated the importance of auxin binding for all known ABP1-mediated postembryonic developmental processes, including morphology of leaf epidermal cells, root growth and root meristem

activity, andvascular tissue differentiation. Taken together, these findings demonstrated that auxin binding to ABP1 is central to its function, supporting the role of ABP1 as auxin receptor.

INTRODUCTION

The plant hormone auxin, a key regulator of plant growth and development, controls fundamental cellular processes, such as cell division, expansion, and differentiation, but its overall role in plant development is still not fully understood (Bennett and Leyser, 2014). Anyway, cellular auxin levels have been shown to be perceived by multiple auxin receptor/coreceptor systems, of which one is the well characterized nucleus-localized S-PHASE KINASE-ASSOCIATED PROTEIN1–CULLIN1–F-BOX (SCF) TRANSPORT INHIBITOR RESPONSE1 (TIR1) coreceptor system that mediates auxin-dependent transcription (Ruegger et al., 1998; Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Calderon Villalobos et al., 2012). In contrast, the putative receptor AUXIN-BINDING PROTEIN1 (ABP1) is associated mainly with fast nontranscriptional auxin effects (Rück et al., 1993; Steffens et al., 2001; Xu et al., 2010; Robert et al., 2010).

Although ABP1 is mostly localized at the endoplasmic reticulum (ER) (Jones and Herman, 1993), the physiological roles have been characterized for a small ABP1 fraction at the cell surface (Robert et al., 2010, Xu et al., 2010, Xu et al., 2014). This cell surface activity of ABP1 has recently been reinforced by the identification of the plasma membrane (PM)-localized transmembrane receptor-like kinase family (TMK) as a docking station for ABP1 that transmits the signal from the extracellular space to the cytosol (Dai et al., 2013; Xu et al., 2014). Further downstream components include small Rho of plants (ROP) GTPases and their interacting ROP-interacting CRIB motif-containing (RIC) proteins that can be activated in an auxin-dependent manner, translocating to the PM vicinity as a consequence (Xu et al., 2010, Xu et al., 2014).

Despite the proposed crosstalk between the TIR1 and ABP1 pathways in the gene expression regulation (Tromas et al., 2013; Paque at al., 2014), ABP1 typically mediates fast, nontranscriptional effects. Early studies have demonstrated that APB1 is involved in the rapid regulation of the membrane potential and ion fluxes at the PM and that it mediates the auxin-induced cell swelling and cell elongation (Gehring et al., 1998; Steffens et al., 2001; Yamagami et al., 2004). The recently identified cellular roles for ABP1 include cell morphogenesis, cytoskeleton rearrangement (Xu et al., 2010, Chen et al., 2012, Nagawa et al.,

2012; Chen et al., 2014), and clathrin-mediated endocytosis (Robert et al., 2010). At the tissue level, ABP1 acts as a coordinator of cell division and expansion (Braun et al., 2008; Tromas et al., 2009).

The developmental roles of ABP1 are less clear, because ABP1 is a single-copy gene in *Arabidopsis thaliana* and its complete loss of function has been reported to be embryo lethal (Chen et al., 2001). Partially conditional loss- and gain-of-function alleles show a number of postembryonic developmental defects in processes, such as root meristem maintenance, shoot and root organogenesis, and vascular tissue development (David et al., 2007; Braun et al., 2008; Tromas et al., 2009). All these observations demonstrate the crucial importance of ABP1 for a multitude of processes in plants. Decades of biochemical studies clearly established a high-affinity binding of auxin to ABP1 (Venis and Napier, 1995; Napier et al., 2002) and revealed a protein structure including an auxin-binding pocket (Woo et al., 2002). However, the exact role of auxin binding to ABP1 for its critical cellular and developmental roles remains to be unraveled.

Here, we addressed this issue by targeted mutagenesis of the auxin-binding pocket. A number of different *abp1* mutants with amino acids substitutions were evaluated for their importance during ABP1-mediated cellular processes. Our results demonstrate that auxin binding plays a crucial role for proper ABP1 protein function.

RESULTS and DISCUSSION

Design of mutant ABP1 variants defective in auxin binding

The auxin-binding protein ABP1 has been identified decades ago in different plant species (Hertel et al., 1972, Löbler and Klambt, 1985) and its structure has already been characterized by crystallization and the auxin-binding pocket has been detected as well (Chen at al., 2001, Woo et al., 2002). Nevertheless, little is known about the significance of the auxin binding for the cellular functions and developmental roles of ABP1.

To address this question, a series of mutations were generated. Single amino acids important for the formation of the auxin-binding pocket (Fig. 1*A*) were mutated based on the known crystal structure (Woo et al., 2002). Given the conserved stability of the β-barrel fold (Woo et al., 2002) and the selected amino acid substitutions, the overall protein structure would be probably not be compromised. As the metal core seems to be most important for the auxin binding (Woo et al., 2002), two disrupting ABP1 variants, ABP1-H59A (ABP1-M1X) and ABP1-H59A/H61A (ABP1-M2X) were prepared (Fig. 1*B*).

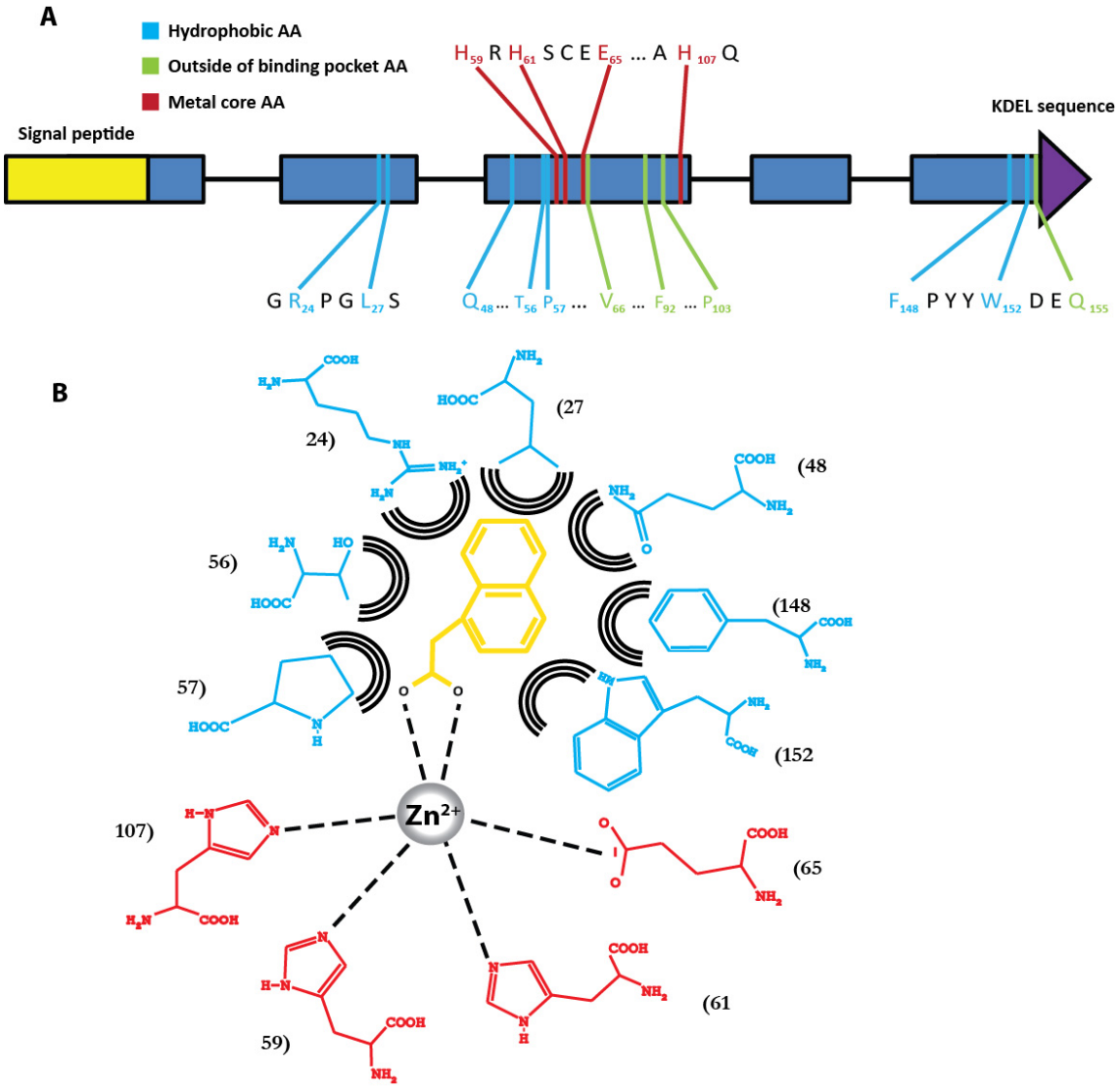

Figure 1. Structure of the ABP1-binding pocket. (A) Schematic structure of ABP1 with amino acid positions that participate in the formation of the binding pocket. Residues interacting with the zinc ion are shown in red, hydrophobic residues stabilizing the aromatic ring system of active auxin are shown in blue. Positions of amino acids that were used for preparation of neutral mutations are indicated in green. The N-terminal signal sequence for delivering ABP1 to the apoplast is indicated in yellow and the C-terminal ER retention motif KDEL is indicated in purple. (B) Simplified representation of the interactions between amino acids from the ABP1-binding pocket and the auxin molecule. Numbering is based on the *Arabidopsis* protein sequence.

Additionally, we mutagenized seven largely hydrophobic amino acids that play a role in the stabilization of the indole or aromatic rings of the auxin molecule. Seven single (R24K, L27V, Q48D, T56V, P57L, F148L and W152Y) and three double (R24K/L27V, T56V/P57L, and F148L/W152Y) mutants (Fig. 1*B*) were generated to evaluate their role in the ABP1 activity and to test the hypothesis that auxin binding is necessary for the ABP1 acivity. As negative control, four random amino acids outside the binding pocket were chosen and mutation variants were prepared (ABP1-V66A, ABP1-F92L, ABP1-P103L, and

ABP1-Q155D). These *abp1* mutant variants were then evaluated for their impact on the cellular and developmental roles of ABP1.

Downstream ROP2/RIC4 activation by *abp1*-binding mutants

Next, we tested the ability of the auxin-binding-defective variants to activate downstream signaling processes. As the Rho GTPases, ROP2 and ROP6, have been shown to be activated within a few minutes by the ABP1-dependent auxin signaling (Xu et al., 2010), leading to translocation of the interacting partners, RIC4 and RIC1, from the cytosol to the PM, *Arabidopsis* protoplasts were cotransfected with *35S::RIC4-RFP* and with the corresponding *35S::ABP1-GFP* constructs possessing different mutations. Of the protoplasts transfected with the wild-type ABP1, 90% showed no RIC4 activation, manifested by the RFP signal remaining predominantly intracellular. However, after auxin treatment, RIC4 was activated and translocated to the PM (Xu et al., 2010) (Fig. 2, *A*, *B*, and *E*).

In the *abp1* auxin-binding mutant variants, RIC4 activation was highly reduced, particularly in the ABP1-M1X and ABP1-M2X mutant variants, in which up to 45% and 70% of the cells for M1X and M2X, respectively, still had a RIC4 cytosolic localization (Fig. 2, *C*, *D*, and *E*). In the other *abp1* mutant variants with mutations in hydrophobic amino acids that are responsible for the interactinon with indole or aromatic rings of auxin molecules, we observed a slight increase of 20% in the proportion of cells with a cytosolic RIC4 localization, but never as high as in ABP1-M1X or ABP1-M2X (Fig. 2, *F*, *G*, and *H*). The neutral mutant variants ABP1-V66A, ABP1-F92L, ABP1-P103L, and ABP1-Q155D did not differ in the RIC4 translocation when compared to wild-type ABP1 protein.

Hence, the mutations in the auxin-binding pocket interfere with the ABP1 capability to activate/translocate the RIC4 protein. The most important amino acid residues are those that reside in the metal core. Seven mostly hydrophobic amino acids interacting with indole or aromatic rings of auxin molecules play a rather minor role in this process.

Endocytosis inhibition by *abp1* mutant variants in BY-2 cells

Regulation of the clathrin-mediated endocytosis is one of the rapid auxin effects ascribed to the ABP1 function (Robert et al., 2010). Auxin, via ABP1, inhibits endocytosis of some PM-residing proteins, including internalization of PIN-FORMED (PIN) auxin efflux carriers (Robert et al., 2010; Čovanová et al., 2013). By this mechanism, auxin is supposed to regulate its own transport (Paciorek et al., 2005). For evaluation of the *abp1* auxin-binding mutations, we cotransfected Bright Yellow 2 [BY-2]) tobacco (*Nicotiana tabacum*) suspension-cultured

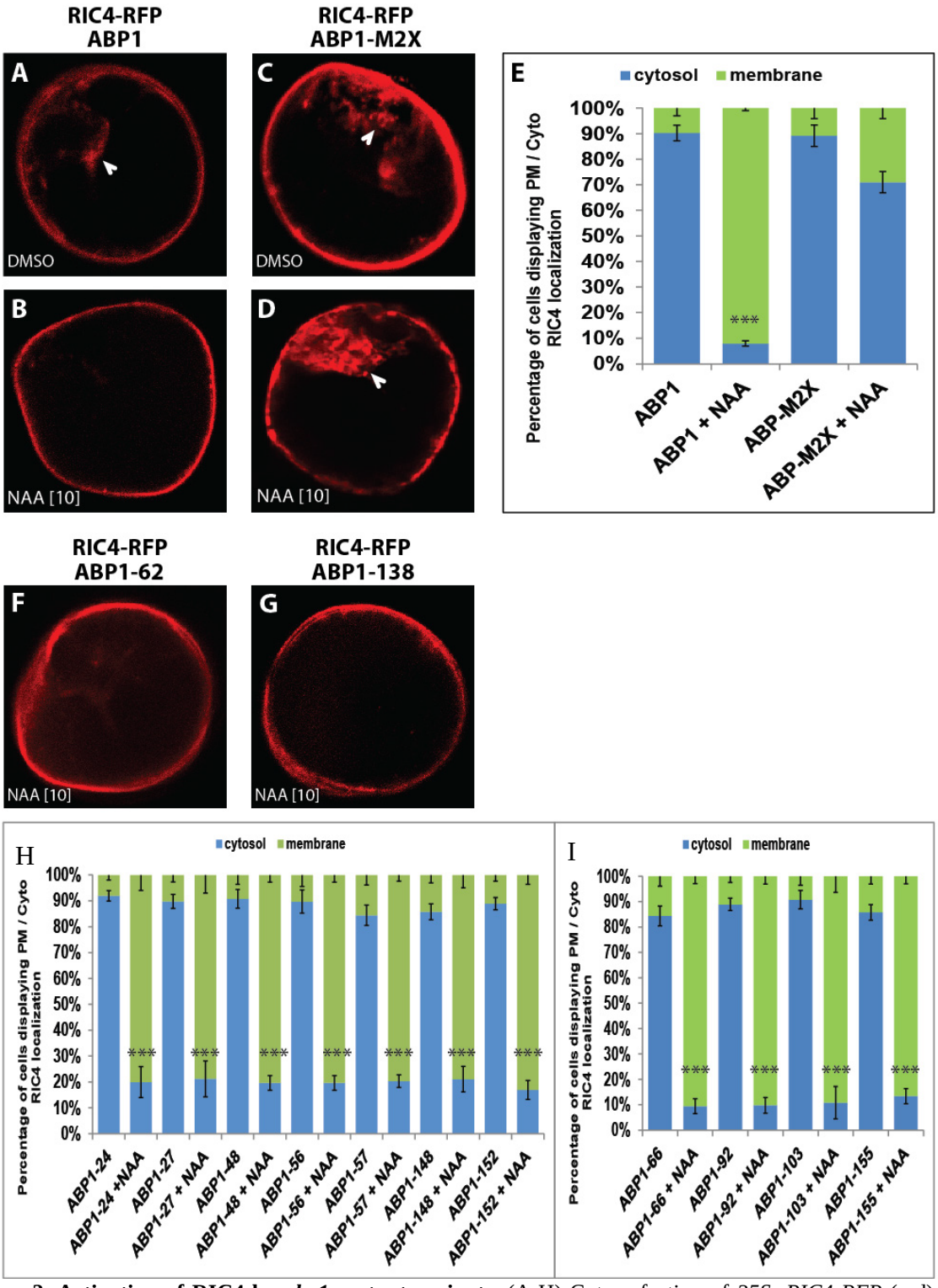

Figure 2. Activation of RIC4 by *abp1* mutant variants. (A-H) Cotransfection of *35S::RIC4-RFP* (red) with the particular *35S::ABP1-GFP* variant in *Arabidopsis* protoplast cells. In the control situation, RIC4-RFP is localized in the cytosol (A and C). After auxin treatment, ABP1-GFP activated RIC4-RFP that translocated to the PM (B). The ABP1-GFP-M2X mutant variant mostly failed to activate RIC4-RFP in the presence of auxin (D). (E) Percentage of cells showing a cytosolic or PM localization of RIC4-RFP. Representative pictures of activated RIC4-RFP by the *abp1* mutant variant with mutation in hydrophobic amino acids (F) and with neutral mutation (G). (H) Percentage of cells showing cytosolic or PM localization of RIC4-RFP in mutant variants with mutation in hydrophobic amino acids. (I) Percentage of cells showing cytosolic or PM localization of RIC4-RFP in mutant variants with mutation in neutral amino acids aoutside of binding pocket. Three independent experiments were done and at least 15 protoplasts for each were counted. Arrows indicate the RIC4-RFP cytosolic localization. Student's T-test was calculated for the comparison of number of cell showing cytosolic

RIC4 localization between non-treated and treated samples of each construct (*** p<0.001). Error bars represent SE.

cells with PIN1 (*35S:PIN1-RFP*) and particularly with the *35S::ABP1-GFP* constructs possessing different mutations and analyzed the amount of cells showing PIN1 internalization. Wild-type ABP1 promotes endocytosis of PIN1 from the PM and, consistently, more than 60% of cells had severely internalized PIN1.

This process was greatly inhibited by addiition of auxin, when over 80% of cells showed no or a very weak internalization (Robert et al., 2010, Nagawa et al., 2012; Fig. 3 *C, D* and *G*). However, no change in PIN1 internalization capability was observed when BY-2 cells were cotransfected with the ABP1-M2X construct. Even in the presence of auxin; this *abp1* version still promoted PIN1 accumulation inside the cell (Fig. 3, *E, F*, and *G*). Notably, a very small amount of PIN1-RFP was endocytosed, when the cell culture was transfected with this construct only, implying the importance of ABP1 in coordinating PIN internalization (Fig. 3, *A, B,* and *G*). Similarly, we analyzed other *abp1* versions bearing mutations in the hydrophobic residues. In all single and double mutant variants, the proportion of cells with inhibited PIN1 internalization after auxin treatment was slightly higher with maximally up to 35% than that of the ABP1 wild-type variant; however, the auxin insensitivity was not as pronounced as in the ABP1-M2X version (Fig. S1, *A, B,* and *D*). In contrast, none of the neutral mutations outside the binding pocket showed changes in the PIN1 internalization rate after auxin treatment when compared to the control (Fig. S1, *A, C,* and *E*). All together, the auxin-mediated inhibitory effect on the PIN endocytosis was related to mutations in the auxin-binding pocket and the decrease was the highest in mutant variants with mutations in the metal core.

Endocytosis inhibition by *abp1* mutant variants in *Arabidopsis* roots

To test the effect of auxin-binding mutations on endocytosis in *Arabidopsis*, we introduced all the above mentioned constructs into the *Arabidopsis* Col-0 background via *Agrobacterium*-mediated transformation. Most experiments were done with the *ABP1-M2X* overexpression line because this construct showed the most severe auxin insensitivity in protoplast and BY-2 assays. Three independent lines with expression levels similar to that of *35S::ABP1* (Rakusová, unpublished data) were chosen for the analysis. As a control, the *35S::ABP1* line (Xu et al., 2014; Rakusová, unpublished data) was used with the corresponding *ABP1* expression level with phenotypes similar to the *35S::ABP1-GFP* line (Robert et al., 2010; Xu et al., 2014; H. Rakusová, unpublished data).

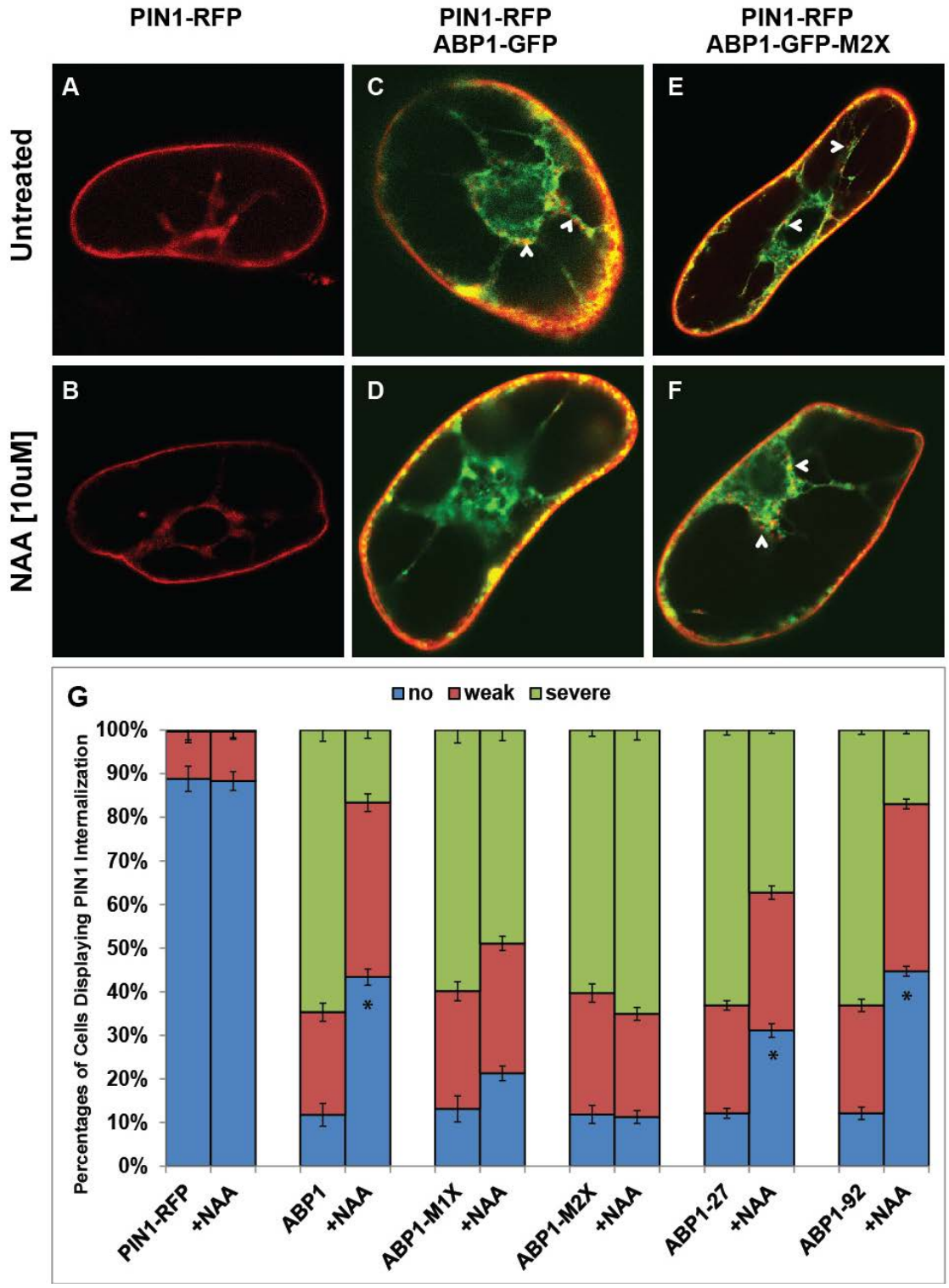

Figure 3. Inhibition of endocytosis in tobacco BY-2 cells by *abp1* mutant variants. (A and B) Transfection of tobacco BY-2 cells with PIN-RFP cannot be internalized in the presence of auxin without ABP1 and localize to the PM and ER. (C-F) Cotransfection of tobacco BY-2 cells with *35S:PIN1-RFP* (red) and the particular *35S::ABP1-GFP* variant (green). ABP1-GFP-dependent (green) promotion of PIN1-RFP (red) internalization (C) is significantly reduced after auxin treatment (D). Internalization of PIN1-RFP cannot be inhibited by auxin in the presence of the ABP1-GFP-M2X mutant variant (E and F). Three independent experiments were done and at least 20 cells for each were counted. Arrows indicate PIN protein internalization. (G) Percentage of cells displaying severe (green), mild (red), or not detectable (blue) PIN1-RFP internalization. Student's T-test was calculated for the comparison of number of cell showing no PIN internalization between non-treated and treated samples of each construct (* $p << 0.001$). Error bars represent SE.

The fungal toxin brefeldin A (BFA) is a useful tool to investigate PIN trafficking and endocytosis. BFA treatments lead to the accumulation of internalized PIN proteins in pronounced intracellular aggregates – so-called BFA bodies, because BFA inhibits preferentially the recycling of PIN proteins to the PM (Geldner et al., 2001, Kleine-Vehn et al., 2008). PIN protein immunodetection in the wild type, treated with 25 μM BFA revealed accumulation of PIN proteins in BFA bodies.

Auxin can inhibit this internalization and stabilizes PIN proteins at the PM (Paciorek et al., 2005) (Fig. 4, *A, D,* and *G*). In *35S::ABP1* roots, the BFA-induced PIN internalization was slightly higher than of the wild type and formation of BFA bodies was almost completely inhibited by auxin in both the wild type and the *35S::ABP1* overexpression line (Fig. 4, *B, E,* and *G*). Mutations in the auxin-binding pocket did not influence the internalization of PIN1 and formation of BFA bodies (Fig. 4, *C, F,* and *G*). The inhibitory effect of auxin on the BFA body formation was not observed in the *35S::ABP1-M2X* line, as manifested by the presence of pronounced PIN1-containing BFA bodies (Fig. 4, *D, F,* and *G*). In addition, we tested PEO-IAA (Hayashi et al., 2008), an auxin analog that inhibits endocytosis, but does not activate auxin-mediated transcriptional responses (Robert et al., 2010). PIN1 immunodetection in wild-type and *35S::ABP1* overexpression seedlings cotreated with PEO-IAA and BFA inhibited PIN1 internalization similarly to auxin (Fig. S2, *A, B,* and *G*). In contrast, *35S::ABP1-M2X* seedlings showed a persistent presence of BFA bodies after cotreatment with PEO-IAA and BFA (Fig. S2, *C* and *G*). Similar observations were made when seedlings were cotreated with BFA and auxinole, which is an auxin analog with structure and function similar to those of PEO-IAA (Fig. S2, *D-H*) (Hayashi et al., 2012). These results revealed that auxin and its analogs as well that preferentially inhibit endocytosis cannot restrain PIN internalization in the *35S::ABP1-M2X* mutant variant.

Involvement of ABP1 in the regulation of the clathrin association at the PM and TGN/early endosomes

PIN proteins are internalized by the endocytic machinery that involves the coating protein clathrin (Dhonukshe et al., 2007). ABP1 acts as a positive regulator in clathrin recruitment and association with the PM and, thus, promotes endocytosis (Robert et al., 2010; Chen et al., 2012; Wang et al., 2013). We investigated the auxin effect on the levels of the membrane-associated clathrin heavy chains (CHCs) by using anti-CHC antibodies. In the wild type, *trans*-Golgi network/early endosomes (TGN/EEs) and PM-associated CHC signals increased

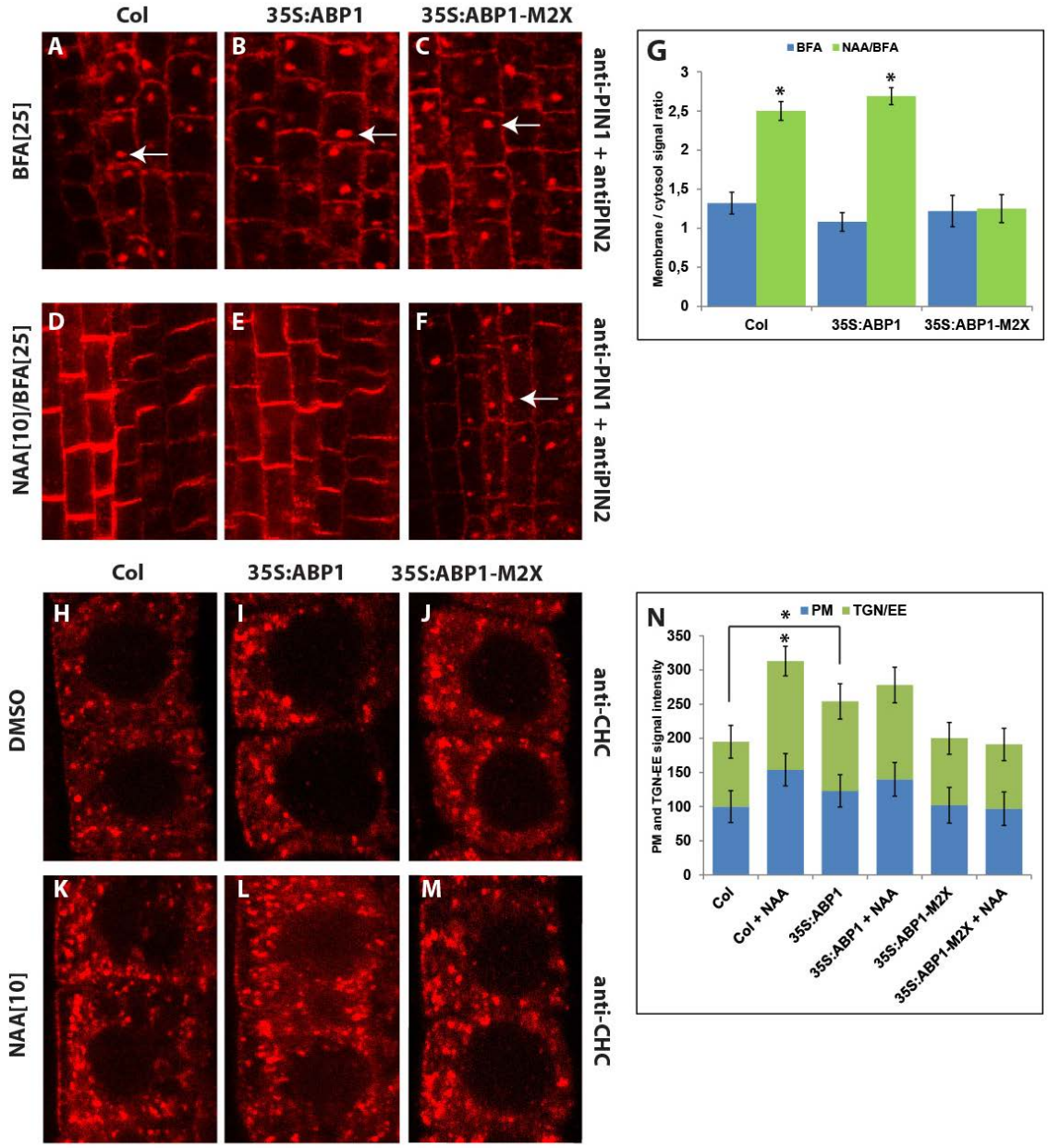

Figure 4. Involvement of the *abp1* mutant variants in endocytosis. (A-G) BFA-induced internalization of PIN1 and PIN2 leads to the formation of BFA bodies (A-C) that can be inhibited by auxin pretreatment (D and E). In *35S::ABP1-M2X* seedlings, auxin cannot inhibit the BFA body formation (F). (G) Quantification of the signal ratio at the PM and in the cytosol. Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (* $p<0.01$). Error bars represent SE. (H-N) Auxin effects on the clathrin association with PM and TGN/EE. Increase of the signal in wild-type seedlings after treatment with 10 µM auxin (K) compared to the solvent-treated control (H). In *35S::ABP1* seedlings, signal increased even without treatment (I) and slightly after auxin (L). *35S::ABP1-M2X* seedlings showed no increase with solvent (J) and resistance toward auxin treatment (M). (N) Quantification of the signal intensity of clathrin associated with PM or TGN/EE. Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (* $p<0.01$). Error bars represent SE. Three independent experiments were done and at least 50 cells for each were counted.

after auxin treatment. The intensity of the CHC signal at the PM and TGN/EE was 50% higher than that of the mock controls (Fig. 4, *H*, *I*, and *N*).

Interestingly, the CHC signal at the PM and TGN/EE increased in the *35S::ABP1* overexpression line already without auxin treatment and the increase after the auxin treatment was not as pronounced as in the wild type (Fig. 4, *J, K*, and *N*). In contrast, in the *ABP1-M2X* overexpression line, the CHC signal did not increase and the line was also resistant to auxin treatments (Fig. 4, *L-N*).

Altogether, our results suggest that ABP1 regulates the clathrin association with the PM and TGN/EEs in an auxin-dependent manner.

ABP1-mediated auxin effect on PIN polarization in *Arabidopsis*

Another prominent cellular auxin effect is the change in PIN polarity (Sauer et al., 2006) that seems to require both TIR1 and ABP1 signaling (unpublished data) as manifested by the basal-to-outer lateral relocation of PIN2 in the cortex and basal-to-inner lateral relocation of PIN1 in the endodermis of *Arabidopsis* roots after auxin treatment (Sauer et al., 2006). In the wild type, lateralization of PIN proteins occurred after 4 hours of auxin treatment; the lateral-to-basal signal ratio of PIN1 in endodermis increased up to 50% and up to 25% for PIN2 in the cortical cell layer (Fig. 5, *A*, *G*, and *H*).

The ABP1 involvement is noticeable by the PIN lateralization in the *ABP1* overexpression line already without any auxin treatment. Such phenotype could not be enhanced by additional auxin treatments (Fig. 5, *B, E, G*, and *H*). In contrast, overexpression of *ABP1-M2X* did not cause any PIN lateralization that occurred only after auxin treatment (Fig. 5, *E-H*), possibly due to the presence of the wild-type ABP1 allele in the Col-0 background and also probably due to the involvement of the second auxin signaling pathway SCF^{TIR1}. Hence, the PIN polarization process is maintained via ABP1 in an auxin-dependent manner and the ability of auxin to bind the ABP1 molecule is a crucial part of this regulation.

Auxin-mediated ABP1 secretion to the cell surface

How exactly does the predominantly ER-localized ABP1 arrive to the cell surface where it is supposed to operate? The question has remained unanswered for decades (Bargman et al., 2013). Recently, the PM-related ABP1 mode of action has been emphasized by the identification of the ABP1-interacting partner, the TMK receptor-like kinase at the cell surface (Xu et al., 2014) and auxin itself seems to promote the ABP1 secretion to the cell surface (Chen, unpublished data). To assess how the ABP1 secretion to the cell surface is influenced by auxin binding, we used transmission electron microscopy (TEM) with

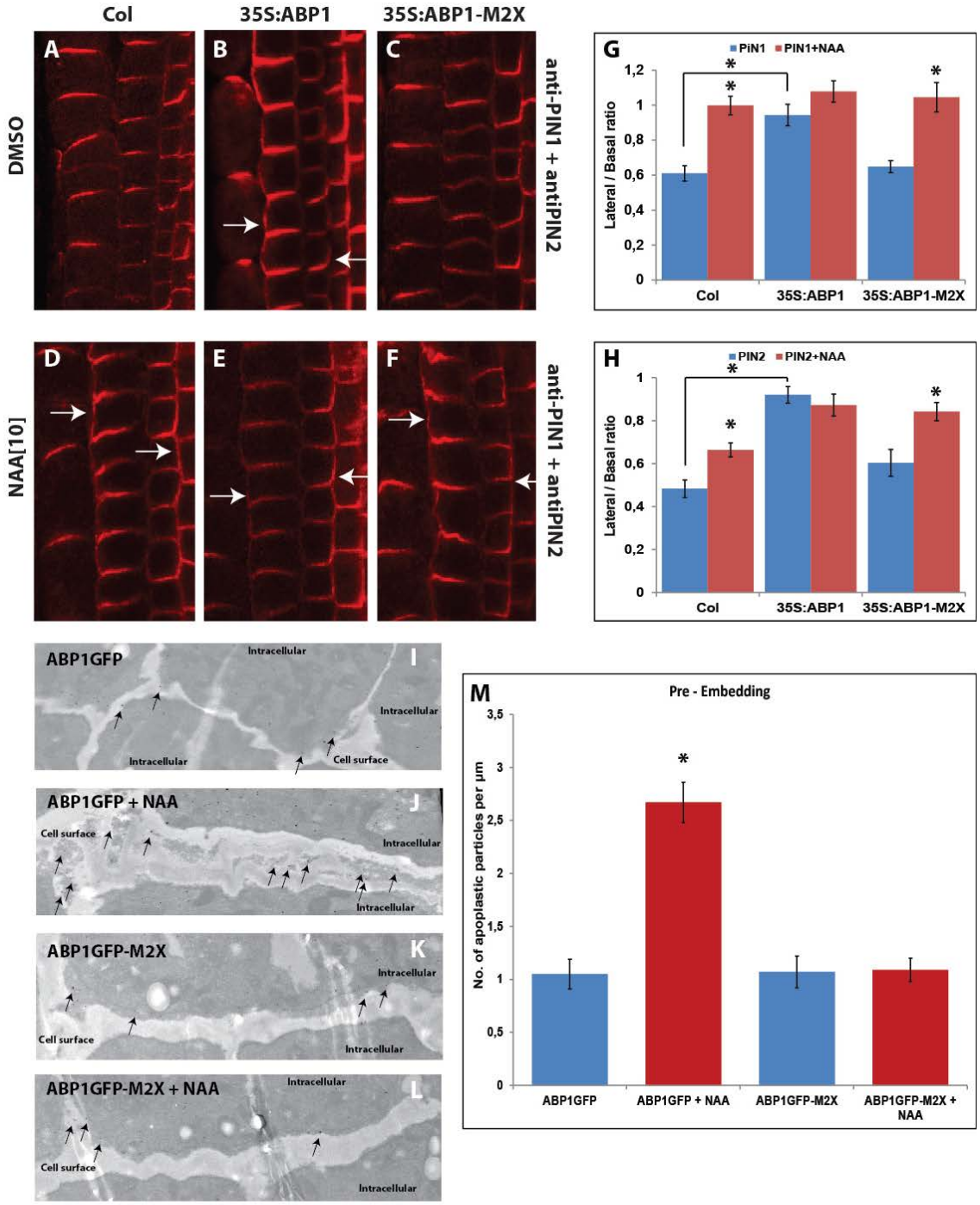

Figure 5. Auxin-dependent PIN polarization by *abp1* mutant variants. (A-F) Lateralization of PIN1 and PIN2 in *Arabidopsis* roots after 4 h of auxin treatment (10 µM). *35S::ABP1* seedlings exhibited lateralization when treated with solvent (B) when compared to the wild type (A) and *35S::ABP1-M2X* (C). Auxin induced lateralization of PIN proteins in the wild type (D) and *35S::ABP1-M2X* (F), but did not have a pronounced phenotype in *35S::ABP1* (E). (G and H) Quantification of auxin-dependent lateralization of the PIN1 (G) and PIN2 proteins (H). Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (* $p<0.01$). Error bars represent SE. Experiments were done independently 3 times and at least 50 cells per experiment were counted. Auxin-mediated secretion of ABP1 to the cell surface. (I-L) Pre-embedding TEM showed increased number of ABP1-labeled golden particles with apoplastic localization in

35S::ABP1-GFP seedlings after auxin treatment (10 μM) (J) compared to the untreated control (I). In *35S::ABP1-GFP-M2X* seedlings, the amount of golden particles in the apoplast did not differ in the untreated control (K) and the auxin-etreated seedlings (L). (M) Quantification of ABP1-labeled golden particles localized in the apoplast. Fifteen cells were counted per line. Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (* $p<0.001$). Error bars represent SE.

immunogold detection of ABP1 in *35S::ABP1-GFP* and *35S::ABP1-GFP-M2X* (with an α-GFP antibody).

These GFP-tagged lines exhibit phenotypes similar to those of the *35S::ABP1* lines (Xu et al., 2014). We used the pre-embedding method that works with almost intact cell structures, preventing the migration of epitopes through the membranes and allowing a better determination of native protein localization (Baude et al., 1995). The immunogold ABP1-GFP signals were found predominantly at the ER with a minor fraction at the cell surface. Quantification of gold particles at the cell surface revealed a 2.0-2.5-fold increase of the cell surface-localized ABP1 after NAA treatment in the *35S::ABP1:GFP* (Fig. 5, *I, J,* and *M*). In contrast, in the *35S::ABP1-GFP-M2X* line, auxin had no effect on the secretion of ABP1-GFP-M2X (Fig. 5, *K, L,* and *M*).

Interestingly, the elevated number of golden particles in the apoplast is not caused by the increased *de novo* protein synthesis. Based on qPCR data, the activity levels of the ABP1 protein measured at various time points after auxin treatment (0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h) did not differ (data not shown).

As mentioned before, the pre-embedding method is superior for epitope position determination, but is not ideal for quantification; therefore, we also performed TEM with post-embedding immunogold detection of endogenous ABP1 in the wild type (with an α-ABP1 antibody) and in *35S::ABP1-GFP* and *35S::ABP1-GFP-M2X* (with an α-GFP antibody). The immunogold ABP1 signals exhibited the same localization pattern as in the previous experiment. Quantification of gold particles at the cell surface revealed a 2.5–3.0-fold increase of the cell surface-localized ABP1 after NAA treatment of the wild type and *35S::ABP1-GFP*, but not of the *35S::ABP1-GFP-M2X* line, confirming the results obtained with pre-embedding TEM (Fig. S2, *I-O*). An increased number of gold-labelled ABP1 particles at the cell surface in the auxin-treated samples consistently demonstrated the promotional effect of auxin on the ABP1 secretion from the ER to the apoplast. This effect was completely abolished in the *abp1* auxin-binding mutant, thus revealing the importance of auxin binding for the ABP1 secretion.

Morphological phenotypes of plants expressing the auxin-binding *abp1* mutants

ABP1 has been shown to take part in many important developmental processes (Braun et al., 2008; Tromas et al., 2009; Xu et al., 2010, 2014). Among others, it mediates the auxin-dependent pavement cell interdigitation (Xu et al., 2010). The number of lobes in pavement cells increased up to 25% after auxin treatment in the wild type or in seedlings of the *35S::ABP1* overexpression line. In contrast, in the *abp1* auxin-inding mutant (ABP1-M2X), the number of lobes in pavement cells did not change even after auxin treatment (Fig. S3, *A-E*).

Seedlings overexpressing the wild-type ABP1 had a reduced root length, decreased root meristem size, increased lateral roots number, and defects in cotyledon vasculature when compared to the wild type (Fig. S3, *F-M*). These lines also showed a decrease in apical dominance, resulting in more branching. All these aberrant phenotypes were significantly reduced in the *35S::ABP1-M2X* overexpression lines. Altogether, these observations suggest that auxin binding to ABP1 is crucial for performing its developmental roles.

Complementation of the *abp1* knockout mutant

Despite many years of research on ABP1, only one apparently full knockout mutant allele has been characterized so far, which is embryo lethal (Chen et al., 2001). This phenotype was reported to be rescued by introducing the *35S::ABP1* overexpression construct into the genome. To evaluate the functionality of our constructs, we used the same approach and via *Agrobacterium*-mediated transformation we introduced several different constructs into the *abp1*/ABP1 heterozygous mutant. However, none of our constructs, i.e. *35S::ABP1-GFP*, *35S::ABP1-M2X*, *35S::ABP1-GFP-M2X*, and even *35S::ABP1*, was able to rescue the embryo phenotype, because the appearance of white aberrant seeds in the siliques still remained (Fig. S3*N*). Genotyping also confirmed the presence of a functional wild-type allele in all progenies.

In our subsequent attempt, the native ABP1 promoter was used to bring the expression to a level similar tot that *in planta*. We used two versions of the native promoter, a long one (1585 bp; adapted from Klode et al., 2011) and a short one (708 bp, until the start codon of the next gene). We screened more than three independent lines with an expression lower than, higher than, or equal to that of the wild type. None of these variants (with or without a tag or mutation) was able to rescue the embryo-lethal phenotype (Fig. S3*N*). The same negative result was observed when the native promoter with the genomic ABP1 sequence and 3'-untranslated region was cloned together in one piece and transformed into the *abp1/ABP1*

heterozygous line. Taken together, we were unable to complement the *abp1* embryo-lethal phenotype with any construct, preventing us to analyze the importance of auxin binding for the ABP1 developmental roles in the absence of the wild-type allele.

CONCLUSION

ABP1 had been identified as a first protein that binds auxin and had been suggested as receptor responsible for mediating a broad range of auxin responses (Bargman et al., 2013). However, the auxin-binding role of ABP1 has not been assessed until now.

To provide insights into this question, we prepared a series of different *abp1* variants containing mutations in the metal core or in the seventh hydrophobic amino acids in the binding pocket (Woo et al., 2002). In two assays, activation of the downstream components ROP2/RIC4 of the ABP1 signaling pathway in *Arabidopsis* protoplasts (Xu et al., 2010) and inhibition of the PIN internalization in tobacco BY-2 suspension cells (Robert et al., 2010), the ABP1-M2X mutant variant exhibited the most serious defects. This auxin-binding mutant variant failed to activate RIC4 and to translocate it from the cytosol to the PM and to inhibit the PIN internalization in the presence of auxin. In these assays, other mutant variants possessing mutations in hydrophobic amino acids had only mild defects or no defects in respect of the control mutations.

To exactly quantify the binding ability of prepared *abp1* mutant variants, the auxin-binding assay was carried out that had supposedly to be performed on the heterologously expressed and purified protein with the use of radio-labelled IAA (Bauly et al., 2000). Unfortunately, our attempts to overexpress and purify the ABP1 in lines of *Escherichia coli, Nicotiana benthamania,* or *Spodoptera frugiperda* insect cells were so far unsuccessful. Therefore, at this stage we cannot provide the direct evidence of the binding ability of the *abp1*-binding mutants.

The overexpression phenotypes of the *ABP1-M2X* mutant variant were consequently analyzed *in planta*. BFA-induced PIN internalization in the *35S::ABP1* line was slightly increased, but the result was not as convincing as in previous work (Robert et al., 2010), because a different quantification method was used. Instead of counting the number of BFA bodies per cell (Robert at al., 2010), the signal at the PM and in the intracellular part was measured. This *abp1* mutant variant was not influenced, but the formation of BFA bodies could not be inhibited by the addition of auxin or its analogs PEO-IAA and auxinole. Association of clathrin with the PM and TGN/EE is essential for clathrin-mediated endocytosis (Wang et al.,

2013). Auxin positively regulates this process by increasing the association and thus elevating the CHC signal intensity. In the *35S::ABP1* overexpression line, the signal of CHC at the PM and TGN/EE was increased already without auxin treatment. In contrast, the *35S::ABP1-M2X* overexpression line showed no primary increase of the CHC signal intensity and resistance to auxin treatment. The rapid increase of CHC levels after auxin treatment is not caused by *de novo* protein synthesis (Wang et al., 2013), but the exact mechanism still needs to be unraveled.

The auxin-mediated polarization of PIN proteins (Sauer et al., 2006) is regulated in an ABP1- and TIR1-dependent manner. Interestingly, in the *35S::ABP1* line, lateralization of PIN proteins occurred even without auxin treatment and could not be enhanced by addition of auxin. In contrast, PIN polarization was visible in the *35S::ABP1-M2X* overexpression line only after auxin treatment. Secretion of ABP1 from the ER to the cell surface seems to be mediated by auxin. We showed by two TEM methods that in the *35S::ABP1* overexpression line, the ABP1-labelled golden particles increased in the apoplast after auxin treatment and that in the *35S::ABP1-M2X* line this process was abolished. This information raises another question: where does the signal for the secretion of the ER-localized ABP1 to the apoplast come from? Further investigation will be needed to address this important question. A challenging task will be also to assess the rapid increase of the ABP1 signal in the apoplast without *de novo* protein synthesis. One of the options is the repressed degradation mechanism of ABP1, drawing the attention to the AtRma2 E3 ubiquitin ligase (Son et al., 2010) that has been shown to be involved in the cellular regulation of the ABP1 expression levels.

ABP1 plays an important role in several developmental processes (Braun et al., 2008; Tromas et al., 2009; Xu et al., 2010). The number of lobes in the leaf epidermal PC is regulated in an auxin-dependent manner (Xu et al., 2010). Auxin increased the number of lobes in the wild type and in the *35S::ABP1* overexpression line, but decreased in the *35S::ABP1-M2X* mutant line. From the other morphological phenotypes observed in the *35S::ABP1* overexpression line, such as reduced root length and root meristem size or defects in cotyledon vasculature (Rakusová, unpublished data), the *35S::ABP1-M2X* mutant line exhibited significantly reduced phenotypes compared to the *35S:ABP1* line. A reason might be the presence of the wild-type ABP1 allele, because we had to introduce our mutant variants into the Col-0 background due to our inability to complement the *abp1* mutant allele (Chen et al., 2001). The other conditional *abp1* mutant lines, such as SS12S6 or SS12K9, could not be used because the immunomodulation mechanism would target also constructs introduced into these lines. The failure to complement any construct did not allow us to analyze the importance of the

auxin binding for the ABP1-mediated developmental processes without the presence of the wild-type ABP1 protein.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Seedlings of *Arabidopsis thaliana* (L.) Heyhn., accession Columbia (Col-0), were vernalized for 2 days in the dark at 4^{o}C and grown on vertical half-strength Murashige and Skoog (0.5 MS) plates containing with 1% sucrose and 0.8% agar at 18^{o}C in a 16-h light/8h- dark photoperiod for 5 days. Liquid 0.5 MS medium was used for all chemical treatments. The transgenic lines and constructs used have been described previously: *35S::ABP1-GFP* construct (Robert et al., 2010), *35S::ABP1* line (Xu et al., 2014), *abp1/ABP1* heterozygous mutant line (Chen at al., 2001), and *35S::PIN1-RFP* construct (Robert et al., 2010).

Drug treatments

Five-day-old seedlings were incubated with the following chemicals: 25 μM BFA dissolved in DMSO (Sigma-Aldrich) for 90 min, 10 μM NAA dissolved in DMSO (Sigma-Aldrich) for 30 min for pretreatments and with 25 μM BFA/10 μM NAA for 90 min for cotreatments, 50 μM PEO-IAA dissolved in DMSO for 30 min (Hayashi et al., 2008) for pretreatments and 25 μM BFA/50 μM PEO-IAA for 90 min for cotreatments, 10 μM auxinole dissolved in DMSO for 30 min (Hayashi et al., 2012) for pretreatments and 25 μM BFA/10 μM auxinole for 90 min for cotreatments, and 10 μM NAA for 4 h. In control treatments, equal amounts of solvent were used.

Construct preparation and transformation

The *35S::ABP1g*, *shortABP1::ABP1g*, *longABP1::ABP1g*, and *longABP1::ABP1g::ABP1-3'UTR* plasmids were constructed with the Gateway cloning technology (www.invitrogen.com). Genomic fragments of the *ABP1* gene were cloned into the donor vector *pDONR221* and the *shortABP1* promoter (708 bp upstream of ATG) and *longABP1* promoter (1585 bp upstream of ATG; adapted from Klode et al., 2011) were cloned into the *pDONRP4P1r* vector. The *35S::ABP1g* construct was created by recombining *ABP1* in *pDONR221* into *pB7GW2*. The expression clones containing the native ABP1 promoter variants were generated by recombining these fragments into the expression vector *pB7m24GW,3*. *35S::RIC4-RFP* was constructed by recombining the *RIC4* genomic fragment from *pDONR221* into the *p2GWR7* destination vector.

The *ABP1-M1X* and *ABP1-M2X* constructs were made by substituting the modified fragment by classical cloning via *Sac*I and *Pas*I in the *35S::ABP1*, *shortABP1::ABP1*, *longABP1::ABP1*, *longABP1::ABP1::ABP1-3'UTR*, and *35S::ABP1GFP* vectors. The mutations R59K, L62V, Q83D, T91V, P92L, F186L, W190Y, R59K/L62V, T91V/P92L, F186L/W190Y, V101A, F127L, P138L, and Q193D in ABP1 were done by site-direct mutagenesis PCR with modified primers.

The resulting constructs were transformed into *Arabidopsis* (Col-0) and into *abp1/ABP1* heterozygous plants by floral dipping in *Agrobacterium tumefaciens* liquid cultures. Transformants were selected on phosphinothricin-containing plates. From each construct, at least three independent lines with similar expression levels were chosen and analyzed. All primers and prepared fragments used for cloning are summarized in Table S1.

Genotyping and qRT-PCR

The ABP1 T-DNA insertion line was genotyped with the right border primer for the Wisconsin T-DNA lines in combination with the ABP1-specific primers (Table S1). To test the *ABP1* gene expression level in the T-DNA insertion lines and other transformants, qRT-PCR was performed. Five-day-old seedlings were harvested, RNA extracted, and cDNAsynthezised. Of a 1:10 cDNA dilution, 5 µl was used in a 20-µl qRT-PCR reaction containing 1× DyNAmoTM SYBR® Green Mastermix (FINNZYMES). Real-time PCR reactions were run in triplicate. Gene expression was calculated with the 2-ΔΔCT method (Livak and Schmittgen, 2001). Tubulin was used as endogenous control for the relative quantification of the *ABP1* gene expression. Primers used for qRT-PCR are summarized in Table S1.

Transmission Electron Microscopy (TEM)

High-pressure freezing and freeze-substitution for immunogold labeling

Root tips of 3-day-old seedlings of *Arabidopsis* were excised, immersed in 5% (w/v) sucrose in growth medium, frozen immediately in a high-pressure freezing machine (HPM010; Bal-Tec, Balzers, Liechtenstein), and stored in liquid nitrogen until further use. Freeze substitution was carried out in an EM AFS device (Leica Microsystems, Vienna, Austria). Over a period of 4 days, root tips were freeze-substituted in anhydrous acetone containing 0.25% (v/v) glutaraldehyde and 0.1% (w/v) uranyl acetate as follows: -80°C for 48 h, gradual warming to -60°C over a 10-h period, -60°C for 16 h, gradual warming to -30°C over a 10-h period, and -30°C for 16 h. At -30°C, samples were rinsed 3 times in acetone for 20 min each and

gradually warmed to 4°C over a 4-h period. Samples were rinsed in dry ethanol (4 times for 10 min each) and infiltrated in LR-White (#62662; Fluka, Buchs, Switzerland) overnight at room temperature on a shaking platform. Samples were then brought into gelatin capsules, fully filled, tightly capped, and polymerized at 50°C for 24 h. Ultrathin sections (70-80 nm) were sliced with an ultramicrotome (Leica EM UC7), collected on formvar-coated nickel slot grids, and processed for immunogold cytochemistry. Sections were incubated in 50 mM glycine in Tris-buffered saline (TBS; 50 mM, 0.9% NaCl, pH 7.4) for 20 min at room temperature (RT) for quenching free aldehyde groups, followed by incubation in 2% bovine serum albumin (BSA), 10% normal goat serum, and 1% cold water fish-skin gelatin in TBS containing 0.1% Triton X-100 (TBS-T) for 60 min at RT to block nonspecific binding sites. Primary antibodies were then applied at a concentration of 1:100 in TBS-T containing 2% BSA overnight at 4°C. After rinsing in TBS-T (4 times for 10 min each), goat anti-rabbit immunoglobulins coupled to 10-nm gold particles were applied (British BioCell Int., Cardiff, UK) diluted 1:50 in TBS-T containing 2% BSA and 0.05% polyethylene glycol for 90 min at RT. Sections were rinsed in double-distilled water and air dried. Specificity of immunolabeling was controlled by omitting the primary antibodies after application of the full set of secondary antibodies.

Sections were contrast enhanced by means of 1% (w/v) uranyl acetate in water for 20 min at RT and 2.66% (w/v) lead nitrate in sodium citrate for 5 min at RT. Sections were examined in a TECNAI 10 TEM operated at 80 kV, equipped with a Morada CCD camera (Soft Imaging Systems, Münster, Germany). Whole images were level adjusted, sharpened, and cropped in Photoshop (Adobe) without manipulating any specific feature.

Quantification was done by counting the number of golden particles in the intracellular space and dividing by the length of the membrane. Per line, 10-15 pictures were analyzed.

Preembedding immunoelectron microscopy

Four-day-old seedlings were fixed with 4% paraformaldehyde + 0.05% glutaraldehyde in phosphate buffered saline (PBS) for 1 h in vacuum and afterward washed 4× for 10 min with PBS. Cell walls were digested with 1.5% driselase solution in PBS at 37^{o}C for 30 min and, consequently, washed 3× for 5 min with PBS. Seedlings were incubated in 50 mM glycine in PBS for 1 h at RT for quenching free aldehyde groups, followed by incubation in 2% BSA + 1% fish gelatin in PBS for 2 h at RT (or overnight at 4^{o}C) for blocking nonspecific binding sites. Primary antibodies were applied in PBS containing 2% BSA for 4 h at RT (or overnight at 4°C). Antibodies anti-GFP (Sigma-Aldrich) were 1:1000 diluted. After the seedlings had

been washed 3× for 15 minutes in PBS, they were incubated with nanogold®-conjugated Fab' fragments (1:100 in PBS containing 2% BSA) for 2 h at RT. Nanogold particles were amplified with silver with the HQ Silver™ Enhancement Kit (Nanoprobes Inc.) for 5-7 min at RT with control under light microscopy. Samples were washed in MilliQ water, post-fixed in 1% glutaraldehyde in PBS for 10 min at RT, contrast-enhanced with 2% osmium tetroxide in PBS (for 40 min at RT in the dark) and 1% uranyl acetate in 50% ethanol (for 30 min at RT in the dark), and embedded in epoxy resin (Durcupan ACM; Fluca) on greased glass slides. Root tips were dissected and re-embedded in epoxy resin. Serial ultrathin sections (70-80 nm) were sliced with an ultramicrotome (Leica Microsystems VT1000S) and collected on formvar-coated copper slot grids. Ultrathin sections were examined in a FEI Tecnai 10 TEM (operated at 80 kV accelerating voltage), equipped with an OSIS Megaview III camera (Olympus Soft Imaging Systems, Muenster, Germany). Whole images were level-adjusted, sharpened, and cropped in Photoshop (Adobe) without manipulation of any specific feature. Quantification was done by counting the number of golden particles in the intracellular space and dividing by the length of the membrane. Per line, 10-15 pictures were analyzed.

Immunodetection and microscopy

Arabidopsis roots were analyzed by immunofluorescence as described (Sauer et al., 2006). The anti-PIN1 antibody (1:1000) (Benková et al., 2003), the anti-PIN2 antibody (1:1000) (Abas et al., 2006), and the anti-CHC antibody (1:400) (Kim et al., 2001) were used as well as the fluorochrome-conjugated secondary antibodies Alexa488 and the anti-rabbit-Cy3 (1:600) (Dianova). Live-cell microscopy was done on a Zeiss 710 confocal microscope and pictures were analyzed by ImageJ (ImageJ; National Institutes of Health; http://rsb.info.nih.gov/ij). BFA bodies and CHC were quantified by measuring the PM and intracellular signal ratio and lateralization by measuring the signal on the basal or apical membranes and by comparing it to the signal on lateral membranes.

Transient transformation of tobacco BY-2 cells

Of 3-day-old cells, 10 ml was harvested on filter paper by vacuum filtration and kept on solid BY-2 medium. The cells were transformed via particle bombardment with a PDS-1000/He biolistic system (Biorad) according to the manufacturer's recommendations. To coat the gold particles with DNA, 2 ml of plasmid DNA (0.05 mg/ml of each to be transformed construct) was added to 6.25 ml of 1.6-mm diameter gold particles and the suspension was supplemented with 2.5 ml spermidine (0.1 M stock solution) and 6.25 ml $CaCl_2$ (2.5 M stock

solution). The particles were pelleted by centrifugation and washed twice with 70% and 100% ethanol. The pellet was suspended in 10 ml of 100% ethanol. Cells were bombarded under a pressure of 1100 psi with the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad). After transformation, 1 ml of auxin-free medium (mock) or enriched with 10 mM NAA was added to the cells. The plates were sealed with parafilm and kept in the dark for 18 h at 25^{o}C. Samples were imaged via confocal microscopy (Zeiss 710) and analyzed as described (Robert et al., 2010). Experiments were done in triplicate for all prepared constructs and each time between 15-20 cells were analyzed. Cells were divided into three groups based on the number of intracellular particles containing PIN proteins: 0-3 “no internalization”, 4-10 “weak internalization”, >10 “severe internalization”.

Phenotypical analysis

Root length, hypocotyl length, and lateral root density were measured by the Java-based ImageJ application (National Institutes of Health; http://rsb.info.nih.gov/ij). At least 20 seedlings were measured in three independent experiments, giving the same statistically significant results. The statistical significance was evaluated with Student's *t*-test.

The pavement cell shape from *Arabidopsis* cotyledons was imaged directly on a confocal microscopy (Leica SP2) after the cotyledons had been treated with propidium iodide for visualization of the cell outline. At least 10 cotyledons and 30 cells from each were analyzed from each line and the number of lobes and the cell size were measured in ImageJ. The experiment was repeated twice.

RIC4 activation assay

Changes in the RFP-RIC4 localization to the PM were monitored in isolated protoplasts. Protoplasts were isolated from an *Arabidopsis* suspension culture (PSB-L) as described (Goossens and Pauwels, 2013). Four micrograms of the *35S::RFP-RIC4* construct, *35S::ABP1GFP,* or particular *abp1* mutant variant was introduced into protoplasts by PEG-mediated transformation (Mathur and Koncz, 1998). Typically, approximately 70% of the protoplasts were transformed and cells showing both signals were imaged by a Zeiss 710 confocal microscope. Evaluation was done based on the localization of the RIC4-RFP signal, thus dividing cells into two groups with the signal either at the PM or in the cytosol. From each transfection, 10-15 cells showing both signals were evaluated and the experiment was repeated 3 times.

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SUPPLEMENTARY FIGURES

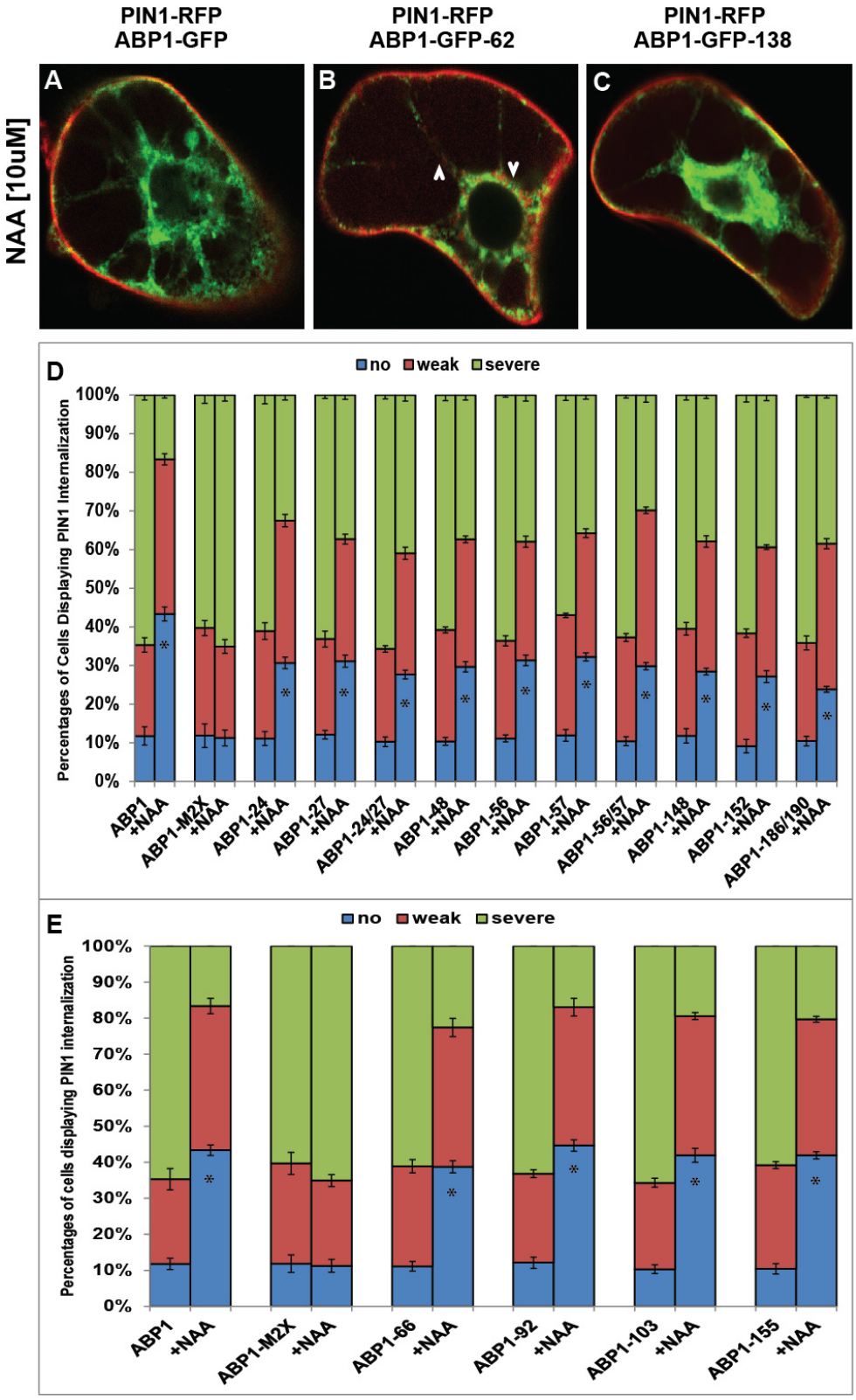

Figure S1. Involvment of seven hydrophobic amino acids on inhibition of endocytosis. (A-C) Representative pictures of internalization inhibition of PIN1-RFP (in red) after auxin treatment (10 μM) in the presence of *35S::ABP1-GFP* (A) and neutral mutation (C), but mutations in hydrophobic amino acid showed presence of PIN internalization after auxin treatment (B). (D and E). Percentage of cells displaying severe (green), mild (red), or not detectable (blue) PIN1-RFP internalization in presence of mutants with hydrophobic amino acid

mutations (D) and of mutants with neutral mutations (E). Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (* $p<0.001$). Error bars represent SE. 3 independent experiments were performed and at least 20 cells per each were counted. Arrows indicate PIN protein internalization.

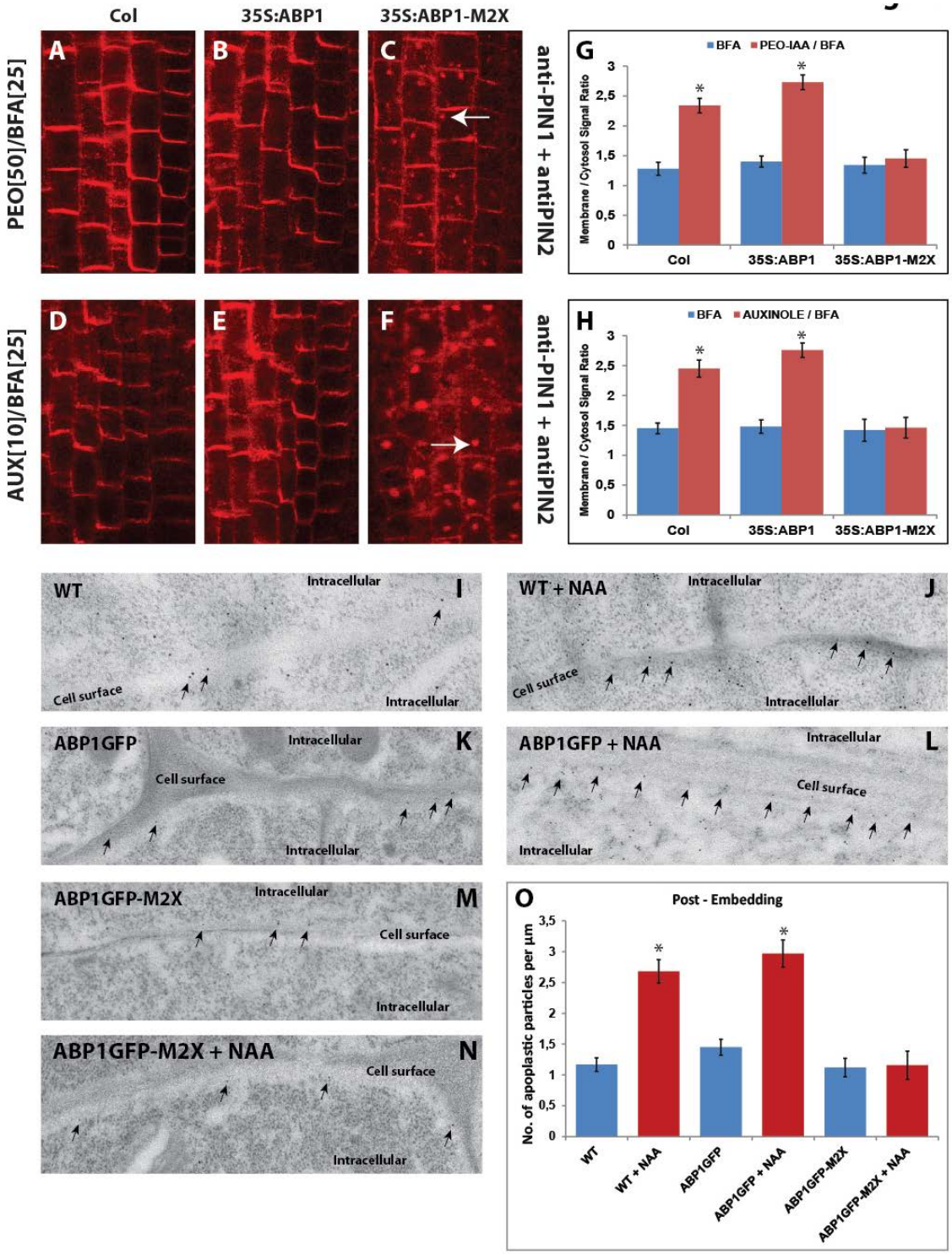

Figure S2. Inhibition of PIN endocytosis in *Arabidopsis* by auxin analogs. (A-C) PEO-IAA can inhibit formation of BFA bodies in WT (A) and in *35S::ABP1* (B), but not in *35S::ABP1-M2X* seedlings (C). BFA-induced PIN endocytosis can be inhibited by auxinole in WT (D) and in *35S::ABP1* (E), but cannot be in *35S::ABP1-M2X* (F) seedlings. (G and H) Quantification of signal ratio between PM and cytosol for PEO-IAA (G) and for auxinole (H). Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (* $p<0.001$). Error bars represent SE. Secretion of ABP1 to the cell surface mediated by auxin. (I-N) Post-embedding TEM exhibited increased number of ABP1-labeled golden particles with apoplastic localization in WT (J) *35S::ABP1-GFP* seedlings after auxin treatment (10 µM NAA) (K) compared to untreated WT control (I) and *35S::ABP1-GFP* (L). *35S::ABP1-GFP-M2X* seedlings showed no difference in amount of golden particles in apoplast in untreated control (M) and auxin treated seedlings (N). (O) Quantification of ABP1-labeled golden particles localized in the apoplast. Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (* $p<0.001$). Error bars represent SE.

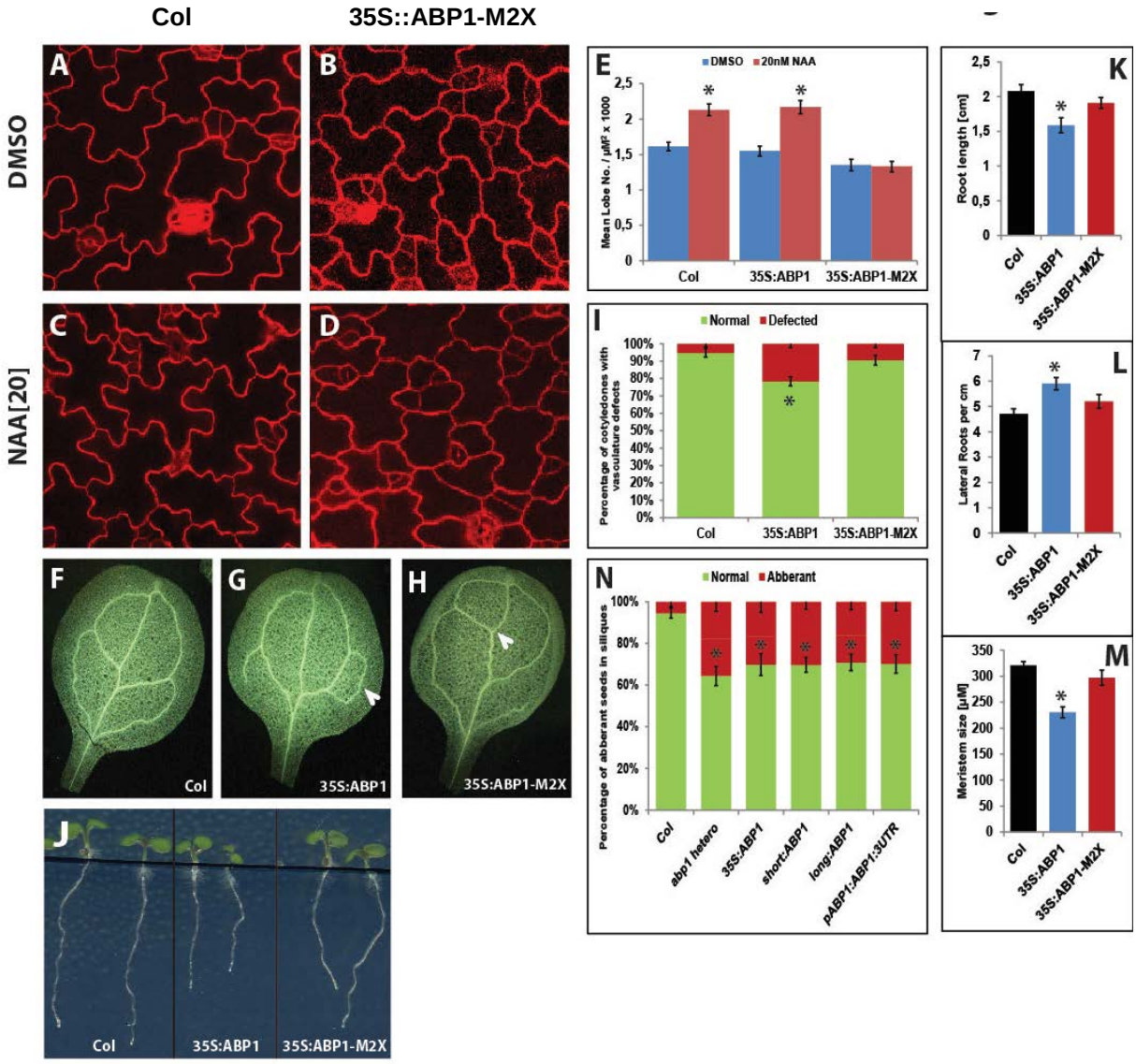

Figure S3. Morphological defects of *abp1* auxin binding mutants. (A-D) Induction of pavement cells interdigitation by auxin treatment (20 nM NAA) appeared in WT seedlings (C) compared to untreated control (A), but not in *35S::ABP1-M2X* seedlings (B and D). (E) Quantification of mean lobe number of leaf pavement cells. Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples (* $p<0.01$). Error bars represent SE. Experiment was done 2 times, each time 100 cells per line were counted. Error bars represent SE. (F-H) Cotyledon vasculature defects in WT (F), *35S::ABP1* (G) and *35S::ABP1-M2X* (H) seedlings. (I) Quantification of cotyledone number with vasculature defects. Student's T-test was calculated for the comparison to the control (Col-0) (* $p<0.05$). Error bars represent SE. (J). Quantification of root length (K), lateral roots (L) and meristem size (M). Student's T-test was calculated for the comparison to the control (Col-0) (* $p<0.001$). Error bars represent SE (N) Quantification of aberrant seeds in siliques in different complementation lines. Student's T-test was calculated for the comparison to the control (Col-0) (* $p<0.01$). Error bars represent SE.

ABP1-M1X	GAGCTCCTTGTCCCATCAATGGCTTGCCAATCGTGAGGAATATTAGT GACCTTCCTCAGGATAACTATGGAAGACCAGGTCTTTCCCACATGAC TGTTGCTGGCTCCGTATTGCATGGAATGAAAGAGGTTGAAATATGGC TTCAGACATTTGCTCCAGGTTCAGAGACACCAATT**GTC**AGG**C**A**C**TCC TGTGAAGAGGTTTTTGTTGTCCTAAAGGGCAGTGGTACTCTGTATCT CGCTGAAACACATGGAAATTTCCCTGGG
ABP1-M2X	GAGCTCCTTGTCCCATCAATGGCTTGCCAATCGTGAGGAATATTAGT GACCTTCCTCAGGATAACTATGGAAGACCAGGTCTTTCCCACATGAC TGTTGCTGGCTCCGTATTGCATGGAATGAAAGAGGTTGAAATATGGC TTCAGACATTTGCTCCAGGTTCAGAGACACCAATT**GTC**AGG**GTC**TCC TGTGAAGAGGTTTTTGTTGTCCTAAAGGGCAGTGGTACTCTGTATCT CGCTGAAACACATGGAAATTTCCCTGGG
ABP1_Fw	ATGATCGTACTTTCTGTTGGTTCC
ABP1_Rv	TTAAAGCTCGTCTTTTTGTGATTCTTG
ABP1_Rv_ΔKDEL	TTATTGTGATTCTTGAATGCATTGCTC
ABP1_ R ΔKDEL ΔSTOP	TTGTGATTCTTGAATGCATTGCTCATC
prom_long_ABP1_Fw	AGTCTCGGAATACCAAGAAC
prom_long_ABP1_Rv	TTTCTCGATGCTTCGACGAAC
prom_short_ABP1_Fw	TTACCTGCACAAAAACGGACC
prom_short_ABP1_Rv	TTTCTCGATGCTTCGACGAAC
ABP1-3'UTR_Rv	TGGTGGTTCATATGAGATCGGACC
ABP1_R59K/L62V_Fw	TCCCACATGACTGTTGCTGGCTC
ABP1_R59K_Rv	AAGACCTGGTTTTCCATAGTTATCCTGAG
ABP1_L62V_Rv	AACACCTGGTCTTCCATAGTTATCCTG
ABP1_R59K/L62V_Rv	AACACCTGGTTTTCCATAGTTATCCTGAG
ABP1_Q83D_Fw	ACATTTGCTCCAGGTTCAGAGACAC
ABP1_Q83D_Rv	GTCAAGCCATATTTCAACCTCTTTCATTCC
ABP1_T91V/P92L_Fw	ATTCACAGGCACTCCTGTGAAGAG
ABP1_T91V_Rv	TGGTACCTCTGAACCTGGAGCAAATG
ABP1_P92L_Rv	TAGTGTCTCTGAACCTGGAGCAAATG
ABP1_T91V/P92L_Rv	TAGTACCTCTGAACCTGGAGCAAATG
ABP1_F186L_Fw	CCTTACTATTGGGATGAGCAATGCATTC
ABP1_F186L_Rv	GAGCTTCAGCCTTGCAGCAGTG
ABP1_W190Y_Fw	GATGAGCAATGCATTCAAGAATCACAAAAAGAC
ABP1_W190Y_Rv	GTAATAGTAAGGGAACTTCAGCCTTGC
ABP1_F186L/W190Y_Fw	CTATTACGATGAGCAATGCATTCAAGAATC
ABP1_F186L/W190Y_Rv	TAAGGGAGCTTCAGCCTTGCAGC
ABP1_V101A_Fw	TTTGTTGTCCTAAAGGGCAGTG
ABP1_V101A_Rv	AGCCTCTTCACAGGAGTGC
ABP1_F127L_Fw	AATCTTTGCCAACAGTACAATTCATATTC
ABP1_F127L_Rv	GGAAGTTCGATTGGTTTCCCAG
ABP1_P138L_Fw	TTCTGATCAATGATGCTCATCAG
ABP1_P138L_Rv	TATGAATTGTACTGTTGGCAAAGATTG
ABP1_Q193D_Fw	ATGAGGACTGCATTCAAGAATCAC
ABP1_Q193D_Rv	CCCAATAGTAAGGGAACTTCAG
abp1-2_Fw	ATTTTATAATAACGCTGCGGACATCTAC
abp1-2_Rv	TCCCAACAGTTGCGCACCTGAATG
T-DNA_Rv	GTATCTACGTAGTGTCACAAAACCTCAAC

Table S1. Primers and synthesized fragments used for construct preparation.

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Chapter 3.

Auxin mediates secretion of its receptor ABP1 through SKU5 interacting partners in Arabidopsis

Adapted from

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PG and XC initiated the project and designed most of the experiments, XC and PG carried out most of the experiments. RDR performed electron microscopy, KW developed software for analyzing electron microscopy pictures, JC helped with ROP2/ROP6 activity detection, DL helped with pavement cells quantification, HR donated TME on RPS5A::ABP1GFP, BC helped with technical support and HL with TEM. All authors analysed and discussed the data; XC, PG, CPR, ZY and JF wrote the manuscript and all authors saw and commented on the manuscript..

Auxin mediates secretion of its receptor ABP1 through SKU5 interacting partners in *Arabidopsis*

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Abstract

Auxin Binding Protein1 (ABP1) is one of the first characterized proteins that binds auxin and has been implied as an extracellular receptor to mediate a broad range of auxin responses. ABP1 predominately resides in the endoplasmic reticulum (ER) but a minor, physiologically relevant fraction escapes from the ER to the extracellular space by a yet unknown mechanism. Here, we identify the ER-localized and cell surface-docking glycosylphosphatidylinositol (GPI)-anchored protein SKEWED5 (SKU5) which binds ABP1 both at the ER and on the cell surface, in an auxin-dependent manner. Deficiency of SKU5/SKSs (SKU5 similar homologues) shows largely overlapping defects with ABP1-inactivation lines. The secretion of SKU5 to the cell surface is triggered by ABP1-dependent auxin signaling; meanwhile ABP1 secretion to the cell surface is promoted by SKU5/SKSs-involved auxin signaling. These findings identified a novel mechanism of auxin signaling, whereby SKU5 and its

homologues promote the possibility of extracellular auxin perception via increase the secretion of ABP1 to the extracellular matrix.

INTRODUCTION

Auxin, one of the major plant hormones, is essential to coordinate diverse processes of plant development, such as embryogenesis, tropic response, shoot branching, vascular development, apical dominance, flowering and fruit ripening (Mockaitis and Estelle, 2008). These are result of regulations at the cellular level including cell expansion, division and differentiation (Chapman and Estelle, 2009). The effects of auxin on plant development have been largely attributed to transcriptional regulations governed by the Transport Inhibitor Response1/Auxin-Related F-BOX (TIR1/AFB) - auxin/indole-3-acetic acid (Aux/IAA) signaling pathway (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). However, at the cell surface, the rapid responses of auxin including ion fluxes, membrane hyperpolarization, endocytosis and cytoskeleton rely on Auxin Binding Protein1 (ABP1)-mediated signaling (Cleland et al., 1977; Gehring et al., 1998; Hager et al., 1991; Thiel et al., 1993; Yamagami et al., 2004)(Chen et al., 2014).

Arabidopsis ABP1 is a single copy gene, whose deficiency leads to severe phenotypic defects such as embryo lethality in the null *abp1* mutant (Chen et al., 2001). ABP1 has been proposed for a long time as a putative auxin receptor (Jones and Venis, 1989; Lobler and Klambt, 1985; Tian et al., 1995), as it binds auxin with high affinity (Jones and Venis, 1989). Until recent discoveries uncover part of this mysterious signaling pathway, e.g. ABP1 activates Rho small GTPases (Rho-like guanosine triphosphatases from plants)-mediated signalings for leave pavement cells interdigitation (Xu et al., 2010); ABP1 mediates auxin inhibition on clathrin-dependent endocytosis (Robert et al., 2010); as well as ABP1 is essential for cell wall formation (Tromas et al., 2013).

The plant cell surface and apoplast (pH 5.5) are ideal environments for promoting high binding affinity of ABP1 to auxin (Shimomura et al., 1986; Tian et al., 1995). Immunocytochemisty in conjunction with electron microscopy detected a small population of ABP1 at the plasma membrane (PM) where its physiological role has been demonstrated (Diekmann et al., 1995; Jones and Herman, 1993). Due to the lack of transmembrane domain, ABP1 is unlikely to transmit auxin signal from the exterior through the PM. Recently, the PM-localized ABP1 interacting proteins, the Trans-Membrane Kinase (TMK) receptor-like kinases (Xu et al., 2014) which couple with ABP1 on the cell surface have been identified to

transduce extracellular auxin signal to the cytoplasm (Xu et al., 2014). However, ABP1 contains a C-terminal ER retention sequence of KDEL (Lys-Asp-Glu-Leu) motif (Hesse et al., 1989; Inohara et al., 1989). Numerous visualizations also show that most ABP1 localizes to the endoplasmic reticulum (ER) (Henderson et al., 1997; Jones and Venis, 1989; Napier et al., 1992), where the auxin binding is limited by the pH of ER lumen (Tian et al., 1995). Thus, the amount of ABP1 on the cell surface where auxin binds ABP1 with high affinity determines the efficiency of following auxin signal transduction events.

While the mechanism on how ER-located ABP1 escapes to the cell surface remains unclear. Therefore, interacting partners that transport ABP1 to the membrane fractions deserve our attentions.

In this study, we described the GPI-anchored SKEWED5 (SKU5) which binds ABP1 in an auxin –dependent manner. We revealed a mechanism of ABP1 secretion to the cell surface along with its potential physiological role. We further discovered that auxin itself regulates ABP1 escape from the ER by promoting interaction between ABP1 and SKU5 as well as its interacting partners SKU5 Similar homologues (SKSs).

RESULTS

ABP1 Associates with SKU5 and SKS4 Proteins in *Arabidopsis*

Previously, the GPI-anchored protein, the C-terminal Binding Protein 1 (CBP1) had been identified as a putative interactor of ABP1 in maize (Shimomura, 2006). *Arabidopsis* SKU5 is a homolog of the maize CBP1. To assess the possible interaction between *Arabidopsis* SKU5 and ABP1, we carried out a bimolecular fluorescence complementation (BiFC) assay (Boruc et al., 2010). However, we failed to observe the reconstituted fluorescence, with the exception of the occasionally false positive signal in *N. benthamiana* leaf cells by coexpression of *35S::SKU5-GN* and *35S::ABP1-GC* (Figure 1A) or *35S::SKU5-GC* and *35S::ABP1-GN* (Table S1).

The dimeric structure of ABP1 (Woo et al., 2002) implies that its interacting partners may be dimeric as well. In the *Arabidopsis* genome, 18 *SKU5*-similar genes (SKSs) share 42-65% amino acid sequence similarity with SKU5. According to the phylogenetic analysis, SKS1 and SKS2 exhibit the closest sequence identity with SKU5 (Figure S1A), but SKU5 is highly coexpressed with SKS4 based on the ATTED-II database (Figure S1B). We tried all the combinations among ABP1, SKU5, SKS4, SKS1, and SKS2 in the BiFC assay and only

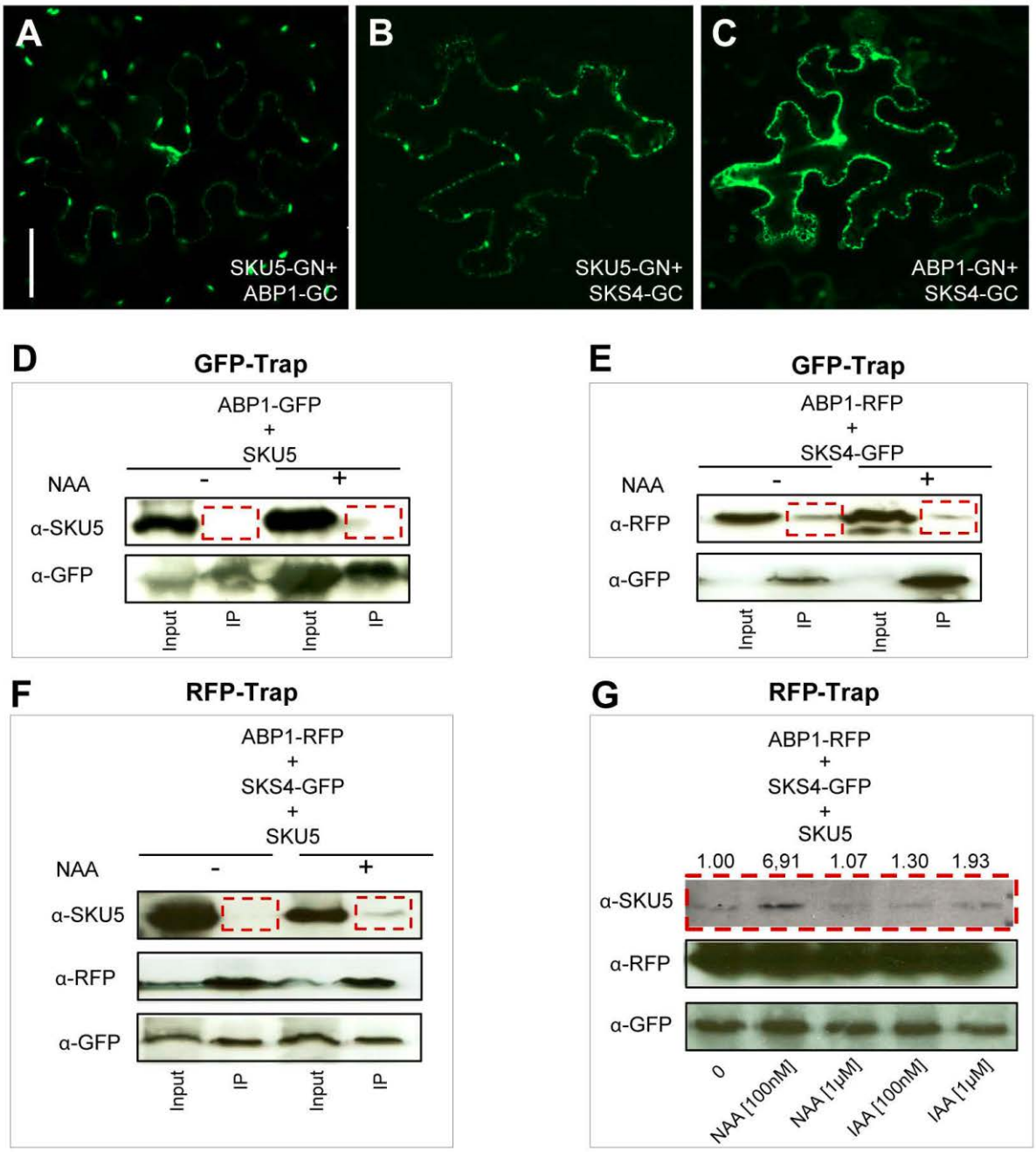

Figure 1. SKU5 Interacts with ABP1 in an Auxin-dependent Manner. (A-C) Interactions of ABP1 and SKU5, SKU5 and SKS4, SKS4 and ABP1 were detected by BiFc assay in N. benthamiana epidermal leave (GN=GFP N-terminal part, GC=GFP C-terminal part).
(D-E) CO-IP confirmation of the interactions among SKU5, SKS4 and ABP1 from infiltrated N. benthamiana protein extracts. Without (-NAA) or with NAA application (+NAA, 1μM, 1h), ABP1 was captured by GFP-Trap beads, and the total extracted protein (input) and IP-SKU5 proteins were analyzed by immunobolt with SKU5 antibody (α-SKU5) (D). The interaction between SKS4 and ABP1 was performed in the similar way (E). Immunoprecipitated proteins were highlighted by red dot frame.
(F-G) CO-IP confirmation of the interactions between ABP1 and SKU5 in the presence of overexpressed SKS4. Proteins extracted from N. benthamiana leaves expressing the combination of 35S::ABP1:RFP, 35S::SKS4:GFP and 35S::SKU5 with NAA or IAA (100nM or 1μM) treatment. ABP1 was captured by RFP-Trap beads. IP-SKS4 and SKU5 proteins were analyzed with α-GFP and α-SKU5, respectively. NAA/IAA treatment was performed for 1h (F) or 3h (G).
SKU5 proteins which were immunoprecipitated were highlighted by red dot frame (F-G), and the relative protein level of IP-SKU5 was quantified as the number showed (G).

observed the positive signal on the ABP1-SKS4 pair (Figure 1B, C, Table S1), suggesting a possible association between the *Arabidopsis* SKS4 and ABP1.

Next, we performed a co-immunoprecipitation (Co-IP) assay with ABP1-GFP as bait in the infiltrated *N. benthamiana* cells and, consistently with the BiFC result, we could also not detect the interaction between ABP1 and SKU5 (Figure 1D). Consequently, the Co-IP experiments with the co-expressing ABP1-RFP and SKS4-GFP proteins confirmed that ABP1 was efficiently co-precipitated with the SKS4 protein (Figure 1E, S1C).

To test whether SKS4 and SKU5 might collaborate to bind ABP1, we co-infiltrated the three constructs *35S::SKU5*, *35S::ABP1-RFP,* and *35S::SKS4-GFP* into *N. benthamiana.* By means of the Co-IP assay with ABP1-RFP or SKS4-GFP as bait, we failed to discover the co-precipitated SKU5 protein (Figure 1F, S1D).

ABP1 Interacts with SKU5 in an Auxin-dependent Manner

As an auxin-binding protein, ABP1 has been proven to bind synthetic auxin 1-naphthaleneacetic acid (NAA) or the natural auxin IAA with high affinity (Brown and Jones, 1994; Jones and Venis, 1989), it raises the possibility that auxin could influence the ABP1 and SKU5 association. To test this hypothesis, we applied exogenous auxin on infiltrated *N. benthamiana* leaves before Co-IP. Without the SKS4 protein, no interaction was detected by co-transfection of SKU5 and ABP1 in NAA-treated plants (Figure 1D). When all three components (*35S::ABP1:RFP*, *35S::SKS4:GFP,* and *35S::SKU5*) were co-expressed together in the presence of auxin, a clear interaction band was observed (Figure 1F, Figure S1D), indicating that auxin promotes the association of ABP1, SKS4, and SKU5.

To confirm the results of the auxin-dependent ABP1 and SKS4-SKU5 association, we used auxin analogs (NAA and IAA) at different concentrations for a 3-h pretreatment before Co-IP detection. The observed binding affinity between ABP1 and SKU5 was the strongest after application of 100 nM NAA, but decreased with the exposure to higher concentration of NAA (1 μM) (Figure 1G). IAA slightly induced the association of ABP1 with SKS4 and SKU5 (Figure 1G). These results showed that SKU5 interacts with ABP1 in an auxin-dependent manner, requiring the assistance of the SKS4 protein.

ABP1 and SKU5/SKSs Inactivations Share Similar Morphological Phenotypes

The complex formation between ABP1 and SKU5-SKS4 implied the involvement of SKSs in the ABP1-mediated processes. Therefore, we tested the auxin sensitivity of the *sku5* mutant

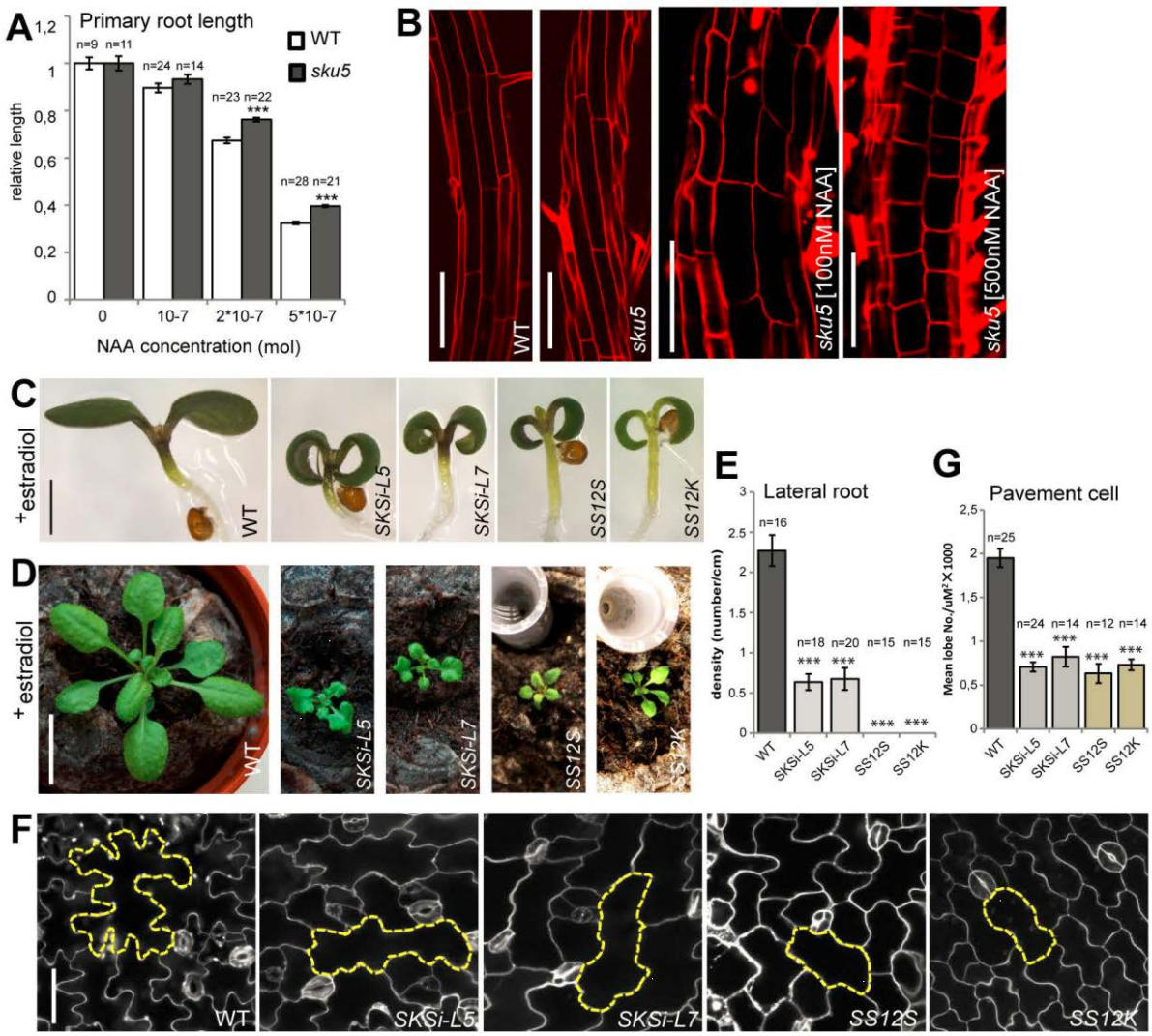

Figure 2. Deficiency of SKU5/SKSs Simulates the Morphological Phenotypes of ABP1-inactivation Lines.
(A) The primary root length of WT and *sku5* was measured when grown on NAA containing mediums at different concentrations. Student's T-test was calculated for *sku5* mutant in comparison to WT at each NAA concentration (*** $p<0.001$).
(B) The phenotype of *sku5* mutant having skewed root was partially rescued when grown on NAA (100nM, 500nM)-containing medium, and the cellular morphology was confirmed by PI staining in the elongation zone.
(C-D) Grown on 2μM 17-β-estradiol containing medium for 5 days (C) or sprayed by 17-β-estradiol (2μM) for 20days (D), *SKSi-L5* and *SKSi-L7* phenocopied ABP1-inactivation lines (SS12S and SS12K).
(E) Grown on 2μM 17-β-estradiol containing medium for 7 days, *SKSi-L5* and *SKSi-L7* had less later roots which were quantified as later root number density (number/mm).
(F-G) Pavement cell (PC) interdigitation was defected in *SKSi-L5* and *SKSi-L7* (2μM estradiol induction from germination), SS12S and SS12K (5% ethanol induction from germination). Cotyledon PCs interdigitation was quantified by determining the density of lobes, and the outline of the representative PCs was highlighted as yellow dot lines.
In all panels, error bars are s.e.m determined by student's T-test (*** $p<0.001$). Scale bar: 30 μm (B), 2mm (C), 2cm (D) and 50 μm (F).

that had roots strongly skewed to the left and twisting root cell files (Sedbrook et al., 2002). Auxin inhibition of the primary *root growth was impaired in the* sku5 *mutant* (Figure 2A). Interestingly, exogenous auxin application that rapidly reorientates microtubules (Chen et al., 2014) restored the skewed roots and twisting cell morphology of the *sku5* mutant (Figure 2B,

S2A). These observations support that SKU5 might be involved in the auxin signaling pathway. Despite the SKS4 interaction with ABP1, the *sks4* single mutant did not show any obvious phenotype.

Previously, two conditional transgenic *Arabidopsis* ABP1 inactivation lines (referred to as SS12S and SS12K) and one ABP1 antisense line (ABP1AS) have been described. By exposure of the plants to ethanol vapor, the SS12S6 and SS12K9 lines produced antibodies that bound to ABP1 and inactivated it or ABP1AS generated an antisense RNA that downregulated *de novo* protein synthesis (Braun et al., 2008; David et al., 2007). Repression of the ABP1 activity in these three lines exhibited epinastic cotyledons, retardation of leaf growth, and a decrease in cell expansion (Braun et al., 2008). Next, we compared growth of the *sku5* mutant and ABP1 inactivation lines. The *sku5* roots had a delayed gravitropic response (Figure S2B), resembling that of the ABP1 inactivation lines (Chen et al., 2014). Given the synergistic associations of SKS4 and SKU5 with ABP1, we generated the *sku5sks4* double mutants. The *sku5sks4* seedlings exhibited smaller and darker green cotyledons than the normal cotyledons in the *sku5* and *sks4* single mutants (Figure S2C). Moreover, 19% (71/367) of the *sku5sks4* mutant showed a discontinuous vasculature pattern, as described for the ABP1 inactivation lines (Braun et al., 2008) and 8% (20/367) had an additional vein loop (Figure S2D, E).

To circumvent the possible redundancy of the SKS homologs, we designed artificial microRNAs (amiRNAs) against the conserved domain of SKSs under the control of an estradiol-inducible promoter (Curtis and Grossniklaus, 2003; Zuo et al., 2000). In the presence of estradiol, amiRNAs-targeted *SKS* genes were efficiently silenced in independent amiRNA lines (*SKSi*). Six independent lines were analyzed, among which *SKSi-L5* and *SKSi-L7* were selected as the representative lines for the following analysis (Figure S2F). After estradiol induction, these *SKSi* lines exhibited small, epinastic, and downward curling cotyledons (Figure 2C), a significantly reduced stature (Figure 2D, S2G), and greatly decreased (~65%) lateral roots density when compared to the wild type (WT) (Figure 2E, S2H). All these features were reminiscent of the inactivated ABP1 lines (Figure 2C-E).

ABP1-dependent auxin signaling has been shown to modulate the interdigitated growth of *Arabidopsis* leaf epidermal pavement cells (PCs) to form a jig-saw puzzle shape. In accordance, the PC interdigitation was reduced in the ABP1AS line and the *abp1-5* mutant, which is a weak ABP1 mutation line harboring a point mutation in the auxin-binding pocket (Woo et al., 2002; Xu et al., 2010). Therefore, we also examined the PC interdigitation in the

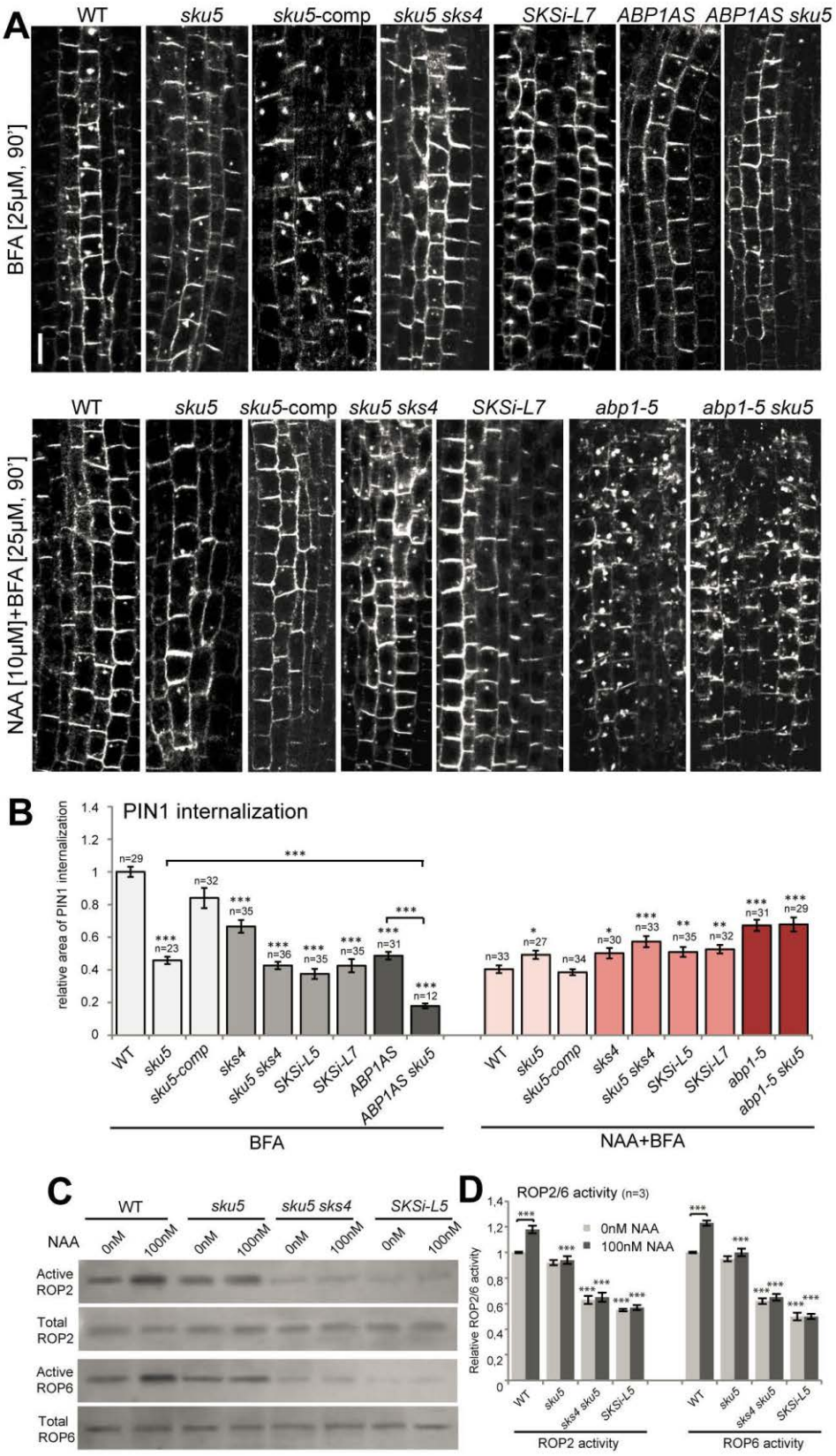

Figure 3. Deficiency of SKU5/SKSs Phenocopy ABP1-inactivation Lines in Regulation of PIN1 Endocytosis. (A-B) WT, *sku5*, *sku5-comp*, *sks4*, *sku5 sks4*, *SKSi-L5*, *SKSi-L7*, ABP1AS and ABP1AS *sku5* were treated by BFA (25 μM); WT, *sku5*, *sku5-comp*, *sks4*, *sku5 sks4*, *SKSi-L5*, *SKSi-L7*, *abp1-5* and *abp1-5 sku5* were treated by NAA (10 μM) plus BFA (25 μM). Relative area of PIN-containing BFA bodies was quantified as the chart showed (B). Scale bar: 5μm.

(C-D) GTP-bound active ROP2 or ROP6 and total ROP2 or ROP6 (GDP and GTP forms) were analyzed in WT, *sku5*, *sku5 sks4* and *SKSi-L5* lines (with 0.5μM estrodial induction), without or with 100nM NAA treatment for 2 min (C). In the chart, the activity of GTP-bound ROP2 and ROP6 in *sku5* mutant was comparable to WT (t test, $p > 0.1$). Auxin increased the activity of GTP-bound ROP2/6 in WT (*** $p < 0.001$) but not in *sku5*, *sku5 sks4* and *SKSi-L5* ($p > 0.1$). T-test was calculated for the comparison between mutants and WT (n=3, *** $p<0.001$) (D).
In all panels, error bars represent s.e.m determined by student's T-test (*$p<0.05$, ** $p<0.01$, *** $p<0.001$).

SKS-deficient and ABP1 inactivation lines. As expected, the SS12S and SS12K lines also exhibited a reduced PC interdigitation, resembling the defects in ABP1AS and *abp1-5* (Figure 2F, G) (Xu et al., 2010). Very similar phenotypes were also found in the *sku5sks4* and *SKSi* lines (Figure 2F, G, S2I). In addition, auxin application increased interdigitation in the WT, but not in the tested *sku5*, *sku5sks4* and *SKSi-L5* lines (Figure S2I), similar to the ABP1 inactivation lines.

Taken together, inactivation of SKU5/SKSs and ABP1 showed largely overlapping defects in auxin-mediated growth and morphology, suggesting that SKU5/SKSs and ABP1 are involved in a common process, consistent with their physical interaction.

ABP1 and SKU5/SKSs Inactivations are Defective in the Auxin Regulation of Endocytosis

One of the well typified fast auxin responses mediated by the ABP1 signaling is the inhibition of clathrin-dependent endocytosis (Paciorek et al., 2005; Robert et al., 2010). The polarly localized auxin PIN-FORMED efflux carriers (PINs) are among the best characterized cargos in clathrin-dependent endocytosis that undergo constitutive endocytic cycling (Dhonukshe et al., 2007; Kitakura et al., 2011; Kleine-Vehn et al., 2011). To visualize the PIN endocytosis, we inhibited their recycling to the plasma membrane (PM) by using the fungal toxin, brefeldin A (BFA), thus inducing the constitutive internalization of PINs in the aggregates termed BFA bodies (Geldner et al., 2001). PIN internalization and clathrin-mediated endocytosis are both inhibited by auxin via the ABP1 signaling; correspondingly, the ABP1 inactivation lines result in endocytosis inhibition (Robert et al., 2010).

The *sku5* mutant, similarly to the ABP1 inactivation lines, showed a decreased BFA-induced PIN1 internalization, which was restored in the complementation line, designated *sku5-comp* (*pSKU5::SKU5:GFP* in the *sku5* mutant background) (Figure 3A, B). Accordingly, the *sku5* mutant, but not the *sku5-comp* line, was less sensitive to auxin effects on the BFA-induced PIN internalization, as manifested by pronounced intracellular PIN accumulations after NAA+BFA treatment (Figure 3A, B). Similar to the *sku5* single mutant, the *sku5sks4* double

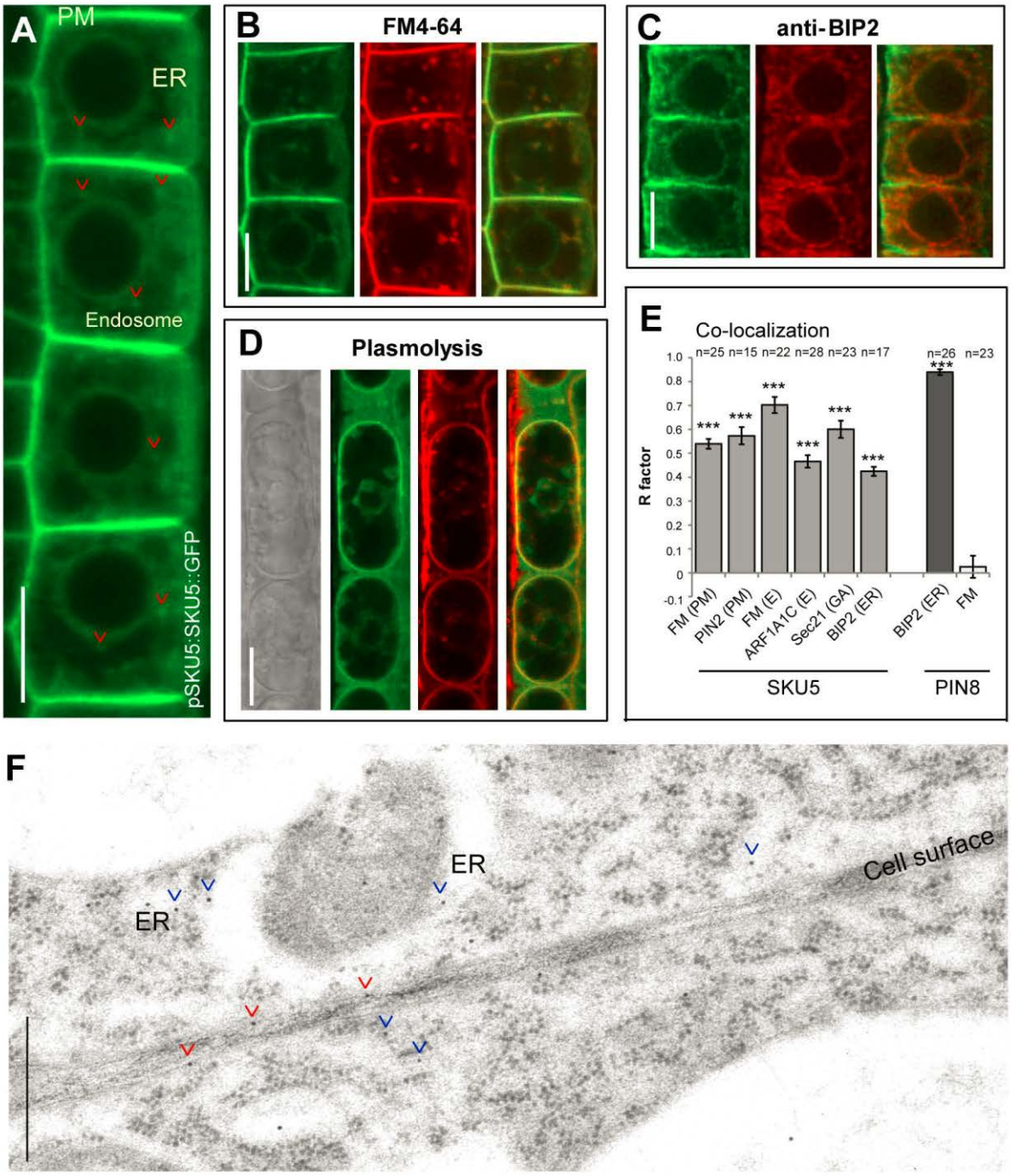

Figure 4. SKU5 Localizes in PM, Endosome, Golgi, ER and Apoplast. (A-E) Live imaging of *pSKU5::SKU5:GFP* showed the localizations of SKU5 in PM, endosome, Golgi, ER and cell surface (A). PM and endosome distributions were shown by FM4-64 staining (red) of *pSKU5::SKU5:GFP* (B). ER distribution was indicated by immunodetection with co-localization of *pSKU5::SKU5:GFP* (α-GFP, green) and ER marker BIP2 (α-BIP2, red) (C). *pSKU5::SKU5:GFP* seedlings were plasmolyzed and showed the apoplastic distribution (green), and the PM was labeled with FM4-64 (red) (D). Co-localization analysis was quantified as co-localization factor R, and co-localization between PIN8 and BIP2, PIN8 and FM4-64 staining was used as the positive and negative control, respectively (the maximum co-localization factor R=1, PM=plasma membrane, E=endosome, GA=Golgi). Student's T-test was calculated in comparison of the negative control (*** $p<0.001$) (E). Error bars represent s.e.m.
(F) Immunogold labeling with α-GFP of high pressure-freeze fixed *pSKU5::SKU5:GFP* root tip showed ER and cell surface localization of SKU5. Cell surface-localized SKU5 was highlighted by red arrowheads and ER-localized SKU5 was marked by blue arrowheads. Scale bar: 5μm (A-D) and 0.2μm (F).

mutants and *SKSi* lines displayed comparable phenotypes as illustrated by the decrease in PIN1-containing BFA bodies and resistance to the auxin inhibitory effect on the PIN1 internalization (Figure 3A, B). Thus, inactivation of SKU5/SKSs led to cellular defects, typical for ABP1 inactivation phenotypes.

Previously, the SS12S, SS12K, and ABP1AS lines have been shown to exhibit a decrease in PIN1 endocytosis, whereas the *abp1-5* mutant line was resistant to auxin with respect to its inhibitory effect on the PIN internalization (Robert et al., 2010). To gain further insight into the genetic relationship between ABP1 and SKU5, we generated the double mutants of ABP1AS*sku5* and *abp1-5sku5*. Phenotypes associated with ABP1AS (Robert et al., 2010) or *sku5* were enhanced in the ABP1AS*sku5* double mutant that further reduced the BFA-induced PIN internalization (Figure 3A, B). In contrast, the *sku5abp1*-5 double mutant conferred insensitivity to auxin, as illustrated by the concomitant NAA and BFA treatment that resulted in a phenotype similar to that of the *abp1-5* single mutant (Figure 3A, B). These observations suggest that SKU5 acts together with ABP1 to regulate the auxin signaling for cellular processes, such as endocytosis.

SKU5/SKSs are Required for the Activation of Processes Downstream of ABP1

Auxin, via the ABP1 signaling, has been show to activate the downstream Rho GTPases, ROP2 and ROP6, that promote the development of interdigitated PCs (Xu et al., 2010). Therefore, we tested whether the SKU5/SKSs function is required for the activity of the ROP2/ROP6 GTPases. The activation of ROP2 and ROP6, as monitored by the presence of their active GTP-bound forms, was gradually impaired in *sku5*, *sks4sku5* and *SKSi-L5* when compared to the WT (Figure 3C, D). Furthermore, the ROP2 and ROP6 activities increased significantly 1.2 to 1.3-fold in the WT after NAA application, whereas this increase was largely abolished in *sku5*, *sks4sku5*, and *SKSi-L5* plants (Figure 3C, D). These results suggest that the SKU5/SKSs function is required for the auxin-dependent activation of ROP2/ROP6 downstream from ABP1.

Given the evidence that ABP1 acts genetically upstream of TIR1/AFBs-AUX/IAA, inactivation of ABP1 leads to the downregulation and degradation of AUX/IAA repressors (Braun et al., 2008; Tromas et al., 2013). To test the involvement of SKSs in the downstream events of the ABP1 signaling, we analyzed the transcript levels of the *AUX/IAA* genes, including *IAA1*, *IAA2*, *IAA5*, *IAA17*, and *IAA19* in the *SKSi* lines. Compared to the WT, the expression of the *IAA* genes dramatically decreased in the *SKSi-L5* and *SKSi-L7* lines (Figure S3). Additionally, ABP1 is crucial for the cell cycle regulation and, thus, the transcript levels

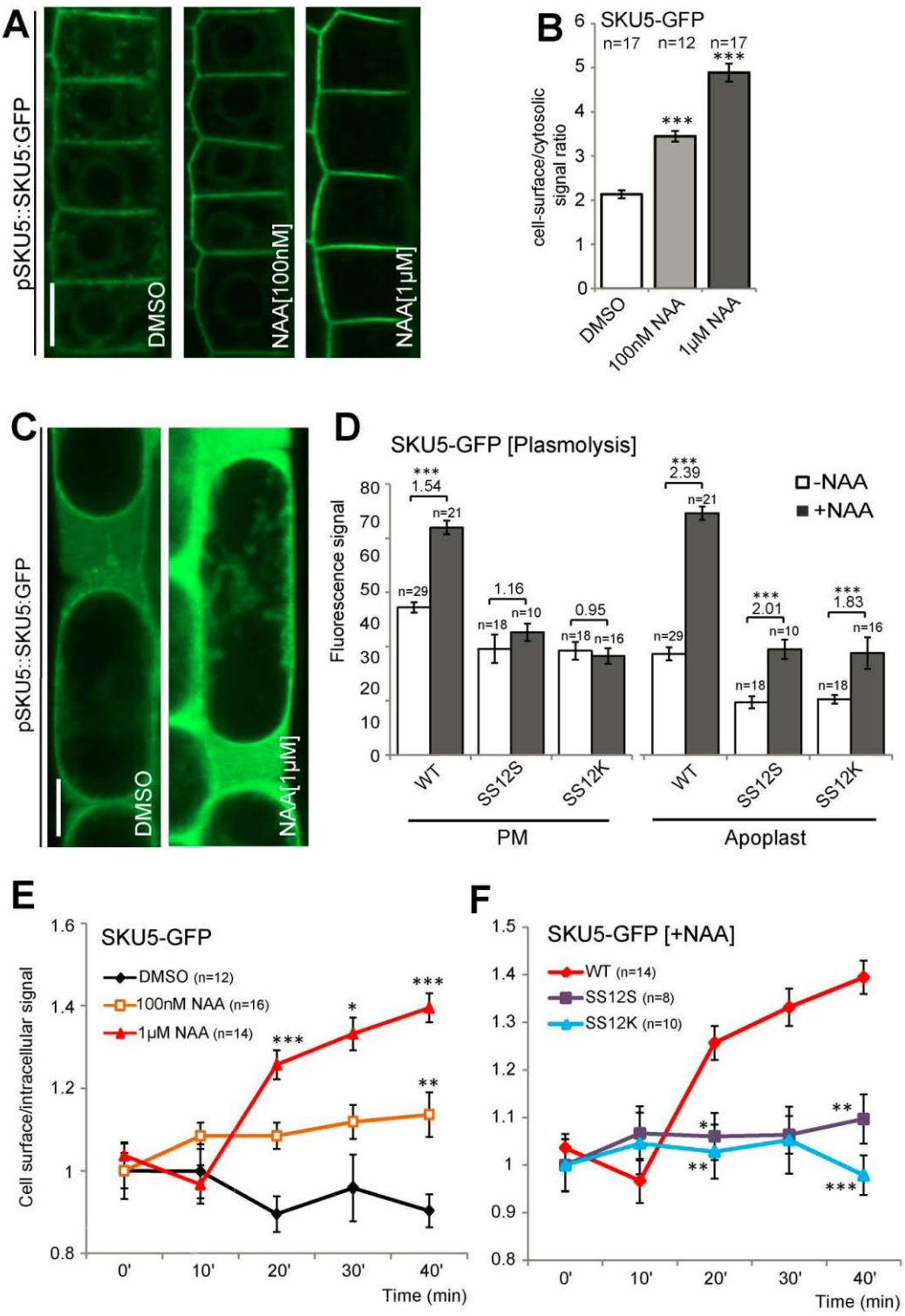

Figure 5. Cell surface Localization of SKU5 is Induced by ABP1-dependent Auxin Signaling. (A-B) Cell surface localization of SKU5 was induced by auxin. *pSKU5::SKU5:GFP* seedlings were treated with 100nM or 1μM NAA for 40min compared with DMSO treatment (A), and the signal on the cell surface was quantified (B). (C-D) The secretion of SKU5 to the apoplast was promoted by auxin, via an ABP1-dependent manner. *pSKU5::SKU5:GFP* expressing in WT, SS12S and SS12K were treated with 1μM NAA for 2h, and then plasmolyzed (Figure S5D). GFP signal at PM and in the apoplast was individually quantified, and the signal ratio between NAA-treated/untreated in each genotype was indicated above the columns (D).
In all panels, error bars are s.e.m. determined by student's T-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) and scale bars are 5μm.

(E) Time-lapse observation (Figure S5A, every 10min, 40min in total) showed that the intracellular SKU5 were secreted to the cell surface after NAA (100nM or 1μM) treatment. The signal on the cell surface in comparison with the intracellular one was measured (DMSO at 0min=1). Student's T-test was calculated for the signal between NAA-treated and DMSO-treated at each time point (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).
(F) Time-lapse observation (Figure S5A-C, every 10min, 40min in total) and quantification showed that auxin effect on SKU5 secretion was attenuated in SS12S and SS12K lines (expressing *pSKU5::SKU5:GFP*) after 1μM NAA treatment. Student's T-test was calculated for the signal between WT and SS12S/K at each time point (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

of cell cycle-related *CYCD* genes, including *CYCD3.1* and *CYCD6.1* are downregulated in ABP1 inactivation line (David et al., 2007; Tromas et al., 2009). Consistently, the *SKSi* lines showed a 20%-30% decreased expression of *CYCD3.1* and *CYCD6.1* (Figure S3), confirming that the SKU5/SKS function is required for the processes downstream of the ABP1 action.

SKU5 Localizes Along the Secretory Pathway, Including the ER and Apoplast

Next, we analyzed the subcellular distribution of SKU5 in relation to the ABP1 localization. Previously, SKU5 had been localized at the cell surface (Sedbrook et al., 2002). To obtain a more detailed insight into the SKU5 distribution, we performed live imaging combined with immunodetection in transgenic lines expressing the functional *pSKU5::SKU5:GFP* construct (Sedbrook et al., 2002). The SKU5-GFP signal was associated mostly with the cell surface and partially with the intracellular compartments, including perinuclear fluorescence, indicative of the ER localization (Figure 4A). High colocalization with the PM staining by a short incubation with FM4-64 (colocalization factor R=0.54) (Jelinkova et al., 2010; Ueda et al., 2001) or the PM marker PIN2 (R=0.57) (Wisniewska et al., 2006) with the SKU5-GFP signal (Figure 4B, 4E, S4) confirmed the presence of SKU5 at the PM. Notably, after plasmolysis of the root cells that detaches the PM from the cell wall, the SKU5-GFP signal was clearly visible in the apoplast (Figure 4D), indicating that SKU5, like ABP1, is also secreted to the extracellular space. Additionally, the intracellular SKU5-GFP signal prominently colocalized with the ER marker, the lumenal-binding protein 2 (BIP2) (R=0.42) (Muench et al., 1997), and, to some extent, with the internalized FM4-64 signal (R=0.70), *trans*-Golgi network/early endosome (TGN/EE) marker ADP-RIBOSYLATION FACTOR 1 (ARF1A1C) (R=0.47) (Xu and Scheres, 2005), and a Golgi marker γ-subunit of COP (SEC21) (R=0.60) (Movafeghi et al., 1999) (Figure 4B-E, S4), confirming that, besides the ER and cell surface, SKU5 resides also at the TGN/EE and Golgi apparatus, along the secretory pathway.

Transmission electron microscopy (TEM) on immunogold-labeled thin sections of *pSKU5::SKU5:GFP* root tips revealed, consistently with the confocal microscopy results, that

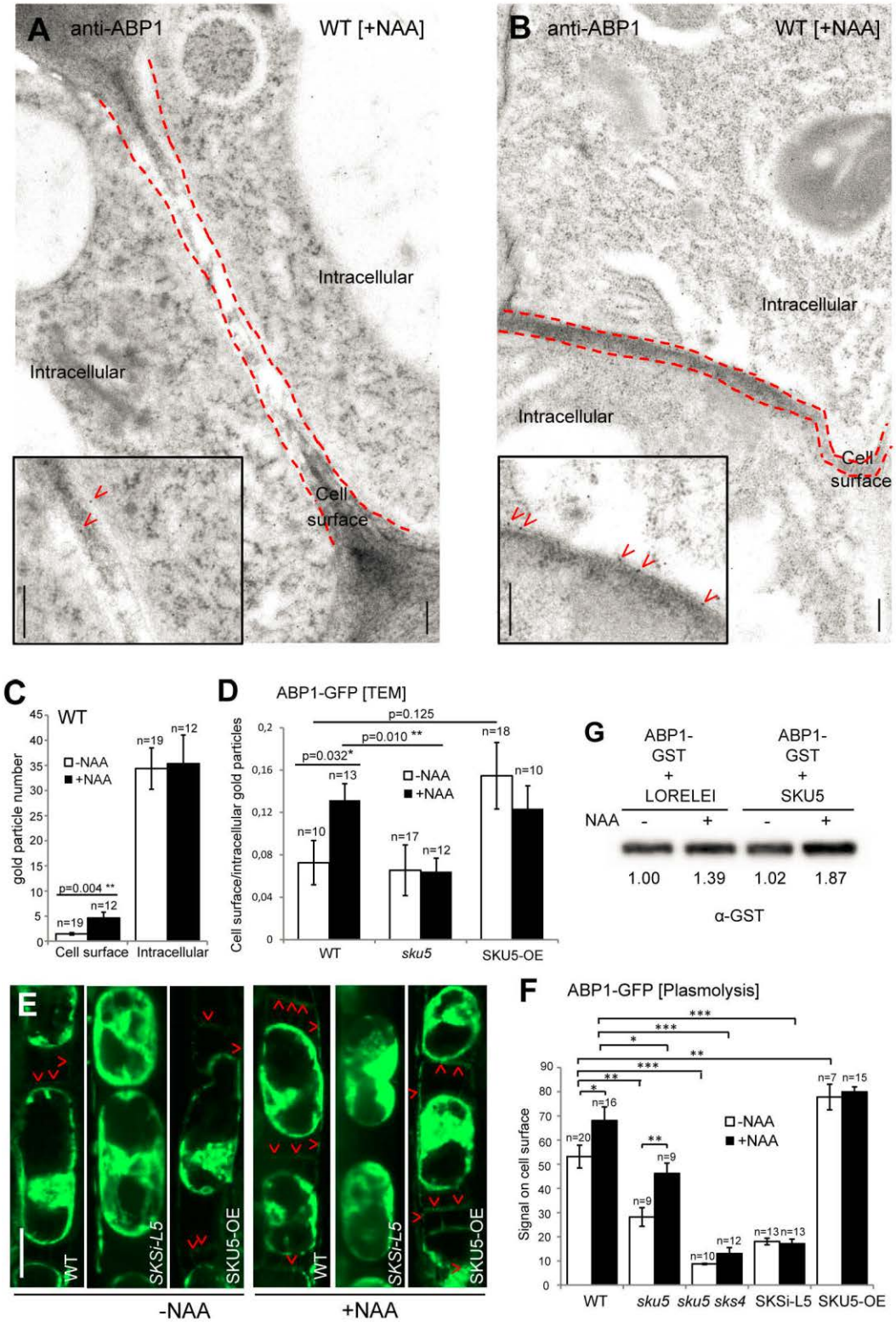

Figure 6. SKU5 Assists ABP1 Secretion to Cell Surface. (A-C) Immunogold labeling with α-ABP1 of untreated or NAA (1μM, 3h)-treated WT seedlings showed more ABP1-gold labeling on the cell surface after NAA treatment (A-B). The gold particles number on the cell surface and in the intracellular space was quantified respectively (C). The boundary between cell surface and intracellular part was marked by red dot lines. Inserts show the enlarged immunogold labeling areas and red arrows head gold particles.
(D) GFP-gold labeled ABP1 proteins were observed and quantified in the root tips of WT, *sku5*, *SKU5-OE* (all expressing *35S::ABP1:GFP*). GFP-labeled ABP1 gold particles on the cell surface compared with the intracellular ones were measured.

(E-F) ABP1 secretion to the cell surface was enhanced in *SKU5-OE* but decreased in *sku5 sks4* mutant and *SKSi-L5* line (all expressing *35S::ABP1:GFP*). After plasmolysis, cell surface-localized ABP1 was visualized in untreated or NAA (1μM, 3h)-treated WT, *sku5 sks4* mutant, *SKSi* and *SKU5-OE* lines (E). Cell surface- localized ABP1 was highlighted by red arrows. The signal of ABP1 on the cell surface was measured in F.
(G) ABP1 protein was detectable in the *Arabidopsis* cell culture medium against GST antibody. Overexpressed LORELEI was used as a control compared with overexpressed SKU5 condition. Each sample was manipulated identically (n=2) and ABP1 protein amount was measured as indicated with numbers.
In all panels, error bars are s.e.m. determined by student's T-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Scale bar: 0.2μm (A) and 5μm (E).

the SKU5-GFP signals were predominantly localized at the ER and on the cell surface (Figure 4F). Thus, SKU5 shows a largely overlapping, but somewhat, broader pattern of the subcellular distribution than ABP1.

SKU5 Secretion to the Cell Surface Requires the ABP1 Auxin Signaling

Given the positive effect of auxin on the SKU5 and ABP1 association, we tested the auxin effect on the SKU5 localization in *pSKU5::SKU5:GFP* seedlings by applying different NAA concentrations (100 nM and 1 μM). Auxin treatment increased the cell surface of the SKU5-GFP signal with a concomitant decrease in the intracellular signal (Figure 5A, B). To distinguish between the PM and the apoplast/cell wall signal, we carried out plasmolysis experiments. A high SKU5-GFP signal was detected both at the PM and the apoplast, especially in the auxin-treated *pSKU5::SKU5:GFP* seedlings (Figure 5C, D). To track the kinetics of the auxin effect on the SKU5 localization, we performed time-lapse imaging on a single row of *pSKU5::SKU5:GFP* root epidermal cells treated with 100 nM or 1 μM NAA. Visualization every 10 min revealed a shift in the SKU5-GFP from the interior to the cell surface (Figure 5E, S5A). These data suggest that auxin promotes SKU5 secretion from the cytoplasm to the cell surface.

To investigate whether the ABP1 function is required for the auxin effect on the SKU5 secretion, we introduced *pSKU5::SKU5:GFP* into the ABP1 inactivation lines. The kinetics of the auxin effect on the SKU5 localization in the ABP1 inactivation lines revealed that the auxin-induced SKU5 secretion was largely impaired in the ABP1 inactivation lines (Figure 5F, S5B, S5C). Consistently, also the plasmolysis experiment showed that the auxin-mediated promotion of the SKU5 signal in the apoplast was compromised after inactivation of the ABP1 function (Figure 5D, S5D).These observations demonstrate that auxin, via ABP1, play a role in promoting the SKU5 secretion to the cell surface.

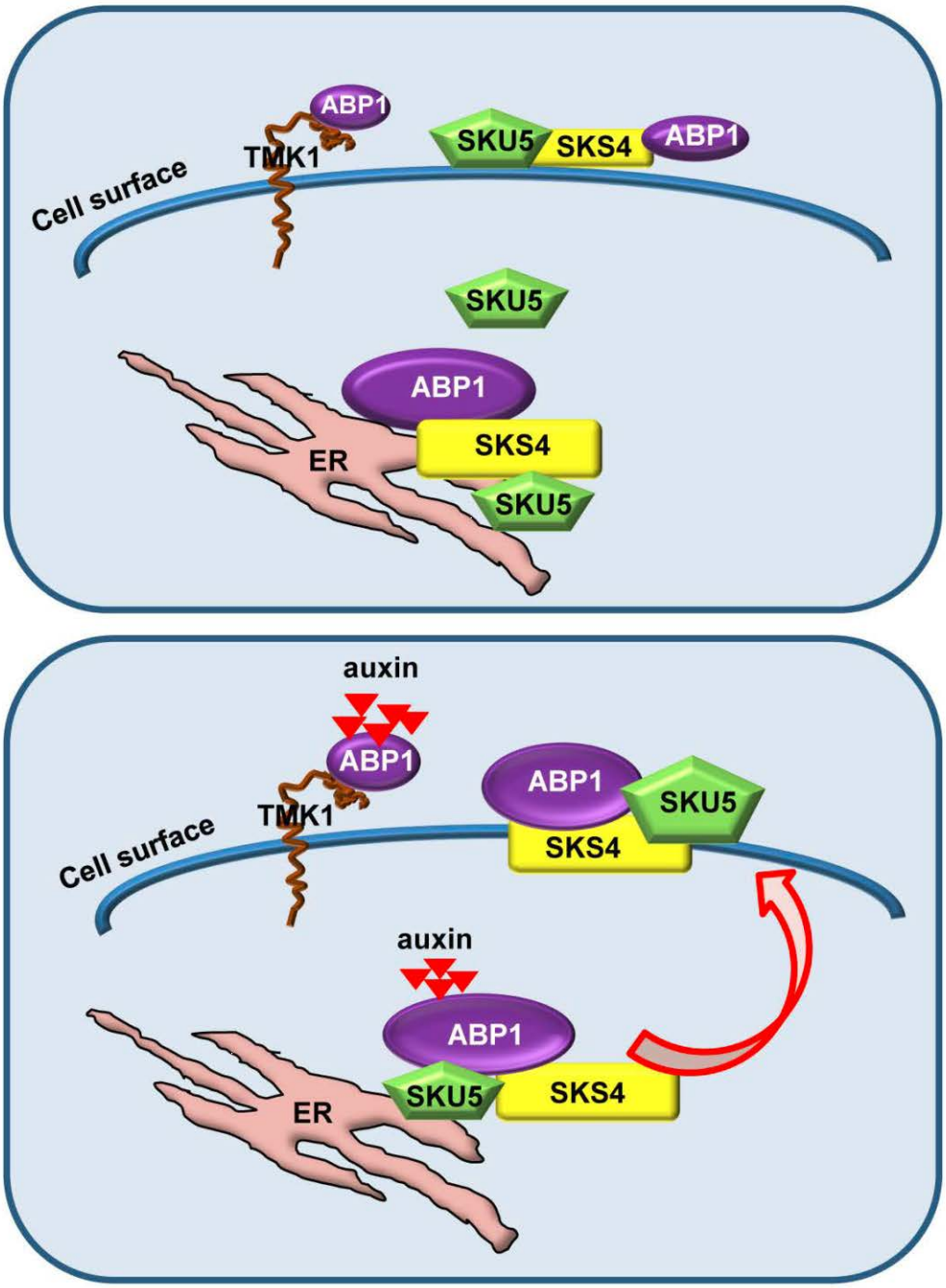

Figure 7. Model: Auxin Mediates Secretion of ABP1 Through SKU5. In the absence of auxin, most ABP1 protein is distributed within ER, and a small proportion locates on the cell surface and associates with TMK1. SKS4 associates but SKU5 disassociates with ABP1. Upon auxin stimulation, ABP1-TMK1 transduces auxin signal from the cell surface to the cytosolic, either (A) SKU5 interacts with ABP1 at ER, thereby promoting the complex of ABP1-SKU5-SKS4 targeting to the cell surface or (B) SKU5 and ABP1 are individually targeted to the cell surface, and SKU5 binds with ABP1-SKS4 on the cell surface.

SKU5 Assists the Auxin-mediated ABP1 Secretion to the Cell Surface

How the portion of the ER-retained ABP1 arrives to the cell surface where it is supposed to act is a lasting enigma (Jones and Herman, 1993; Timpte, 2001) that has recently been

reinforced by the identification of the PM-localized ABP1-interacting TMK receptor-like kinases (Xu et al., 2014). The localization of both ABP1 and SKU5 to a large extent at the ER hinted at an attractive scenario in which ABP1 is secreted with the assistance of SKU5 to the cell surface in an auxin-dependent manner.

To assess whether auxin influences the ABP1 incidence in the apoplast, we performed TEM with immunogold detection of the endogenous ABP1 (with an α-ABP1 antibody) in the WT and of ABP1-GFP (with an α-GFP antibody) in *35S::ABP1:GFP* and *RPS5A::ABP1:GFP*. The immunogold-labeled ABP1 signals were found predominantly at the ER with a minor fraction at the cell surface (Figure 6A, S6A). The number of gold particles of ABP1 on the cell surface was significantly increased (Figure 6A-C, S6A-C) and the quantified ratio of cell surface/intracellular ABP1 signal revealed a 1.8-fold increase in the cell surface-localized ABP1 after NAA treatment (Figure 6D). To provide unbiased, quantitative measures of the gold particle numbers, we developed an image analysis tool that automatically marked the detected particles from the simulated TEM pictures as 'heat maps' (Figure S6D, E). Increased fractions of gold-labeled ABP1 on the cell surface in the auxin-treated samples (Figure S6F) consistently revealed the promoting effect of auxin on the ABP1 secretion. After plasmolysis by separating cell wall and PM, 7-30% cells showed an apoplast-localized ABP1-GFP signal (Figure 6E). Correspondingly, the ABP1-GFP fluorescence in the apoplast increased 1.28-fold after NAA application (Figure 6F). These observations indicate that auxin promotes the secretion of the ABP1 proteins.

To test the SKU5 contribution in this process, we introduced the *sku5* mutant, *sku5sks4* double mutant, *SKSi* line, and *SKU5* overexpression line (*35S::SKU5, SKU5-OE*) into the *35S::ABP1:GFP* line. Due to the unexpected silencing effect of the double 35S promoters (Daxinger et al., 2008), we confirmed two independent overexpressed *SKU5* lines out of 23 lines in the *35S::ABP1:GFP* background (Figure S7A). Live imaging following plasmolysis showed that the ABP1 signals on the cell surface were lower in *sku5, sku5sks4*, and *SKSi* lines, but higher in *SKU5-OE* than those of the WT (Figure 6E, F). Subsequent auxin treatments revealed that the auxin-induced increase in the apoplastic TEM-ABP1 signals was attenuated in the *sku5* mutant (Figure 6D, S7B-E). Consistently, NAA-treated plasmolyzed *sku5, sks4sku5,* and *SKSi* seedlings were less sensitive to the auxin-induced ABP1 apoplastic appearance (Figure 6E, F).

Using another approach, we expressed the ABP1 protein with a GST tag in *Arabidopsis* suspension culture cells under the control of a CaMV 35S promotor. We detected the secreted ABP1-GST in the culture media by western blot and compared cultures overexpressing *SKU5*

with cultures overproducing an unrelated GPI-anchored PM protein LORELEI (Figure 6G). A slightly higher amount of ABP1-GST (1.39 fold) was found in the NAA-containing medium than in the nontreated sample and the NAA-dependent increase in the ABP1-GST secretion was more than double in the cultures overexpressing *SKU5* (1.87 fold) (Figure 6G). Taken together, the above results show that auxin increases the ABP1 secretion to the apoplast and that this process is supported by SKU5 and its homologs.

DISCUSSION

Extracellular signaling molecules, such as phytohormones, typically bind to receptors at the cell surface and trigger signal transduction from the cell periphery to the interior. Most of the fast ABP1-related auxin responses occur on the cell surface, such as membrane hyperpolarization (Cleland et al., 1977), cell expansion (Bauly et al., 2000), endocytosis inhibition (Robert et al., 2010), and ROP activation (Xu et al., 2010). Therefore, the amount of ABP1 on the cell surface, where auxin binds ABP1 with high affinity, determines the efficiency of the following auxin signal transduction events.

Two Cell-surface Auxin Perception Systems: TMKs Versus SKSs

The broad variety of auxin effects requires multiple auxin perception components that coordinate the comprehensive auxin signaling. Besides the auxin events governed by the TIR1/AFB receptor in the nucleus, the auxin signal at the cell periphery apparently requires an ABP1-mediated cell surface perception system. Therefore, the interacting partners of ABP1 that assist ABP1 to attach to the cell surface deserve special attention. Recently, TMK1 has been identified as a transmembrane ABP1 co-receptor, coupling the extracellular auxin signal and its perception by ABP1 to the intracellular space (Xu et al., 2014). Another puzzling question that needs to be addressed is how ABP1 escapes from the ER to the extracellular matrix. The SKU5/SKSs that belong to GPI-anchored proteins are embedded in the lipid bilayer of the membrane and take part in dynamic trafficking between the cell surface and the endomembrane system, harboring the potential ‘ABP1 adaptor’ feature (Lakhan et al., 2009). Here, we showed that SKU5/SKS4 associates with ABP1 and promotes the transportation to the extracellular space. These two discoveries imply that auxin perception on the cell surface is realized by a unique two-component machinery consisting of

a PM-bound transmembrane receptor kinase (TMK) and an ER-extracellular-anchored adaptor (SKS).

The shared signaling of ABP1-TMKs and ABP1-SKSs to activate the downstream ROPs hints at an unknown collaboration of this two-component auxin perception system. Despite the deficiency of the *TMK* and *SKSs* genes, both exhibit the defects of ROP2/ROP6-mediated pavement cell interdigitation, in contrast with the pleiotropic phenotypes of the *tmk* quadruple mutant (Xu et al., 2014) that after *SKSs* inactivation are even closer to the ABP1-inactivation lines, indicating the prominent role of SKSs in the ABP1 signaling.

In comparison with the direct interaction between ABP1 and TMK1 (Xu et al., 2014), SKU5 alone is not sufficient to accomplish the ABP1 association and requires the assistance of its homolog SKS4, implying an essential role for SKS4 and probably all SKSs. Interestingly, despite the constitutive binding of ABP1 by SKS4, the *sks4* loss-of-function mutant does not exhibit any obvious phenotypes. The reason might be the functional redundancy of the SKSs gene family that is supported by severe phenotypes of the *SKSi* lines and also by the dimerization between the SKS homologs that probably determines their association with ABP1.

Auxin is necessary for activation and proper complex formation in these two auxin perception systems. In comparison to the rapid auxin-dependent ABP1-TMK1 binding (2 min), the auxin-induced ABP1-SKS4-SKU5 association is much slower (60 min) (Xu et al., 2014). This difference is probably due to the interval required for the secretory pathway trafficking of the ABP1-SKS4-SKU5 complex to the cell. Thus, we propose a scenario in which, after the auxin stimulus, the small amount of cell surface-located ABP1 binds primarily to TMK1, transmitting an extracellular auxin signal into the cytosol that triggers the SKU5 binding with the ER-resident ABP1, further stimulating the SKU5/SKS-dependent ABP1 secretion into the extracellular space. Based on this hypothesis, *TMK1* might act upstream of *SKU5/SKSs* in the ABP1 signaling pathway. Thus, the comprehensive relationship between SKSs and TMKs deserves additional investigation.

Two ABP1 Docking Spaces: ER Versus Cell Surface

Whereas ABP1 predominantly accumulates within the ER, the functional role of this accumulation in the auxin signaling remains unknown. Recently, the pH in the ER lumen of plant cells has been measured as pH 7.5 (Martiniere et al., 2013), similar to a previously reported pH 7.0 (Tian et al., 1995). At neutral pH, ABP1 exhibits a very low affinity for auxin, ruling out the possibility that auxin binds ABP1 at the ER (Tian et al., 1995).

Mutations of the ER retention signal of ABP1 are insufficient to trigger ABP1 accumulation at the PM (Bauly et al., 2000). As less than 15% of ABP1 ever escapes from the ER (Henderson et al., 1997), this low level suggests that an association with other partners may be necessary to assist in the ABP1 targeting to the cell surface. The SKU5/SKSs interactors undergo vesicular trafficking that originates at the ER and transits through the Golgi apparatus prior to reaching the PM (Foresti and Denecke, 2008; Jürgens, 2004), coinciding with the presumed trafficking routes of ABP1. Therefore, we hypothesize thatthe ER-resident ABP1 may represent an inactive pool of ABP1 awaiting vesicular transport to the extracellular space, mediated by the auxin-dependent activation of the SKSs interactors. Additionally, because ABP1 is also known to interact with the E3 ubiquitin ligase AtRMA2 that resides at the ER and functions as a regulator of the ABP1 degradation (Son et al., 2010), we suppose that the ER lumen serves as an ABP1 storage reservoir to supervise the proper ABP1 action.

Auxin Regulates the SKU5/SKS-dependent ABP1 Secretion

Clathrin-mediated endocytosis is recognized to be inhibited by auxin via ABP1 signaling (Dhonukshe et al., 2007; Robert et al., 2010) and, accordingly, SKU5 internalization is induced after BFA treatments (data not shown), but inhibited by auxin (Figure S6A). Interestingly, BFA also inhibited ABP1 secretion to the culture medium (Jones and Herman, 1993) that might be rescued by auxin. However, the only reported effect of auxin on the ABP1 localization thus far is the promotion of the ABP1 clustering on the cell surface of maize protoplasts (Diekmann et al., 1995). Our results confirm the essential role of auxin in the stimulation of the ABP1 secretion. Based on these findings, we propose the following model: upon auxin starvation, SKS4 constitutively binds to ABP1, but SKU5 dissociates from it; upon auxin stimulation, there are two possibilities: (i) a small ABP1 population firstly couples with TMK1 to transport the auxin signal into the cytoplasm, whereafter SKU5 is recruited by ABP1-SKS4 at the ER, further transporting the ABP1-SKS4-SKU5 complex to the cell surface; (ii) the auxin signal is transduced via TMK1-ABP1 to the cytoplasm, whereafter SKU5 and ABP1 are individually targeted to the cell surface and SKU5 binds with ABP1-SKS4 on the cell surface (Figure 7). Therefore, we assume that the presence of ABP1 on the cell surface might be tightly controlled by the auxin-dependent SKU5 protein that shuttles between cell surface and endomembrane system, in turn, coordinating the auxin perception. Interestingly, our findings identify an apparent auxin-dependent feedback loop of the ABP1-dependent auxin-induced SKU5 secretion and SKU5/SKSs-involved auxin-induced

ABP1 secretion. Both secretions of SKU5 and ABP1 depend on auxin and reciprocally require each other.

Collectively, the discovery of the SKU5/SKS-involved ABP1 secretion provides novel evidence for a long-term argument on how ABP1 targets to the cell surface. Future work on the elucidation of the SKU5/SKSs-based signaling network promises to shed new light on our understanding of the mysterious ABP1 action that impinges on plant developmental processes.

EXPERIMENTAL PROCEDURES

Plant Growth and Phenotype Analysis

Seeds of *Arabidopsis thaliana* were sown on 0.8% agar containing 1/2 Murashige and Skoog media at 22° under 16h light/ 8h dark photoperiod. T-DNA insertion mutant of *sks4* (Salk_080840) was provided from the *Arabidopsis* Information Resource. *sku5* mutant and complementation line (*pSKU5::SKU5:GFP* in *sku5* mutant background, *sku5-comp*) were provided from Sedbrook (Sedbrook et al., 2002). Ethanol induction of conditional lines for ABP1 (SS12S, SS12K and ABP1AS) was performed by exposure of the seedlings to ethanol vapor for various times as indicated for the different assays: the seedlings were exposed to 5% 500µL ethanol for 48 h before cell biological analysis, or they were continuously exposed to 5% ethanol vapor for phenotype observation; when ABP1-inactivation lines were grown in the soil, plants were continuously exposed to 95% ethanol vapor, as described previously (Braun et al., 2008).

For estradiol induction of *SKSi* silenced lines, three-day-old seedlings were transferred to 2µM 17-β-estradiol containing 1/2 MS solid medium for 48h prior cell biological observation, or the germinated seeds were continuously grown on 2µM 17-β-estradiol containing medium (except the other induction concentration as particularly indicated in the text) for the phenotype observation. Once the seedlings were grown in the soil, adult plants were continuously sprayed by 2µM 17-β-estradiol (once a day). Non-induced seedlings were used as controls. Six independent lines were obtained by confirmation with qRT-PCR and two lines (*SKSi-L5* and *SKSi-L7*) were used for each experiment.

For the venation observation, the cotyledons were gradually cleared in 100%- 90%- 70%- 50% ethanol to remove chlorophyll and fixed with a chloral hydrate solution (containing glycerol, chloral hydrate, and water in a ratio of 1:8:2).

Chemical Treatment

BFA (Sigma-Aldrich) and NAA (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO), IAA (Sigma-Aldrich) and 17-β-estradiol (Sigma-Aldrich) were dissolved in ethanol, and FM4-64 (Sigma-Aldrich) was dissolved in distilled water. For BFA treatment, four-day-old seedlings were immersed in 25μM BFA-contained liquid 1/2MS for 90min. For combined treatments with BFA and NAA, four-day-old seedlings were immersed in 10 μM NAA-contained liquid 1/2MS for 30min pretreatment, then transferred to the same medium plus 25μM BFA for another 90min.

BiFc and Agrobacterium Infiltration in *Nicotiana benthamiana* Leave

The gateway vectors used for BiFc and tobacco leave infiltration were described previously in details (Boruc et al., 2010). Briefly, the expressed constructs were transferred into the *Agrobacterium tumefaciens* strain GV3101. The obtained *Agrobacterium* was grown in YEB medium with appropriate antibiotics, harvested by centrifugation at 4000rpm for 5min, and then resuspended in the infiltration buffer (10 mM $MgCl_2$, 10 mM MES pH 5.6, 100 mM acetosyringone) for 2h sharking (Wydro et al., 2006). Before infiltration, the final OD600 was adjusted to 1.0 of each Agrobacterium, and mixed with p19-silencing suppressor in 1:1 ratio (Voinnet et al., 2003). The infiltrated plant was incubated under normal growing conditions and analyzed three to five days after infiltration.

Immunodetection

Immunofluorescence in *Arabidopsis* roots was analyzed as previously described (Sauer et al., 2006). The anti-PIN1 (1:1000) (Robert et al., 2010) was used as the first antibodies, and anti-rabbit-Cy3 (Sigma-Aldrich, 1:600) was used as the secondary antibody.

Confocal Microscopy Observation

Seedlings were mounted on 0.8% agar 1/2MS chamber slides or liquid 1/2MS glass slides containing the indicated concentration of auxin or DMSO, then were immediately imaged. Images were taken by Zeiss LSM 710 or vertical Zeiss LSM 700 confocal microscopes. The settings of excitation and detection were: GFP: 488 nm, 505-550 nm; Cy3: 543 nm, 560 nm; FM4-64:543 nm, 650nm; RFP: 587nm, 610nm; PI: 535nm, 610nm. All the images in a single experiment were captured with the same setting. Quantification of the fluorescence signal was performed by Image J software.

CO-Immunoprecipitation

Protein extracts were prepared from *N. benthamiana* leaves tissue which co-expressed the combination of constructs as described previously (Figure 1D-G; Figure S1C, D) by infiltration-based transient transformation. For different concentration of NAA/IAA treatment, the infiltrated *N. benthamiana* leaves were punched by the hole puncher into same area of pieces before protein extraction, immersing in NAA/IAA contained liquid 1/2MS medium by continuously vacuum pumping for 1h or 3h (according to individual experiment, DMSO-treated infiltrated leaves were used as the control). Then, 1 g of leave tissue was grinded in liquid nitrogen and dissolved in 2 mL of IP buffer (25mM Tris, pH 7.5; 150 mM NaCl; 1mM EDTA; 1% Triton X-100; 1×protease inhibitor cocktail, 1×phosphatase inhibitor). Extracts were centrifuged at 18,000g for 30min at 4°C. The supernatant was incubated overnight with GFP-Trap (Chromotek) or RFP-Trap (Chromotek) at 4°C. IP buffer was used for all washes. Samples were analyzed by SDS-PAGE and immunoblotting with individual first antibody: SKU5 antibody (1:2000) which is synthesized in Eurogenetec company according to the previous description (Sedbrook et al., 2002), ABP1 antibody (1:2000) (Xu et al., 2014), GFP antibody (Santa Cruz Biotech) (1:2000), RFP antibody (MBL) (1:2000) and the secondary antibodies: ECL anti-Rabbit IgG (GE healthcare) (1:10000) and ECL anti-Mouse IgG (GE healthcare) (1:10000) .

ACKNOWLEDGEMENTS

We thank John C. Sedbrook for providing the seeds of *sku5* mutant, *pSKU5::SKU5:GFP* (in *sku5* background) line and *pSKU5::SKU5:GFP* construct, Daniel Van Damme for providing CD3-959 ER-mcherry marker, Qiang Zhu for preparing the manuscript, Kestrel Rogers for correcting grammatical errors, and Baojun Yang, Stéphanie Robert, Lindy Abas, Matyáš Fendrych and Milada Čovanová for helpful discussion of the manuscript.

SUPPLEMENTARY FIGURES

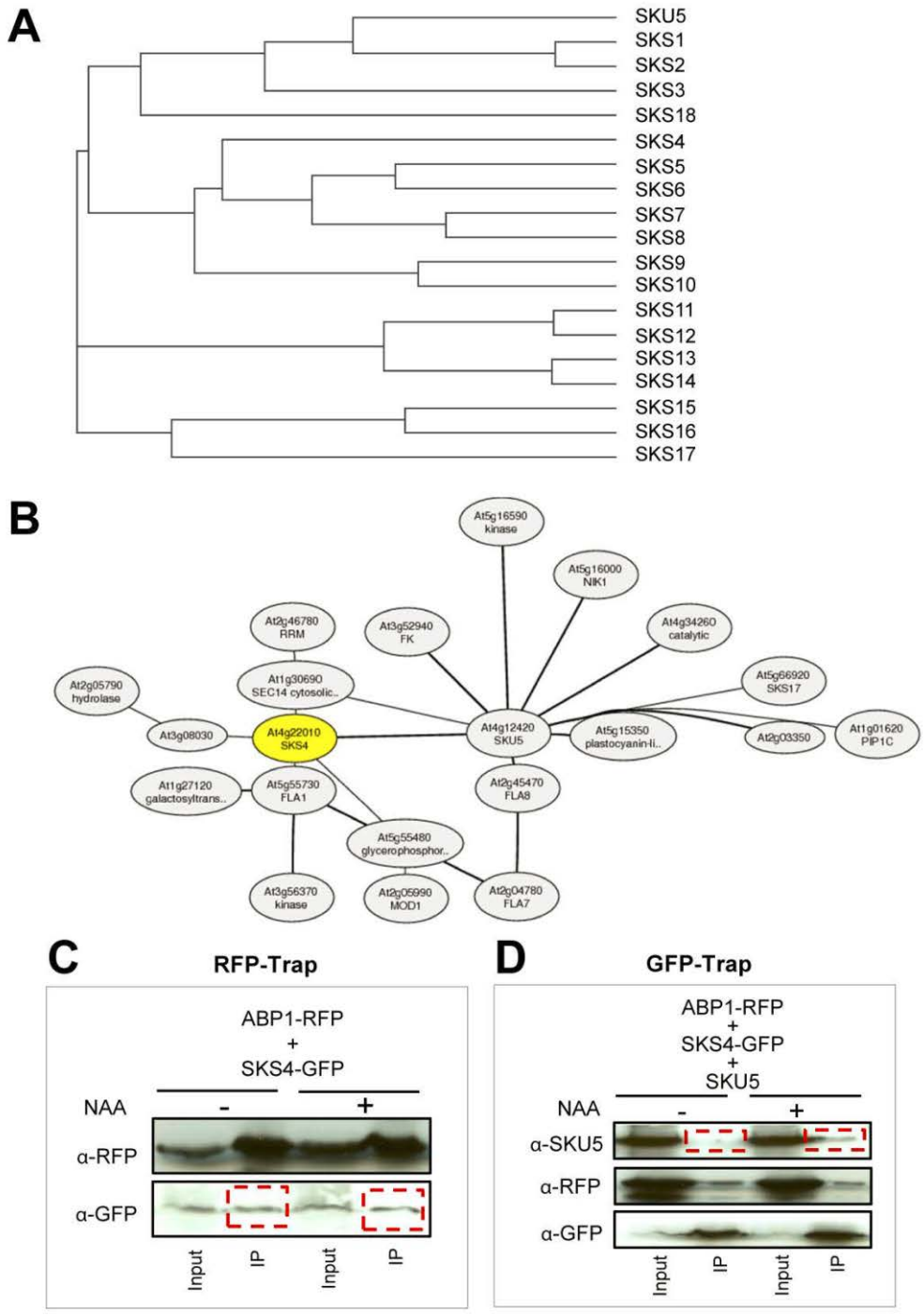

Figure S1. ABP1 Interacts with SKU5 in an Auxin-dependent Manner. (A) Phylogenetic tree analysis of SKS family. (B) Microarray-based co-expression analysis (ATTED-II). Yellow color highlighted the high co-expressing gene *SKS4* with *SKU5*. (C-D) CO-IP confirmation of the interactions between ABP1 and SKS4 (C), ABP1 and SKU5 (D) from infiltrated *N. benthamiana* protein extracts. Without (-NAA) or with NAA application (+NAA, 1μM, 1h), ABP1 was captured by RFP-Trap beads, and total extracted protein (input) and IP-SKS4 proteins were analyzed by GFP antibody (α-GFP) (C). The interaction between ABP1 and SKU5 in the presence of overexpressed SKS4 was performed in the similar way (D). Fig. S1C corresponds to Fig. 1E, Fig. S1D corresponds to Fig. 1F.

Figure S2. Deficiency of SKU5/SKSs Phenocopies ABP1-inactivation Lines. (A) Skewed roots of *sku5* mutant were partially rescued by applying exogenous NAA (100nM, 500nM). Corresponding to Figure 2B. (B) The deviated angles of WT and *sku5* were measured after 2 h, 4 h, 6 h, and 8 h of 135° reorientation. (C-E) Cotyledon morphology and vasculature structure of WT, *sku5*, *sks4* and *sku5 sks4* were shown. The defected vasculatures of *sku5 sks4* were highlighted by arrowheads (D) and quantified as the table (E). (F) Transcript level of 19 *SKS* genes was detected by qRT-PCR in *SKSi-L5* and *SKSi-L7* lines. 2μM 17-β-estradiol induction was performed for 48h prior RNA extraction (individual non-induced lines were used as the controls). The transcript level of individual *SKS* in corresponding non-induced line was standardized as "1", and the relative transcript level is quantified based on individual non-induced lines (n=3). Grey columns highlighted the silenced *SKS* genes. Student's T-test was calculated for the comparison between estradiol-induced and non-induced

transcript levels of each gene (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). (G) Sprayed by 2μM 17-β-estradiol for 30 days, *SKSi-L5* and *SKSi-L7* significantly reduced stature. (H) Grown on 2μM 17-β-estradiol containing medium for 7 days, *SKSi-L5* and *SKSi-L7* had less later roots. Corresponding to Fig. 2E. (I) Auxin activation of pavement cell interdigitation requires SKSs. Compared with WT, *sku5 sks4* double mutant and *SKSi-L5* line (0.5μM 17-β-estradiol induction) displayed less PC interdigitation. Student's T-test was calculated for the comparison between mutants and WT (n = 200 cells). Auxin (20nM) increased the mean density of lobes in WT (*** $p < 0.001$) but not in *sku5, sku5 sks4* and *SKSi-L5* ($p > 0.1$). In all panels, error bars are s.e.m. determined by student's T-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Scale bar: 2 mm (C), 0.5 mm (D), 50 μm (H).

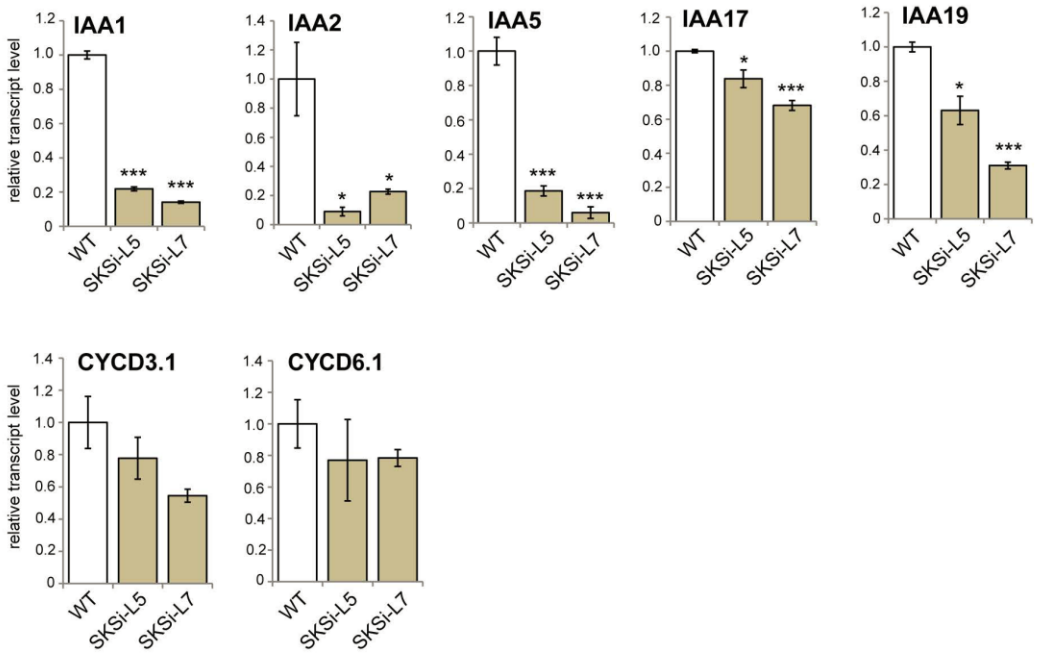

Figure S3. Transcript Level of SKSs, IAAs and CYCDs in *SKSi* Lines. Transcript level of *IAA1*, *IAA2*, *IAA5*, *IAA17*, *IAA19*, *CYCD3.1* and *CYCD6.1* was detected in WT and *SKSi-L5*, *SKSi-L7*. The transcript level is quantified based on individual non-induced lines, and the relative transcript level of WT was standardized as "1" (n=3). Student's T-test was calculated for the comparison between WT and *SKSi-L5*, *SKSi-L7* lines (* $p<0.05$, *** $p<0.001$). In all panels, error bars represent s.e.m..

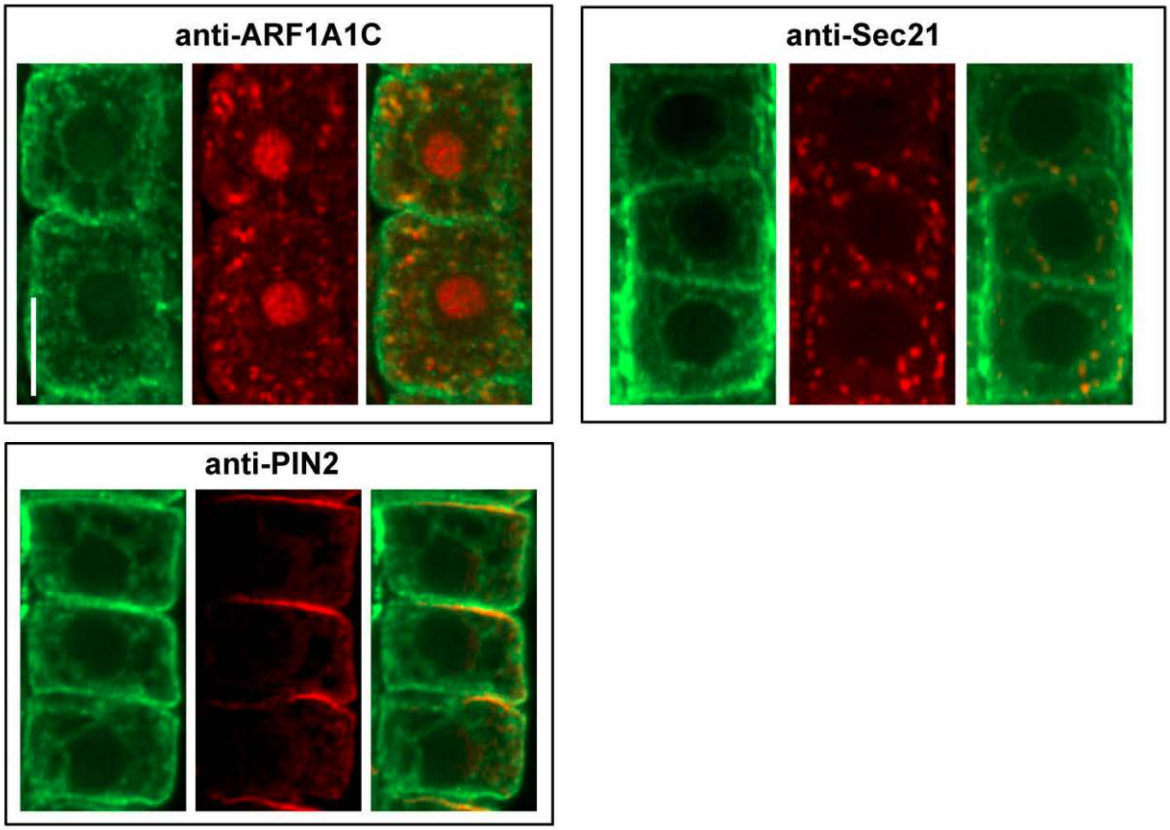

Figure S4. SKU5 Localizes in PM, Endosome, Golgi. The localization of SKU5 in endosome, Golgi, and PM was visualized by immunodetection with co-localization of *pSKU5::SKU5:GFP* (α-GFP, green) and endosome marker ARF1A1C (α-ARF1A1C, red), Golgi marker Sec21(α-Sec21, red) and PM marker PIN2 (α-PIN2, red). Corresponding to Figure 4E. Scale bar: 5μm.

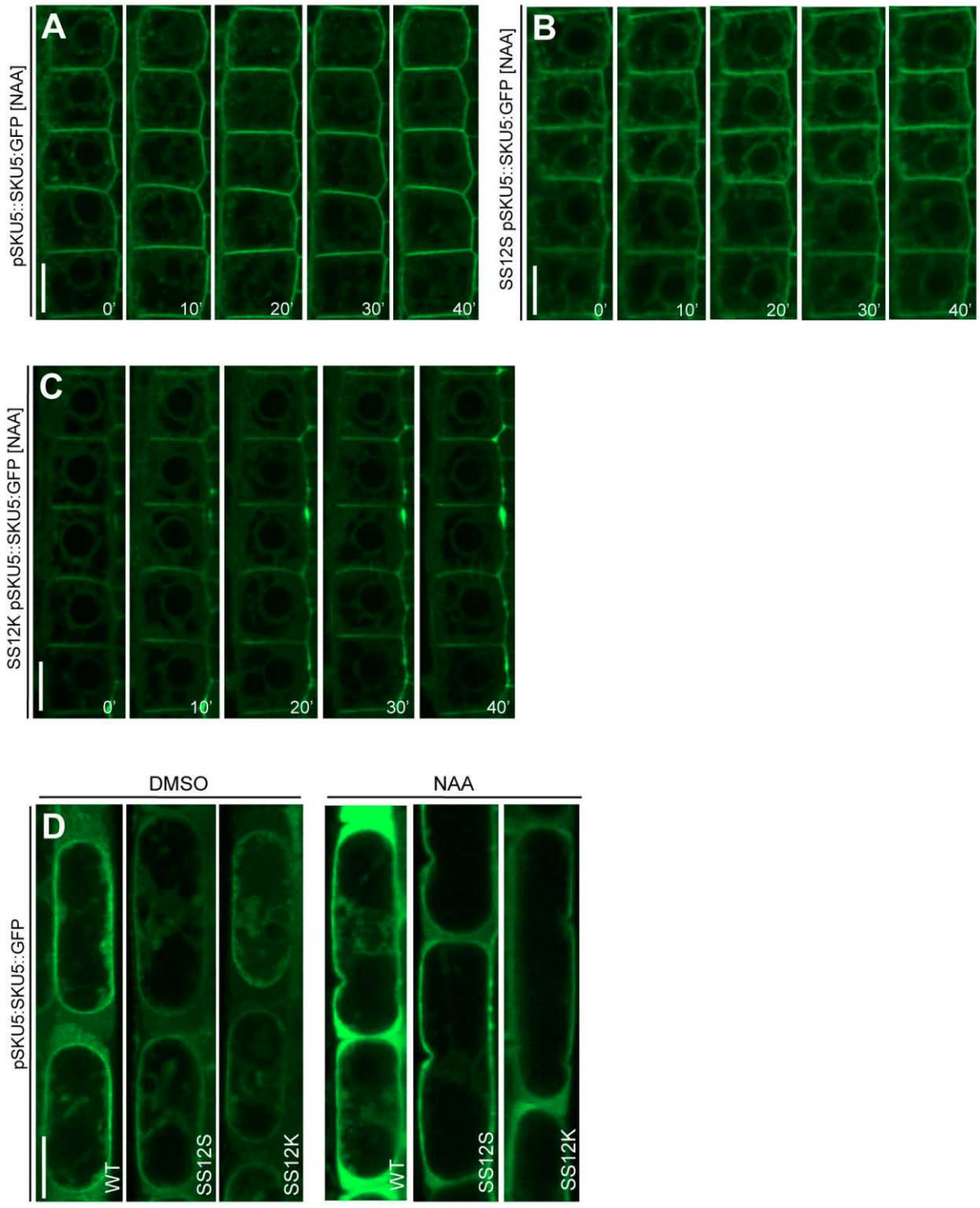

Figure S5. Cell surface Localization of SKU5 is Induced by ABP1-dependent Auxin Signaling. (A-C) Time-lapse observation (every 10min, 40min in total) showed that intracellular SKU5 proteins were secreted to the cell surface after 1μM NAA treatment in WT, while no obvious change of SKU5 translocation were observed in SS12S and SS12K (both expressing *pSKU5::SKU5:GFP*). Corresponding to Figure 5F. (D) PM and apoplast-localized SKU5 were much less in ABP1-inactivation lines following NAA application. WT, SS12S and SS12K lines (expressing *pSKU5::SKU5:GFP*) were treated with DMSO or 1μM NAA for 2h and then plasmolyzed. Corresponding to Figure 5D. In all panels, scale bar: 5μm.

SUPPLEMENTARY TABLES

Table S1. List of tested BiFc combinations showing negative signal.

GFP N-half part	GFP C-half part
SKU5	empty
ABP1	empty
SKS4	empty
Empty	SKU5
Empty	ABP1
Empty	SKS4
ABP1	SKU5
ABP1	SKS1
ABP1	SKS2
SKU5	ABP1
SKS4	ABP1
SKS1	ABP1
SKS2	ABP1
SKU5	SKS4
SKU5	SKS1
SKU5	SKS2
SKS4	SKU5
SKS1	SKU5
SKS2	SKU5

All the BiFc assays in a single expriment were used the same microscope setting.

Table S2. List of primers used for genotyping and Qrt-PCR analysis.

	Forward primers (F) and Reverse primers (R)
SKU5-fulllength	F: GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGGATTTGTTCAAGATCCT R:GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAATGCTGAAGCATCATCAT
SKU5-T-DNA	F: GAAACAATCACAGTCCATCCTG R: GCACCACTTGCTGATACATTCCAT
***abp1-5*-mutation**	F: TGACCTTCCTCAGGATAACTATGG R: CCAACACCTGCAGGTCCTCATGAC
AttB-amiRNA	F: GGGGACAAGTTTGTACAAAAAAGCAGGCTCCCCAAACACACGCTCGGA R: GGGGACCACTTTGTACAAGAAAGCTGGGTCCCCATGGCGATGCCTTAAA
ABP1-N-part	F: GGGGTACCATGATCGTACTTTCTGTTGGTTCCGC R: CCGCTCGAGCCCAGGGAAATTTCCATGTGTTTCAGC
ABP1-C-part	F: CGGGATCCAAACCAATCGAATTTCCAATCTTTGCC R: GCTCTAGATTATTGTGATTCTTGAATGCATTGCTCATC
GFP-glycinelinker	F: CCGCTCGAGGGAGGAGGAGGATCCGGAGGAGGAGGATCCGGAGGAGGAGGATCCATGGTGAGCAAGGGCGAGGAGC R: CGGAATTCGGATCCGGATCCTCCTCCTCCGGATCCTCCTCCTCCGGATCCTCCTCCTCCCTTGTACAGCTCGTCCATGCCGA
NGFP-glycinelinker	R: CGGAATTCGGATCCGGATCCTCCTCCTCCGGATCCTCCTCCTCCGGATCCTCCTCCTCCGGCCATGATATAGACGTTGTGG
CGFP-glycinelinker	F: CCGCTCGAGGGAGGAGGAGGATCCGGAGGAGGAGGATCCGGAGGAGGAGGATCCGACAAGCAGAAGAACGGCATCAAG
NGFP	F:GGGGACAGCTTTCTTGTACAAAGTGGGGATGGTGAGCAAGGGCGAGGAGC
CGFP	F: GGGGACAGCTTTCTTGTACAAAGTGGGGGACAAGCAGAAGAACGGCATCA
SKU5-C-part	F: GGGGACAAGTTTGTACAAAAAAGCAGGCTACGCTTCTCCTCTTGGTGTCCCTCAACAGG R: GGGGACCACTTTGTACAAGAAAGCTGGGTTATGCTGAAGCATCATCATCATCACCAA
ABP1-fulllength	F: GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGATCGTACTTTCTGTTGGTTCC R: GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAAAGCTCGTCTTTTTGTGATTCTTGAATGCATTGC
SKU5-SKS1-2-3-RNAi	I: GATATCCCTGCGGGTTTGGACGGTCTCTCTTTTGTATTCC II: GACCGTCCAAACCCGCAGGGATATCAAAGAGAATCAATGA III: GACCATCCAAACCCGGAGGGATTTCACAGGTCGTGATATG IV: GAAATCCCTCCGGGTTTGGATGGTCTACATATATATTCCT
SKS-conserved-RNAi	I: GATATCGAACCGTATTTAAATGATCCTTTCTCTCTTTTGTATTCC II: GAAAGGATCATTTAAATACGGTTCGATATCAAAGAGAATCAATGA III: GAAAAGATCATTTAATTACGGTTCGATATCACAGGTCGTGATATG IV: GATATCGAACCGTAATTAAATGATCTTTTCTACATATATATTCCT
SKS4-080840-T-DNA	F: GGCTAACCAAGCTCCTCAAGACTATT R: CGAGGCACCGTTCACGGCGTACC
SKS4-fulllength	F: GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGAGAGGTTCTTGCAAAGTCTC R: GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGAATGGCCTAGTGTGGCGGCCTT

SKS1-C-part	F: GGGGACAGCTTTCTTGTACAAAGTGGGGGCGGACCCTTTCGTC TCC R: GGGGACAACTTTGTATAATAAAGTTGTTCAGCAAAATCTGAAC ACCG
SKS2-C-part	F: GGGACAGCTTTCTTGTACAAAGTGGGGGGTGATCCTTACGTCT CTTAC R: GGGGACAACTTTGTATAATAAAGTTGTTCAGCAAAAGGACGAA AACG
LORELEI-fulllength	F: GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGGAGCTGAT ATTATTATTCTTC R: GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAAGTCAACACT AACAAAGCAA
LB4	CGTGTGCCAGGTGCCCACGGAATAGT
LBa1	TGGTTCACGTAGTGGGCCATCG
ACTIN2	F: GTCGTACAACCGGTATTGTGCTG R: CTCTCTCTGTAAGGATCTTCATGAGGT
ACTIN 8	F: CCATCATTTGTTGGAATGGA R: CGGTCTGCAATACCTGAGAA
SKU5-QRT-1	F: AATGTCAGAAACAAGTTAGACGAGGGA R: GAGAAAGGGACTGGAATAATAGC
SKU5-QRT-2	F: CGCCTTCTTTGTCGTCGGTATGG R: CCGACGATGAAACCTTCTGTGGC
SKS1-QRT	F: GTATCCAAATGCGGCGTAACTCG R: GGGAAAGGGATAGGAATAATGTC
SKS2-QRT	F: TCAGATGAGGCGTAACTCGTGGCA R: TCAGGCTCAGTGAACGGAATAGG
SKS3-QRT	F: CGTTTTCTTGTCGGTTACCGGAGCC R: CCAATTACCTGTTGACGAGTGCC
SKS4-QRT	F: AATGGGCAGTTTCCTGGACCTCA R: AGAACGGGACAGGGATGCGAGGAC
SKS5-QRT	F: TTGATGGTTACTCTTTCTGGGTTGT R: TGGTTCACGTAGTGGGCCATCG
SKS6-QRT	F: TATTCCTGGCGGTCCCACTAT R: CTTTGTTTCCCGTTTATCTGT
SKS7-QRT	F: CAGAGCCTTTGTGGAAGTTAT R: ATTGCGTCGTTTAGATTGTAG
SKS8-QRT	F: AACCCTGGAAGCATACCTGAT R: TCCCAACCACGAAGAAAGAGT
SKS9-QRT	F: GAAGCATACCTGACAAGCCGAGAA R: TGGTGGAGCGAGAAATAGCAT
SKS10-QRT	F: TGACAAGCCGAGAAGAGGAGG R: CGAGGTGGTAACTTTGGACGA
SKS11-QRT	F: CCCAGAACAAATCAAGAGCAT R: GCGACGGCAAAGAAGGAGTAA
SKS12-QRT	F: AGAGGAGTGTCCAGTCTTGGCATTT R: TTCTGCGTTCTCCGACCTAAT
SKS13-QRT	F: GGACGAACCAGCAGCCAAGAT R: TCGGAAGCGACAGCGAAGAAG
SKS14-QRT	F: CGGTTTGGTTTCAATGGTGTA R: ACTGCATGGTCTTCTCGTGGT
SKS15-QRT	F: CGGTTTGGTTTCAATGGTGTA R: GTATGGTCCCGTAATGAAATG
SKS16-QRT	F:TATGTTGTTGGATACGGGTCG R: GGCTCGGATTCAGTGTAAAGA
SKS17-QRT	F: ATGACTTCTGGGTCGTTGGAT R: CCACATCCCTTGATTGTCTAA
SKS18-QRT	F: AAAACACCGACTCTTGGAACT

	R: TTGACCGTGAAACAGCATCTA
IAA1-QRT	F: ACCGACCAACATCCAATCTC
	R: TGGACGGAGCTCCATATCTC
IAA2-QRT	F: ATCACCAACCAACATCCAGTC
	R: TGGACGGAGCTCCATATCTC
IAA5-QRT	F: TCCAAGGAACATTTCCCAAG
	R: CCGGAGAAAGAACAGTCTCG
IAA17-QRT	F: CCGGAGAAAGAACAGTCTCG
	R: AGGGTTCTCAGAGACGGTTG
IAA19-QRT	F: GACTCGGGCTTGAGATAACG
	R: GACTCGGGCTTGAGATAACG
CYCD3.1-QRT	F: GCAAGTTGATCCCTTTGACC
	R: CAGCTTGGACTGTTCAACGA
CYCD6.1-QRT	F: GGTGGAAGGTGAGTTTTTTGAT
	R: ACTGGTTTGAGATTTGAGGGA
ABP1-QRT	F: TCGTCGTCTTTTCCGTCGCG
	R: TTGGCAAGCCATTGATGGGACA

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

RNA Extraction and Quantitative Real Time PCR (qRT-PCR)

Whole RNA of seedlings was extracted using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-rad). qRT-PCR analysis was performed using LightCycler® 480 SYBR Green I Master (Roche) following recommendations of the manufacturer. qRT-PCR was carried out in 384-well optical reaction plates by using Perkin Elmer Janus Robot and Roche Lightcycler 480 with heated for 10min to 95°C to activate hot-start *Taq* DNA polymerase, followed by 40 cycles of denaturation for 60s at 95°C and annealing-extension for 60s at 58°C. Expression levels were normalized to the expression levels of *Actin2* or *Actin8*. Specific primers used for gene expression were listed in Table S2. qRT-PCR relative quantification was performed in triplicates on the Lightcycler 480 software combined with the local website (http://qpcr.ista.local).

Used primers, vectors and cloning strategy

The primers used for genotyping, cloning and qRT-PCR are listed in the Table S2. The gateway vectors used for cloning and all the cloning strategies are listed in Table S3.The secretion signal peptides of SKU5, SKS1, SKS2 and SKS4 were predicted online (http://www.cbs.dtu.dk/services/SignalP/), and GFP or splitGFP sequences were inserted in-frame after secretion peptide with Glycine linker (Sedbrook et al., 2002). The sequence of secretion peptides plus GFP or splitGFP of SKU5, SKS4, SKS1 and SKS2 genes were synthesized by Eurofinsgenomics Company for the further cloning. GFP or splitGFP sequences were inserted in-frame in the same position of ABP1 as described previously (Robert et al., 2010). For the generation of overexpressing SKU5 lines (SKU5-OE), the fragment of full-length SKU5 CDS was cloned into the Gateway vector pEN-R2-3×HA-L3 using Gateway® cloning technology (www.invitrogen.com) driven by 35S promoter, then the resultant construct was introduced in 35::ABP1:GFP line by *Agrobacterium*-mediated genetic transformation. 23 independent lines were used for the QRT-PCR analysis and two independent lines were used for the further experiments.

Design and the Generation of the Artifical miRNA

The target region of amiRNA against SKS genes and the designed primers were predicted on the WMD online tool (http://wmd3.weigelworld.org/). The constructs were generated

according to the described primer extension PCR and cloned into gateway vectors pMDC7B under the control of estradiol-inducible ubiquitin promoter (Curtis and Grossniklaus, 2003; Zuo et al., 2000) using Gateway® cloning technology (www.invitrogen.com). Co-expression analysis was performed on the ATTED-II online tool (http://atted.jp/) and phylogenetic tree was established on online website (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Gravitropic response

Four-day-old vertically grown seedlings, under light conditions, were reoriented by 135°, and the angles deviating from the original vertical growing direction of primary roots (defined as 0°) were tracked every 2h till 8h.

Leaf Arabidopsis PC Shape and ROP2 and ROP6 Activity Assays

Epidermal PC outline of *Arabidopsis* cotyledons was imaged on confocal microscopy by propidium iodide (10mg/mL) staining for 2min (Kong et al., 2010) or performed as described previously (Xu et al., 2010). The detection method of ROP2 and ROP6 activity was performed as described (Xu et al., 2010). The relative ROP2 or ROP6 activity level was determined as the amount of GTP-bound ROP2 or ROP6 divided by the amount of total ROP2 or ROP6. The relative ROP activity in different mutants was compared with WT. ROP2 and ROP6 activity in WT without NAA treatment were standardized as ‘‘1’’.

Immunogold and Transmission Electron Microscopy

For immunogold-TEM detection of GFP fusion proteins (*pSKU5::SKU5:GFP*, *35S::ABP1:GFP* and *RPS5A::ABP1:GFP*) or ABP1 protein (WT), four-day-old seedlings were excised, immersed in 20% (w/v) BSA, and frozen immediately in a high-pressure freezer. The following protocol was described previously in details (Feraru et al., 2010). Grids were floated upside down on 25 µl of aliquots of blocking solution (5 % BSA, 1% FSG in PBS) for 20 min followed by a wash step for five times 5 min (1% BSA in PBS), and following incubated in a dilution (1% BSA in PBS) of primary anti-GFP antibody (Abcam, 1:25) or anti-ABP1 antibody (1:100) (Xu et al., 2014) for 120 min. The grids were then incubated with PAG10nm (Cell Biology, Utrecht University) and washed twice for 5 min each time with 0.1% BSA in PBS, PBS, and double-distilled water. Grids were viewed with a JEM1010 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV using Image Plate Technology from Ditabis (Pforzheim, Germany). GFP-immunogold labeling on WT cells was negligible.

Westernblot and Secretion Detection

The constructs of ABP1 with GST tag in combination with SKU5 or LORELEI proteins was transformed into *Agrobacterium LBA4404*. *Arabidopsis* suspension culture were transformed by *Agrobacterium* containing respective construct and cultivated as described previously (Van Leene et al., 2007). Cultures with high transgene expression were gradually scaled up to 500 mL. Cell material was then washed with 2L of media without auxin and grown in auxin free media for another 12 h. Afterwards 1µM NAA was added and cultures were growing for additional 2h. Cell material was separated and the secreted proteins were extracted by TCA-acetone (10% TCA in acetone containing 0.07% DTT) precipitation from the media. The final pellet was suspended in 100 µL protein solubilization buffer (9M urea, 4% CHAPS, 0.5% TritonX-100, 100 mM DTT) (Jones and Herman, 1993; Maldonado et al., 2008). Presence of secreted protein was detected by Western blot and protein amount was specified by GelQuant.NET software and normalized according to sample loading. Two biological repeats were analyzed.

Quantification of Co-localization Factor and Statistics

Zeiss software with co-localization plugin was used for the quantification of co-localization factors. The two-channel merged images were split into two separate pictures with individual channel. The co-localization factor was showed as R value from channel 2 (such as different intracellular markers in Figure 4B-D) vs. channel 1 (such as SKU5-GFP signal) with random ROI (Region of Interest). For all the quantitative data, error bars indicate standard error means (s.e.m.). The number of analyzed samples is indicated as n, and statistical analyses were performed using Student's T-test where *, ** and *** corresponds to p-value <0.05, <0.01 or <0.001, respectively.

Quantification of PIN1 Internalization

Image J was used for the quantification of the area of PIN1-containing BFA bodies. The images of different ecotypes were taken by the same setting (without zoom in or crop). The area of PIN1-containing BFA bodies in WT with BFA treatment was standardized as ''1''.

Image Analysis and Quantification of TEM Immunogold Labeling

For the manual quantification of TEM immunogold labeling, the quantified areas (2µm*4µm) were randomly selected. The ratio of the number of gold particles on the cell wall (4µm distance along the cell surface) was calculated as a percentage of the total number of gold particles in the intracellular area (2µm distance apart from the cell surface) measured. Additionally, The TEM image stacks of *35S::ABP1:GFP* or *RPS5A::ABP1:GFP* seedlings under auxin treatment and control conditions were processed using Matlab inc and Image Processing Toolbox (IPT). Each high resolution image (~2 nm or 20Å) was converted to the binary representation using Otsu's threshold method (Xue and Titterington, 2011). Merged original (grayscale) and binary pictures were processed by the customized Matlab scripts to detect immunogold particles and separate them from the background using optimized, stringent cutoff threshold of 15% in the grayscale range and corresponding heat map representation (see Figure S6D, E). Detected pixels were averaged over 4 neighbors using 'bwconncomp' function from IPT to mark detected particles. The fraction of particles on the cell surface was calculated as the ratio of total number of particles detected on the TEM image to the local number of particles residing in the cell wall and PM. The fraction was averaged over the image stack corresponding to non-treated of treated lines. Statistical significance of the results (p-values) was calculated using T-test (Student's T-test).

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Chapter 4.

Functional characterization of the phosphorylation sites for the biological role of the Arabidopsis PIN3 protein

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Functional characterization of the phosphorylation sites for the biological role of the Arabidopsis PIN3 protein

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Directional intercellular auxin transport is mediated by asymmetrically redistributed of transmembrane carriers PIN-FORMED (PIN) proteins. Eight different PINs in *Arabidopsis* share common structure of ten membrane-spanning helixes and a central hydrophilic loop that differ in size. Phosphorylation of the hydrophilic loop of PIN proteins has been reported to regulate their trafficking and polar localization. Here we investigate the importance of two predicted phosphorylation sites, in which mutations mimicking constitutive dephosphorylated or phosphorylated status were prepared. Mutations of these residues partially disrupted in planta phosphorylation of PIN3 and caused defects in PIN3-mediated developmental processes. We demonstrate that these mutations did not influence the root and hypocotyl length and size of the root meristem, but they have an impact on the tropic response and vacuolar degradation of PIN3 protein. These results suggest that some of the phosphorylation motifs have been functionally conserved among PIN protein members.

INTRODUCTION

The plant hormone auxin IAA controls plant growth and development by modulating fundamental cellular processes, such as cell division, expansion, and differentiation (Mockaitis and Estelle, 2008). Intercellular auxin transport and metabolism are responsible for

changes in auxin concentration, which are reflected in different cellular auxin responses. Auxin transport proteins and their regulators underwent detailed characterization during last years that broaden our knowledge on the polar auxin transport, the auxin gradient formation and mechanism of differential growth and organogenesis (Benková et al., 2003; Grunewald and Friml 2010; Ganguly et al., 2012). Transport of auxin can be divided into two pathways: a long-distance pathway, which is responsible for basipetal auxin transport through the phloem tissue (Swarup et al., 2001; Ljung et al., 2005), and a short-distance pathway that is responsible for cell-to-cell auxin transport and is mediated by specific auxin influx and efflux carriers (Vieten et al., 2007; Vanneste and Friml, 2009). Small portion of apoplastic auxin can get protonated (IAA-H) allowing it to pass through the membrane by diffusion. However, major part of auxin undergoes active transport through the plasma membrane (PM) by one of the three main classes of auxin transporters in *Arabidopsis*: AUX1/LIKE AUX1 (LAX) (Bennett *et al*, 1996; Yang *et al,* 2006), P-glycoproteins (PGP) of the ATP-binding cassette (ABC) transporter family (Geisler et al.,2005), PIN proteins (Petrášek *et al,* 2006; Vieten et al., 2007; Zažímalová et al., 2007) and putative auxin transporters PIN-LIKES (PILS) (Barbez et al., 2013).

PIN proteins, also known as auxin efflux carriers, are localized, with some exceptions, at the plasma membrane (PM) and participate in directional auxin transport (Petrášek et al., 2006). PIN family contains eight different genes that are expressed in an organ- or tissue-specific manner (Vieten et al., 2007; Žažimalová et al., 2007), of which five (PIN1, PIN2, PIN3, PIN4, and PIN7) localize to the PM and possess long hydrophilic loops. From the others, PIN8 localizes to the endoplasmic reticulum (ER) where it presumably facilitate the regulation of the auxin homeostasis in the ER lumen (Ding et al., 2012, Dal Bosco et al., 2012), whereas PIN5 and PIN6 most probably has a dual localization at the PM and at the ER (Mravec et al., 2009; Gaungly et al., 2010; Cazzonelli et al., 2013; Ganguly et al., 2014). The directionality of the auxin transport is determined by a polar, subcellular, localization of PIN auxin efflux carriers (Wiśniewska *et al,* 2006). One of the important cellular regulators of the PIN polar targeting is protein phosphorylation. The apical-to-basal PIN localization is controlled by serine/threonine protein kinase PINOID (PID) (Benjamins et al., 2001; Friml et al., 2004). Apical PIN localization is stimulated in PID overexpression mutant. This leads to disruption of the auxin maxima and to the collapse of the root meristem and causes agravitropic root growth (Benjamins et al., 2001; Friml et al., 2004). On the other hand, in *pid* mutant the apical-to-basal PIN translocation causes deprivation of auxin from the root meristem and pin-like inflorescence phenotype (Reinhardt et al., 2003; Friml et al., 2004).

This regulation is balanced by the PP6-type heterotrimeric phosphate holoenzyme, which consists of a PP2A regulatory A subunit (RETICULOCALBIN1 (RCN1)/PP2AA1, PP2AA2, or PP2AA3) and a catalytic C subunit (PHYTOCHROME-ASSOCIATED SERINE/THREONINE PROTEIN PHOSPHATASE1 [FyPP1] or FyPP3 that facilitates PIN proteins dephosphorylation (Michniewicz et al., 2007; Dai et al., 2012). Several studies demonstrated that phosphorylation of the hydrophilic loop of PIN proteins is required for establishment of PIN polarity during plant growth and development (Michniewicz et al., 2007; Zourelidou et al., 2009; Dhonukshe et al., 2010; Huang et al., 2010; Ding et al., 2011; Zhang et al., 2010; Gaungly et al., 2012, Sasayama et al., 2013).

Recently, another kinase that can phosphorylate PIN proteins has been identified. D6 protein kinase (D6PK) localize to the basal membrane of *Arabidopsis* cells in root and it colocalize with several PIN proteins such as PIN1, PIN2 and PIN4 (Zourelidou et al., 2009). It was shown that D6PK can directly interact with PIN1 protein and phosphorylates it (Zourelidou et al., 2009; Willige et al., 2013; Barbosa et al., 2014).

In this study we decided to examine the potential phosphorylation sites in the PIN3 hydrophilic loop. We test the hypothesis that some of the motives have been functionally conserved among long PIN proteins. We demonstrated their crucial role during PIN3 trafficking, tropic responses and meristematic activity of the root.

RESULTS

Importance of phosphorylation for the gravitropy-mediated PIN3 relocation

Gravistimulation has been shown to induce changes in the polar PIN3 localization in roots where PIN3 relocates toward the bottom side of the columella cells after the gravitropic stimulus (Friml *et al,* 2002a; Harrison and Masson, 2008; Kleine-Vehn *et al,* 2010). Protein phosphorylation by the serine/threonine protein kinase PINOID (PID) and dephosphorylation by the PP2A complex have been implicated in auxin transport and PIN trafficking (Michniewitz et al., 2007; Huang et al., 2010; Zhang et al., 2010). In the hypocotyl, phosphorylation by PID plays an important role in PIN3 polarization during tropic response (Ding et al., 2011; Rakusová et al., 2011). We tested whether phosphorylation and dephosphorylation contribute to the gravity-induced relocation of PIN3 in columella cells.

After 30 minutes of gravistimulation, the PIN3 protein relocates to the basal cell sides following gravity (Fig. 1 A,B,K). We used the PID overexpression line *35S::PID-21* that is responsible for the PIN protein phosphorylation. This line exhibited a significant reduction in

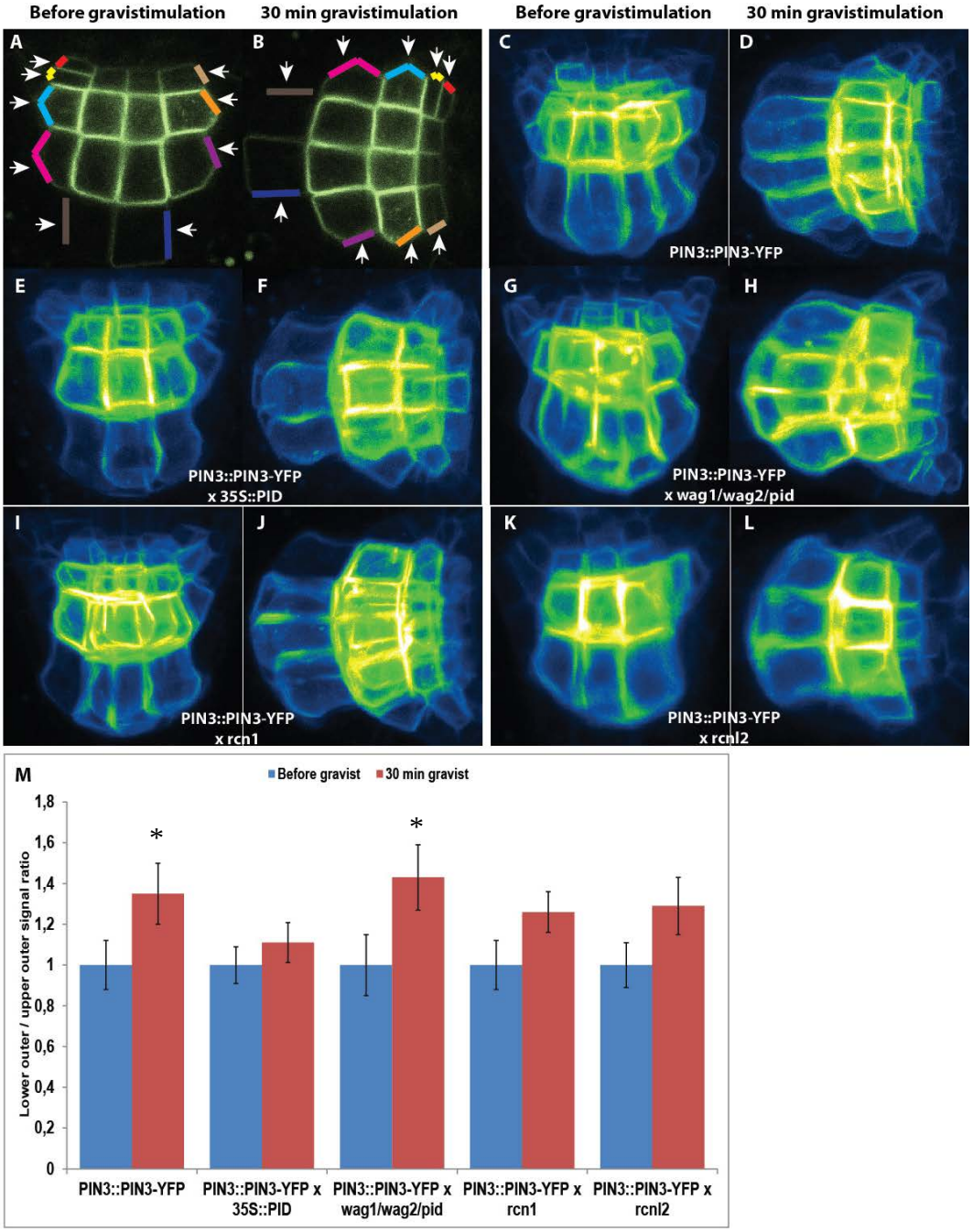

Figure 1. Changes in PIN3 relocalization during gravitropic bending response of *Arabidopsis* root. (A, B) Scheme presenting which membranes were used for quantification. PIN3 protein relocation before and after 30 minutes of gravitropic stimulation in wild type background (C, D) and different phosphorylation mutants: *35S::PID* overexpression line (E, F); *wag1/wag2/pid* triple mutant (G, H); rcn1 single mutant (I, J) and rcnl2 single mutant (K, L). (M) Quantification of gravity-mediated PIN3 relocalization in various phosphorylation mutants. Student's T-test was calculated for the comparison of signal ratio between before and after gravistimulation (* $p<0.05$). Error bars represent SE.

PIN3 relocation (Fig. 1 C,D,K). In a similar way, we tested the influence of the *pid* mutation on this process and we expected an effect opposite to that of the PID overexpression line. The single mutant had only a mild phenotype, whereas the *wag1/wag2/pid* triple mutant that also

lacks the phosphorylation activity of the closest homologs of PID exhibited an increased PIN3 relocation rate after gravitropic stimuli (Fig. 1 E,F,K).

The PP2A protein complex consists of three subunits, RCN1, RCNL1, and RCNL2 and is responsible for the dephosphorylation of PIN proteins. We evaluated the relocation of PIN3 in columella cells in the *rcn1* and *rcnl2* single mutants, because we were not able to obtain the homozygous triple mutant. In both these mutants, the PIN3 relocation was only weakly relocated after gravitropic stimulation (Fig. 1 G-K), most probably due to a functional redundancy between the genes.

Hence, we showed that PIN phosphorylation and dephosphorylation play an important role in gravitropy-mediated PIN3 relocation in the root. As phosphorylated PIN proteins might be quite stable at the PM, their translocation after gravitropic stimuli might be more difficult, whereas dephosphorylated PIN proteins might be more easily internalized and relocated.

Putative phosphorylation residues in the PIN3 hydrophilic loop are required for lateral root formation

Our observations concerning the phosphorylation-dependent gravity-mediated PIN3 relocation in columella cells prompted us to investigate the putative phosphorylation sites of the PIN3 hydrophilic loop and to evaluate their developmental function. Two phosphorylation sites, P1 and P2, both containing three phosphoryliable serines (S226, S243, and S283 for P1 and S316, S317, and S321 for P2) (Fig. 2 A), were chosen based on the phosphorylation prediction software GPS (Xue et al., 2008). The P2 site is analogous to the PIN1 phosphorylation site that plays an important role during the basal-to-apical PIN1 relocalization (Zhang et al., 2010). Four different PIN3 mutant constructs were prepared, in which serines were either substituted by alanines (S226A, S243A, and S283A for P1A and S316A, S317A, and S321A for P2A) to mimic the dephosphorylated state or by aspartic acid to mimic the phosphorylation status (S226D, S243D, and S283D for P1D and S316D, S317D, and S321D for P2D). All mutant variants were cloned under the control of the native promoter and introduced into the wild type and *pin3-4* mutant to evaluate their impact on the PIN3 developmental function. *pin3-4* mutant was successfully complemented with all abovementioned constructs and the plants did not exhibit any developmental defects.

None of the mutant variants exhibited defects in root length or meristem size, but all of them showed a slight reduction in the hypocotyl length of dark-grown seedlings (Fig. 2 B-D). As PIN3 plays an important role during lateral root formation (Marhavý et al., 2013; Peret et al., 2013), we examined the mutation impact on this process.

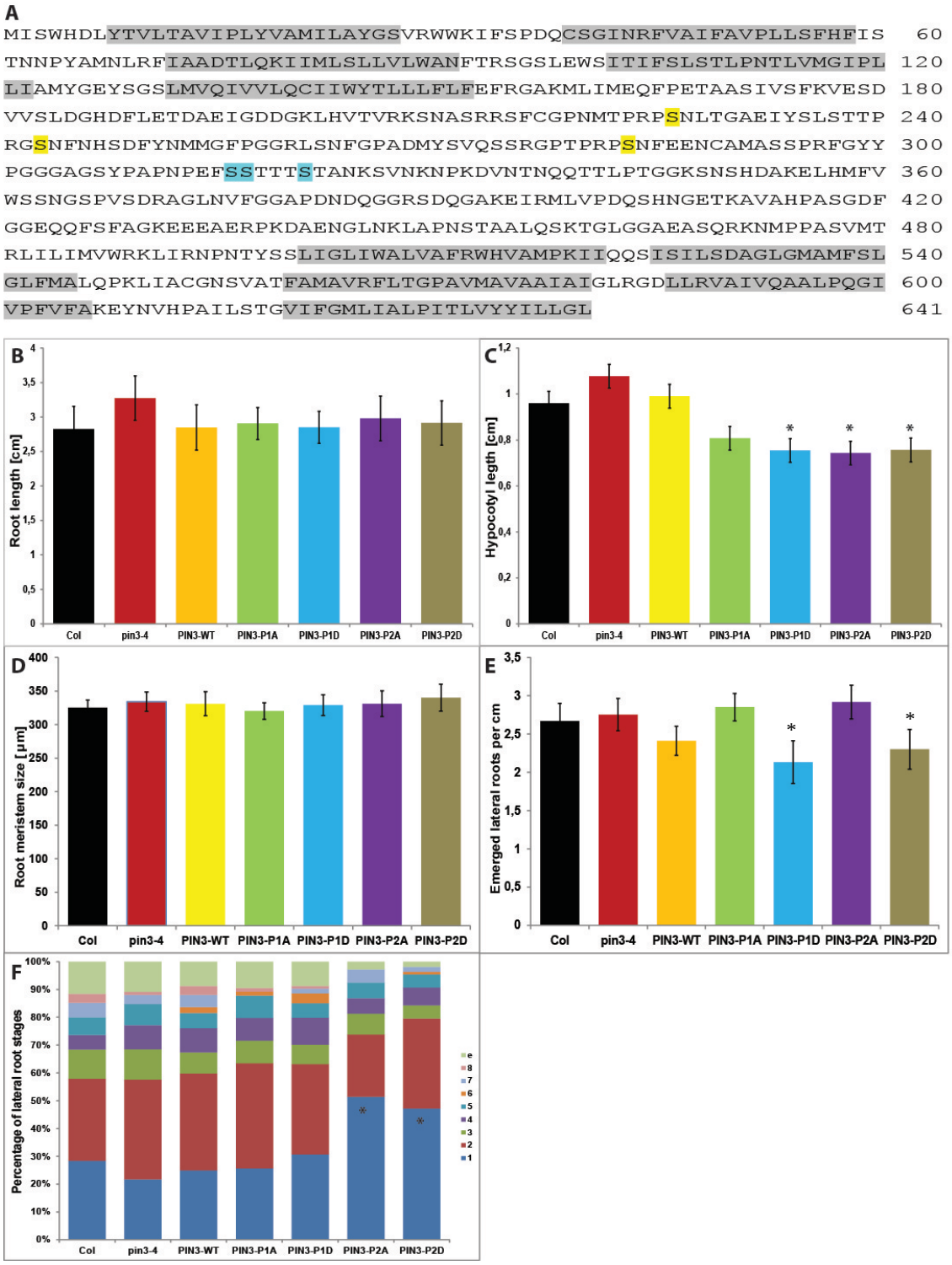

Figure 2. Morphological analysis of PIN3 phospho mutant variants. (A) Positions of mutated amino acids in the sequence of PIN3 protein. S1 site is marked by yellow and S2 by ble colour. Transmembrane domains are highlighted in grey. Phenotypic analysis of PIN3 mutant variants: root length (B); dark grown hypocotyl length (C); root meristem size (D); emerged lateral root number (E) and lateral root stages (F). Experiments were repeated 3 times with >15 root or hypocotyles per sample. Student's T-tests were calculated for the comparison of each line with the control (Col) (* $p<0.05$). Error bars represent SE.

In the mutant variant *PIN3::PIN3-YFP-P2D*, the number of emerged lateral roots was slightly lower than that of the wild type. Analysis of lateral root stages revealed an increased number

of first-stage primordia in this mutant variant (Fig. 2 E,F), but the other mutant variants did not reveal any defects during lateral root formation. This suggests the redundant role of PIN3 during root and hypocotyl growth, albeit important one during the first stages of lateral root development, as observed previously (Marhavý et al., 2013).

Influence of PIN3 phosphorylation on the gravitropic responses of roots and hypocotyls

Next, we studied the effect of gravity-induced relocalization of the PIN3 phosphorylation mutant variants in roots and hypocotyls. On the plant level, all mutant variants were defective in the gravitropic response of the root, but *PIN3::PIN3-YFP-P2D* exhibited the most severe defect, with an almost 40% reduction compared to the wild type (Fig. 3A). We also analyzed the gravitropic response of the PIN3 mutant variants in the *35S::PID* and *wag1/wag2/pid* mutant lines. In the *35S::PID* overexpression line, the response to gravity changes was significantly reduced and the phosphorylation or dephosphorylation status of the P1 and P2 sites did not rescue this phenotype (Fig. 3B). The opposite situation occurred in the *wag1/wag2/pid* triple mutant that lacks activity of PID kinase and its closest homologs, because seedlings of this line responded rapidly to gravistimulation. All PIN3 phosphorylation mutant variants also exhibited this accelerated response to gravity changes (Fig. 3C).

The gravitropic response in dark-grown hypocotyls revealed no defect in the mutant variants except in *PIN3::PIN3-YFP-P2D* that showed a slightly reduced gravitropic growth (Fig. 3D). We tested the influence of the *35S::PID* and *wag1/wag2/pid* mutant lines on the gravitropic growth of hypocotyls. In the *35S::PID* overexpression line, the gravity-induced hypocotyl growth was significantly reduced and did not rescue this phenotype, similarly to the roots of the PIN3 mutant variants (Fig. 3E). The hypergravitropic hypocotyl growth of the *wag1/wag2/pid* triple mutant after gravistimulation was also not rescued by any of the prepared PIN3 phosphorylation variants (Fig. 3F).

We tested also the phototropic hypocotyl bending that is mediated via relocation of the PIN3 protein (Ding et al., 2011), but we did not observe any defects in the phototropic response of any of the PIN3 mutant variant (data not shown). Altogether, the P2 phosphorylation site seems to play a role in gravitropic responses in roots and hypocotyls, but mutations in P1 and P2 could not rescue the PID overexpression or loss-of-function mutants.

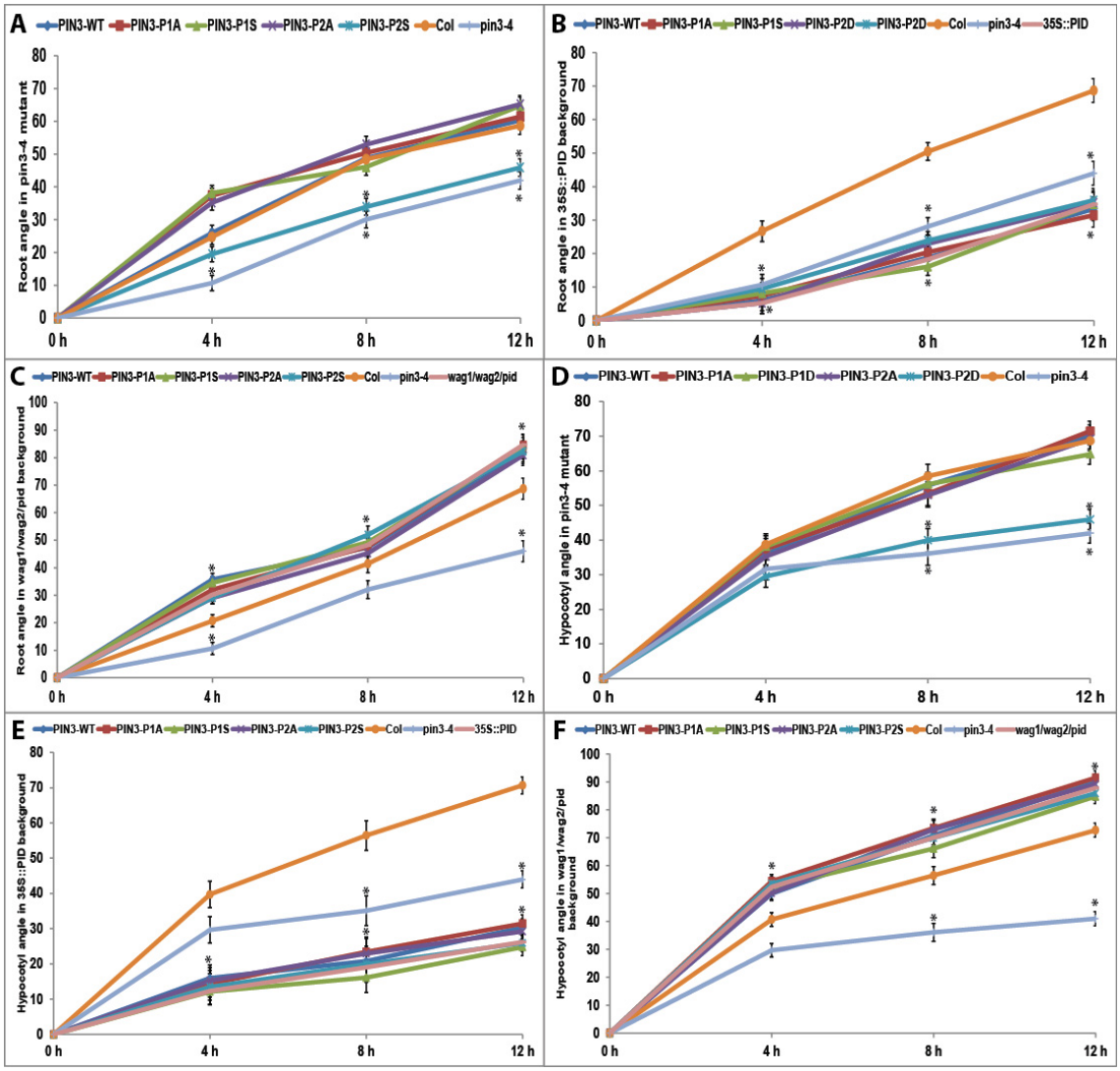

Figure 3. PIN3 phosphorylation sites are required for gravitropic responses. Root bending kinetics of PIN3 mutant variants during gravitropic response (A) in *pin3-4* mutant background; (B) in *pin3-4/35S::PID* double mutant background; (C) in pin3-4/wag1/wag2/pid quadruple mutant background. Hypocotyl bending kinetics of PIN3 mutant variants during gravitropic response (D) in *pin3-4* mutant background; (E) in *pin3-4/35S::PID* double mutant background; (F) in pin3-4/wag1/wag2/pid quadruple mutant background. Root and hypocotyl curvatures were measured every 4 hours and average curvatures were calculated. Values are the average of three biological replicates (n > 10 per time point on each replicate). Student's T-test was calculated for the comparison of each line with the control (Col) (* $p<0.05$). Error bars represent SE.

Gravitropic relocation of the PIN3 protein in roots and hypocotyls of the PIN3 mutant variants

At the cellular level, all PIN3 mutant variants showed the apolar distribution of the PIN3 protein in columella cells. We observed a higher apical-to-lateral signal ratio in the *PIN3::PIN3-YFP-P2D* line than that of the wild type (Fig. 4L). After gravitropic stimulation, the PIN3 protein relocated to the basal cell sides, thus redirecting the auxin flow. The *PIN3::PIN3-YFP-P2D* mutant variant exhibited the most severe defect in the PIN3 relocation in columella cells after a 30-minute gravitropic stimulus, whereas the *PIN3::PIN3-YFP-P1D*

line also had a defective relocation, but not as pronounced as in the P2S mutant variant (Fig. 4K).

In the *35S::PID* overexpression line, the PIN proteins are expected to be more phosphorylated and their relocation during gravitropic stimuli to decrease. The PIN3 phosphorylation mutants exhibited similar phenotypes because their relocation rate decreased in this background (Fig. 4M). Similarly, these mutant variants also failed to rescue the increased relocation rate in the *wag1/wag2/pid* triple mutant, except that the *PIN3::PIN3-YFP-P2D* line showed a slightly diminished phenotype (Fig. 4N).

In hypocotyls, we observed an enhanced signal intensity in the outer lateral membranes of the *PIN3::PIN3-YFP-P1D* and *PIN3::PIN3-YFP-P2D* lines when compared to the control. After gravistimulation, the PIN3 protein relocates from the outer lateral to the inner lateral membranes in the upper cells and from the inner lateral to the outer lateral in the lower cells. Moreover, relocation in the *PIN3::PIN3-YFP-P1D* and *PIN3::PIN3-YFP-P2D* lines decreased after gravitropic stimuli (Fig. 5K-L).

We suppose that PID regulates the gravitropic relocalization of PIN3 in hypocotyls in a manner analogous to that in roots. Overexpression of PID in the *35S::PID* line caused a decrease in the relocation rate of PIN3 after gravistimulation (Fig. 6A). Similarly to roots, also in hypocotyls, none of the PIN3 mutant variant managed to rescue the phenotype (Fig. 6B). In the *wag1/wag2/pid* triple mutant, the phosphorylation rate of PIN proteins should decrease and the relocation rate increase, but of all mutant variant, only the *PIN3::PIN3-YFP-P2D* mutant line had a reduced rate of PIN3 protein relocation after gravitropic stimulus (Fig. 6C).

Long treatments with auxin can relocate the PIN3 protein in endodermal cells of hypocotyls from the outer lateral to the inner lateral membranes, the so-called “inner-lateralization” (Rakusová, unpublished data). We observed a reduced inner-lateralization of the PIN3 protein in *PIN3::PIN3-YFP-P1D* and *PIN3::PIN3-YFP-P2D* after the auxin treatment when compared to the control (Fig. 6D). Hence, these data show that the P2 phosphorylation site participates in the gravitropic-mediated PIN3 relocation, also during inner-lateralization after auxin treatment, but that it, together with the P1 site, cannot fully rescue the PID mutants.

Phosphorylation controls the PIN3 protein stability at the PM

Phosphorylation and dephosphorylation have been proposed to control the abundance of PIN proteins at the PM (Baster et al., 2013), with more stable phosphorylated PIN proteins due to a lower internalization rate. Long dark treatment induces vacuolar targeting of PIN proteins

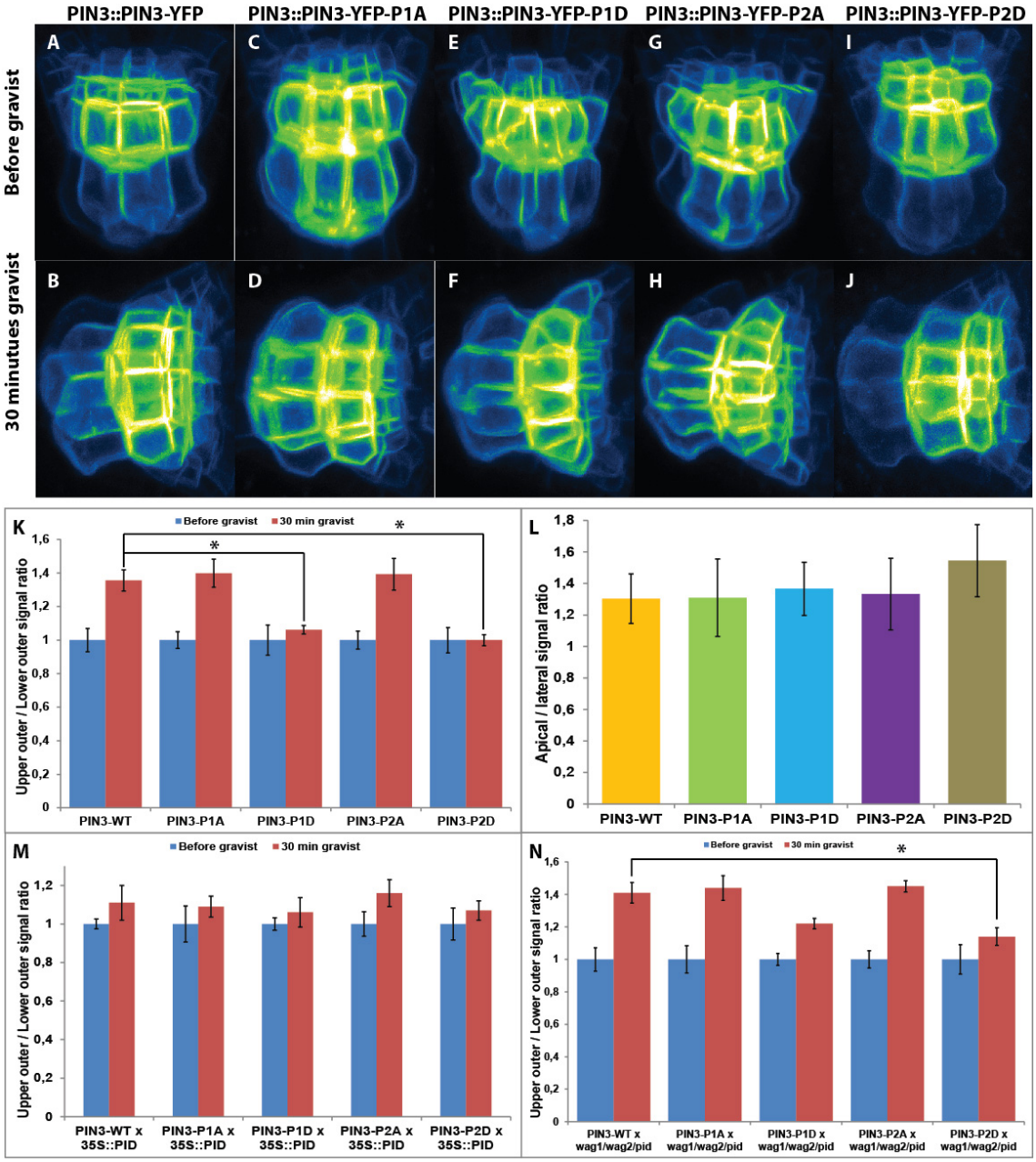

Figure 4. Relocalization of PIN3 protein of PIN3 mutant variants after gravistimulation in root. Localization of PIN3 protein before and after 30 minutes of gravistropic stimulation (A, B) in wild type; (C, D) in *PIN3::PIN3-YFP-P1A* (E, F) in *PIN3::PIN3-YFP-P1D* (G, H) in *PIN3::PIN3-YFP-P2A* (I, J) in *PIN3::PIN3-YFP-P2D*. (K) Quantification of PIN3 signal in columella cells before and after gravistimulation in *pin3-4* background. Signal before gravistimulation was normalized to 1. (L) Quantification of apical-to-lateral PIN3 signal ratio in columella cells. (M) Quantification of PIN3 signal in columella cells before and after gravistimulation in *pin3-4/35S::PID* background. Signal before gravistimulation was normalized to 1. (N) Quantification of PIN3 signal in columella cells before and after gravistimulation in *pin3-4/wag1/wag2/pid* background. Signal before gravistimulation was normalized to 1. Student's T-test was calculated for the comparison of signal ratio between before and after gravistimulation (* $p<0.05$). Error bars represent SE.

from the PM (Kleine-Vehn et al., 2008; Laxmi et al., 2008). We tested whether the PIN3 mutant variants somehow influenced this degradation pathway. After a 12-hour dark treatment, approximately 20% of the PIN3 proteins were internalized from the PM into the vacuole in the wild type, whereas in the *PIN3::PIN3-YFP-P1D* and *PIN3::PIN3-YFP-P2D*

mutant variants, the stability was higher and fewer PIN3 proteins internalized. Other variants exhibited amounts of intracellular signals similar to those of the wild type (Fig. 7 A-F).

The fungal toxin brefeldin A (BFA) is a useful tool to investigate PIN trafficking and endocytosis. BFA treatment leads to accumulation of internalized PIN proteins in pronounced intracellular aggregates, because it inhibits preferentially PIN protein recycling to the PM and form so-called BFA bodies (Geldner et al., 2001, Kleine-Vehn et al., 2008). We investigated whether phosphorylation affects the PIN recycling to the PM. After treating the PIN3 mutant variants with 25 μM BFA, we analyzed the presence of BFA bodies in all the lines, but the phosphorylated mutant variants did not differ from the control (Fig. 7 G-K). Altogether, phosphorylation of the P1 and P2 sites is important for the PIN vacuolar targeting, but apparently it does not play any role in the PIN3 recycling back to the PM.

DISCUSSION

Gravistimulation has been shown to induce changes in the PIN3 localization in roots that promote PIN3 relocation after gravitropic stimuli (Friml et al., 2002; Harrison and Masson, 2008; Kleine-Vehn et al., 2010). Protein phosphorylation by the serine/threonine protein kinase PINOID (PID) and dephosphorylation by the PP2A complex have been implicated in auxin transport and PIN trafficking (Michniewitz et al., 2007; Huang et al., 2010; Zhang et al., 2010, Rakusová et al., 2011).

We demonstrated that PID kinase and PP2A phosphatase are involved in the gravitropic relocation of the PIN3 protein in root columella cells, because the lack of PID increases and of PP2A decreases the PIN3 relocation after gravistimulation, suggesting that the phosphorylated PIN proteins are more stable at the PM and, therefore, their relocalization is hindered. To assess which phosphorylable amino acids are important for the relocation, we chose two sites, P1 and P2, and prepared four different phosphorylated variants. These mutants did not display any obvious phenotypic defects, with the exception of the increased number of first-stage lateral root primordia. This arrest is most probably caused by the necessity to relocate during changes from anticlinal to periclinal divisions (Marhavý et al., 2013). In addition, we observed also that the gravitropic growth of the PIN3 mutant variants had changed. Root and hypocotyl growth after gravistimulation was significantly reduced in the *PIN3::PIN3-YFP-P2D* line. Unfortunately, hypergravitropic growth of roots and hypocotyls in the *wag1/wag2/pid* triple mutant and the reduced gravitropic growth in the

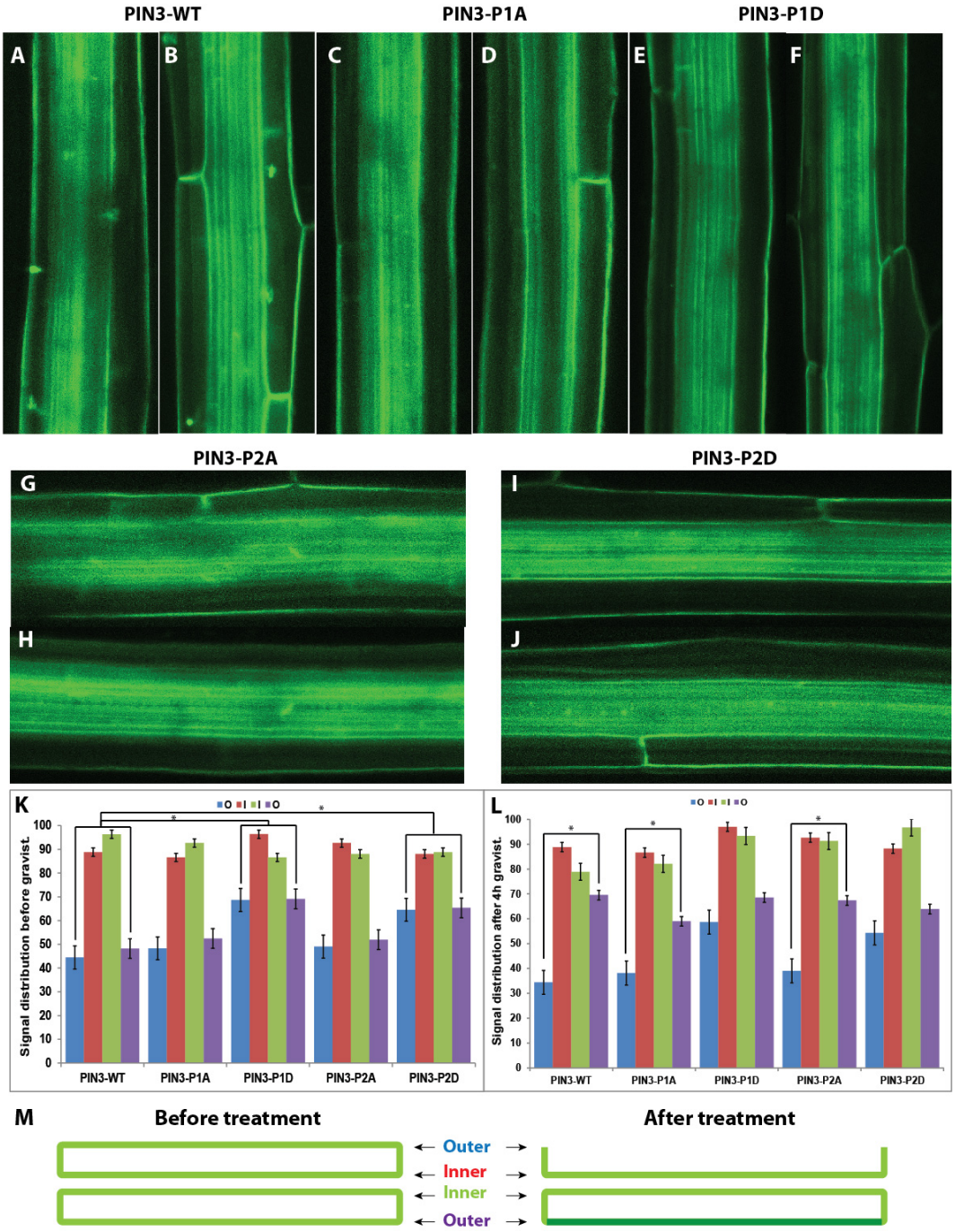

Figure 5. Relocalization of PIN3 protein of PIN3 mutant variants after gravistimulation in hypocotyl. Localization of PIN3 protein before and after 240 minutes of gravistimulation (A, B) in wild type; (C, D) in *PIN3::PIN3-YFP-P1A* (E, F) in *PIN3::PIN3-YFP-P1D* (G, H) in *PIN3::PIN3-YFP-P2A* (I, J) in *PIN3::PIN3-YFP-P2D*. (K) Quantification of PIN3 signal distribution in endodermal cells of hypocotyl. Student's T-test was calculated for the comparison of signal distribution of each line with the control (PIN3-WT) (* $p<0.01$). Error bars represent SE. (L) Quantitative evaluation of gravity-dependent PIN3 relocation in endodermal cells of hypocotyle. Student's T-test was calculated for the comparison of signal distribution between upper outer and lower outer membrane of endodermal cells within each line (* $p<0.01$). Error bars represent SE. (M) Scheme of quantification showing measured membranes.

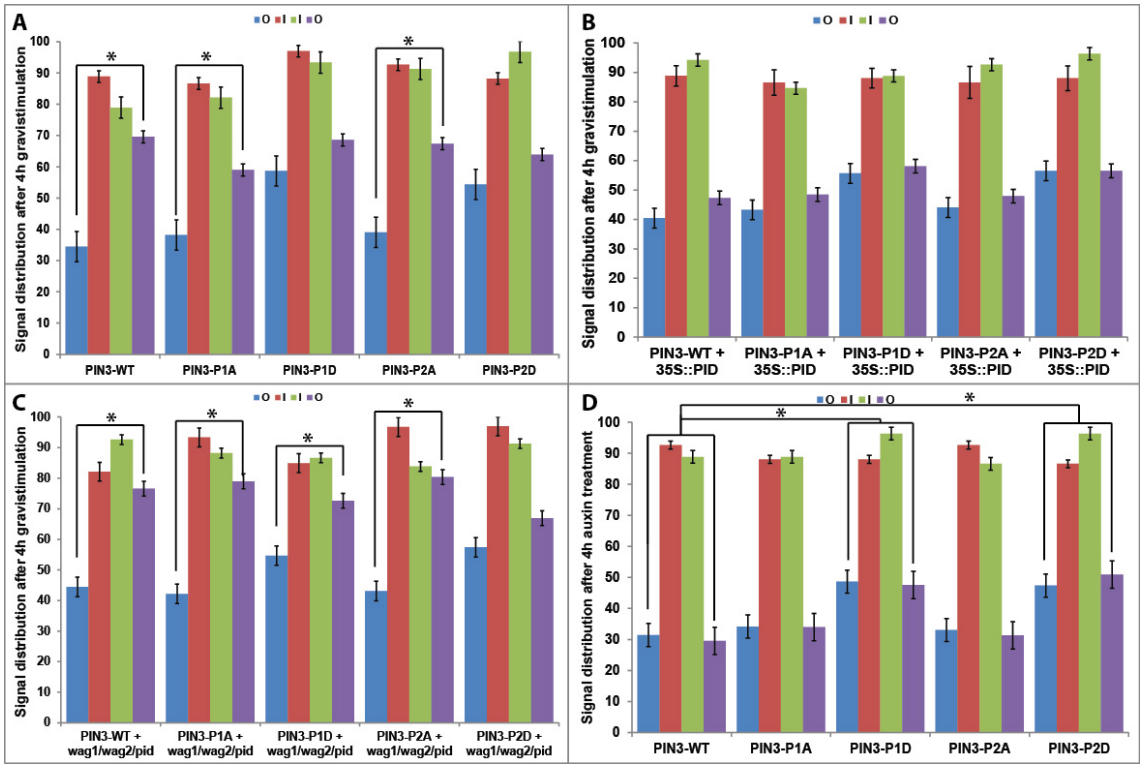

Figure 6. Gravity- and auxin-mediated relocalization of PIN3 protein in hypocotyl. Quantification of PIN3 signal in endodermal cells of hypocotyl after 240 minutes of gravistimulation (A) in *pin3-4* background, (B) in *pin3-4/35S::PID*, (C) in pin3-4/wag1/wag2/pid background (D) Quantification of PIN3 signal in endodermal cells of hypocotyl after 240 minutes of 10 µM auxin treatment in *pin3-4* background. Student's T-test was calculated for the comparison of signal distribution between upper outer and lower outer membrane of endodermal cells within each line (* $p<0.01$). For (D) Student's T-test was calculated for the comparison of signal distribution of each line with the control (PIN3-WT) (* $p<0.01$). Error bars represent SE.

35S::PID overexpression line could not be complemented by any PIN3 phosphorylated mutant variants.

Nevertheless, we closely examined the PIN3 relocation after gravistimulation in these mutant variants and found that the relocation in the *PIN3::PIN3-YFP-P1D* and *PIN3::PIN3-YFP-P2D* lines decreased both in root columella cells and hypocotyls. Similarly, none of the mutant variants was able to complement the phenotypes of *wag1/wag2/pid* and *35S::PID*. We also proposed that phosphorylation control the presence of the PIN proteins at the PM (Baster et al., 2013), because both the *PIN3::PIN3-YFP-P1D* and *PIN3::PIN3-YFP-P2D* lines exhibited a decrease in PIN3 vacuolarization.

These results revealed that the P2 phosphorylation site (adapted from Zhang et al., 2010) is conserved among long PIN proteins and that it is important for the PIN3-mediated gravitropic responses of roots and hypocotyls. Phenotypes of mutations of this site are not completely analogous to the one already published for PIN1 (Zhang et al., 2010); hence, additional phosphorylation sites might be present that may co-operate with the P2 site during gravitropic responses. A similar study about the phosphorylation sites in the PIN3 hydrophilic loop has

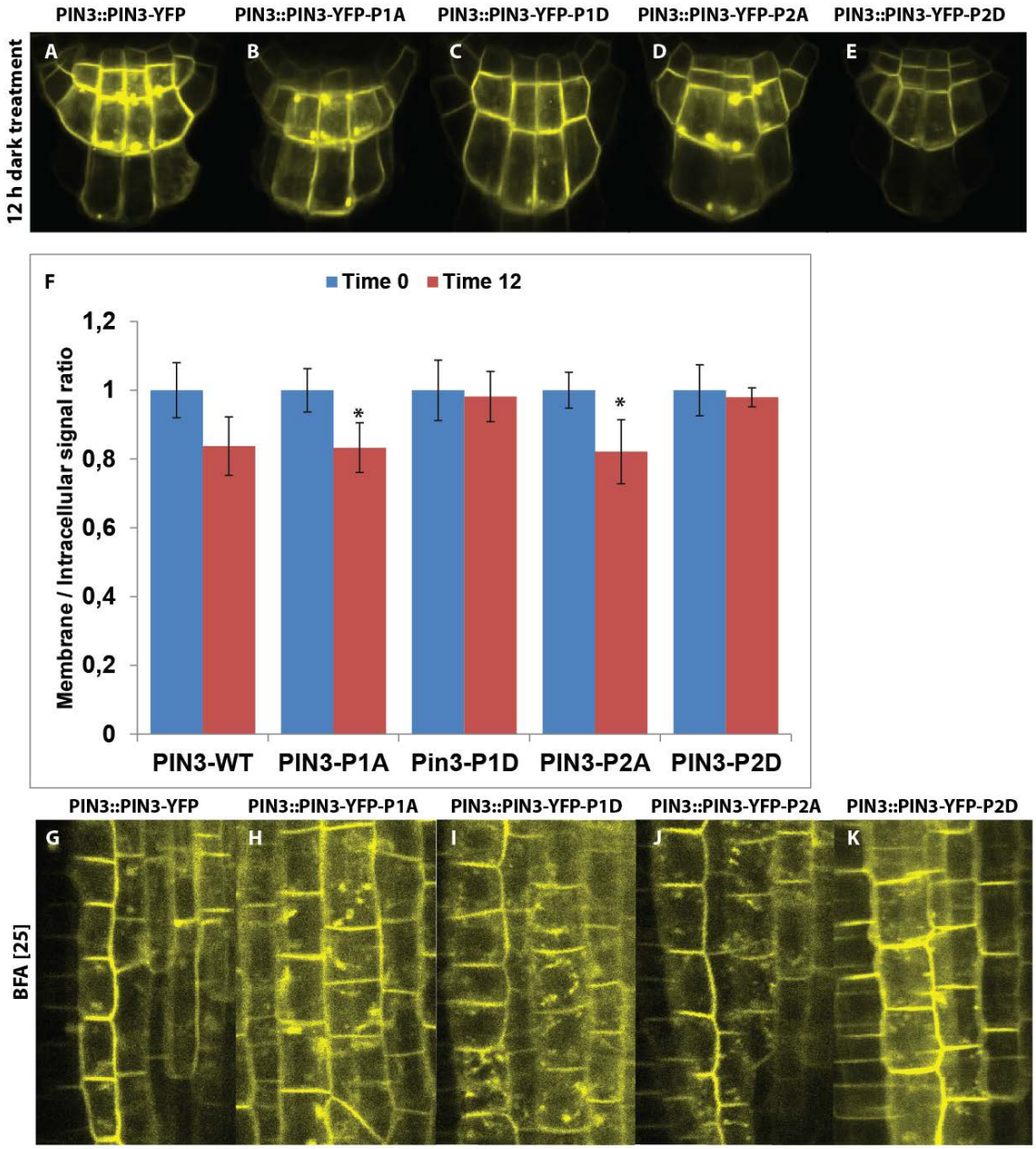

Figure 7. Phosphorylation-mediated stabilization of PIN3 protein at the PM. (A-E) Vacuolarization of PIN3 protein in columella cells in different PIN3 mutant variants after 12h dark treatment. (F). Quantification of dark-induced PIN3 vacuolarization in various phosphorylation mutants. Student's T-test was calculated for the comparison of signal ratio between non-treated and treated samples of each line (* $p<0.01$). Error bars represent SE. (G-K) 90 minutes of 25µM BFA treatment induces internalization of PIN3 protein leading to formation of BFA-bodies in different PIN3 phospho mutant variants.

been published (Ganguly et al., 2012) that identified a different phosphorylation site, M3 ($_{209}$SNASRRSFCGPNMTPRPS$_{226}$), that is important for subcellular trafficking and PIN3-mediated developmental processes, such as auxin efflux activity, root growth, and root gravitropism. In contrast, the PIN3-M3 dephosphorylation-mimicking mutant variant was demonstrated to be still phosphorylated *in vitro* (Ganguly et al., 2012). Thus, mutations of

both the P2 and M3 sites could simultaneously inhibit completely the PIN3 relocation during gravitropic responses, an issue to be unveiled by the future work.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions. The published transgenic and mutant lines were: *PIN3::PIN3-YFP* (Žádníková et al., 2010); *pin3-4* (SALK_005544); *35S::PID-21* (Benjamins *et al*, 2001); *wag1 wag2 pid* (Dhonukshe *et al*, 2010); rcn1 (Michniewitz et al., 2007), rcnl2-2 (Michniewitz et al., 2007). All seeds were grown on agarose plates containing half strength Murashige and Skoog medium. Seeds were vernalized for 3 days at 4 ^{o}C and consequently grown at 18 ^{o}C under 16-h-light/8-h-dark phpotoperiod. For hypocotyl experiments after vernalisation the germination was induced by placing the plates in the light for 5-6 hours that were then transferred to darkness and kept at 19°C for 4 days. For gravitropic stimulations, plates with 4-day-old seedlings were turned 90°, scanned at every timepoint by scanner and the angles were measured by ImageJ. Each experiment was conducted at least in triplicate. For confocal microscopy, a Zeiss LSM 710 confocal scanning microscope modified to vertical position was used.

PIN3 phosphorylation mutagenesis.

The binary vector pK7m42GW containing PIN3::PIN3-YFP sequence (Žádníková et al., 2010) was used for transgene construction. Four different DNA fragments (PIN3-P1A, PIN3-P1D, PIN3-P2A, PIN3-P2D) possessing different mutations (Table S1) were synthesized with XhoI and AegI restriction sites on the ends. Via classical cloning all four of these fragments were introduced into PIN3::PIN3-YFP vector. Transformation of these constructs to the *Arabidopsis* was accomplished via *Agrobacterium tumefaciens* (strain PMP90)-mediated infiltration by floral dip. All transformed lines were analyzed and at least 3 independent transgenic lines for each construct with similar expression level were used in this study.

Quantitative analysis of PIN3 relocalization in root and hypocotyl

Quantification of gravity induce PIN3-YFP relocalization in columella cells was performed by measuring the signal intensity at the apical membranes and comparing with signal intensity of basal membranes of the cells on the periphery of columella (see scheme in Fig. 1). For quantification of the gravity-induced PIN3-YFP relocalization in hypocotyl the rate of PIN3-YFP fluorescence intensity was compared between the external PM sides of endodermal cells (see scheme in Fig. 5). The PIN3 relocation is well visible in the upper endodermal cells, since the lower cell signal is influenced with PIN3-YFP signal in stele. For the auxin-induced

PIN3-YFP relocation the mean fluorescence intensity of PIN3-YFP signal at the inner lateral and the outer lateral membrane of endodermal cells were measured using ImageJ software. Three replicates of at least 10 seedlings with a synchronized germination start were processed. The presented value is the mean of the averages.

Pharmacological treatments. For auxin-mediated inner-lateralization in hypocotyl, seedlings were germinated and grown in dark on mock medium. NAA treatments in the dark was done by transfer and incubation of 4-day-old etiolated seedlings on solid medium supplemented with NAA (10 µM) for 4 hours and consequently imaged by confocal microscope. For BFA-induced internalization of PIN3, 4-day-old seedlings were treated with 25 µM BFA for 90 minutes and imaged by confocal microscope. Dark-induced vacuolarization of PIN3 protein was performed by covering plate with 4-day-old seedlings with aluminium foil for 12 hours and consequently imaged by confocal microscope. For all comparisons, at least three independent experiments were done with the same significant results.

PIN3-P1A	CTCGAGTGGAGCATCACAATCTTTTCCCTCTCCACACTTCCCAACACTCTTGTTATGGGGATTCCTCTCTTGATCGCCA TGTATGGCGAATACTCTGGTTCCCTCATGGTCCAAATCGTCGTCCTCCAGTGTATCATCTGGTACACGCTTCTCCTTTT TCTCTTCGAGTTTCGTGGCGCCAAGATGCTCATCATGGAGCAGTTCCCTGAGACGGCTGCTTCCATTGTTTCTTTCAAA GTCGAATCCGACGTCGTTTCGCTCGACGGCCATGATTTTCTTGAGACCGATGCAGAGATAGGTGACGACGGGAAGCTTC ACGTCACCGTGAGAAAATCCAACGCTTCACGTCGTTCGTTCTGCGGCCCGAACATGACTCCACGGCCGGCAAATCTCAC CGGAGCTGAGATTTATAGTCTCAGCACCACTCCTAGAGGCGCTAATTTCAACCACTCTGATTTTTACAACATGATGGGT TTCCCCGGTGGTCGTCTCTCCAATTTCGGTCCGGCGGATATGTACTCCGTTCAATCATCTAGAGGTCCAACTCCTCGAC CTGCAAACTTCGAGGAGAATTGCGCCATGGCATCCTCCCCGAGATTCGGGTATTACCCTGGAGGAGGAGCCGGGTCTTA TCCGGCTCCGAATCCAGAGTTCTCTTCAACCACCACATCTACCGCCAATAAAAGCGTCAATAAAAACCCGAAAGACGTT AATACGAATCAGCAGACGACTCTTCCAACGGGCGGCAAGTCAAACAGCCATGACGCCAAGGAGCTTCACATGTTCGTCT GGAGCTCAAACGGGTCACCCGTTTCAGACCGGGCGGGTCTTAACGTTTTCGGCGGAGCACCTGACAACGATCAAGGCGG AAGATCTGACCAAGGTGCTAAAGAGATCCGTATGTTAGTCCCAGATCAATCTCACAACGGCGAGACCAAAGGGGGTGGC ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACA AGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGCTGATCTGCACCACCGGCAA GCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATG AAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCA ACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAA GGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAG AAGAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGC AGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGA CCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTG TACAAGGGAGGTGGACCCGGTTAGCAATCTTTAGATAACTTTCTTGATAAGTCACTGCAAATTTTCTCAATTCTGTTTT GTGTGGTGAATATAGCTGTAGCTCATCCAGCAAGTGGAGATTTCGGAGGAGAACAACAATTTAGTTTCGCCGGAAAAGA AGAAGAAGCAGAGAGACCAAAAGACGCCGAGAATGGTCTAAACAAACTTGCTCCAAATTCCACGGCGGCGCTACAATCC AAGACAGGTCTAGGAGGAGCCGAAGCAAGTCAACGAAAAAATATGCCTCCGGCGAGTGTGATGACAAGGCTGATACTGA TAATGGTTTGGAGGAAACTCATCAGAAACCCAAACACTTACTCTAGTCTCATTGGACTTATTTGGGCTCTCGTCGCTTT CCGGTTAGTAAATCAAAATTAATTGTTTTCTTAACTCGAACCAACCGGTT
PIN3-P1D	CTCGAGTGGAGCATCACAATCTTTTCCCTCTCCACACTTCCCAACACTCTTGTTATGGGGATTCCTCTCTTGATCGCCA TGTATGGCGAATACTCTGGTTCCCTCATGGTCCAAATCGTCGTCCTCCAGTGTATCATCTGGTACACGCTTCTCCTTTT TCTCTTCGAGTTTCGTGGCGCCAAGATGCTCATCATGGAGCAGTTCCCTGAGACGGCTGCTTCCATTGTTTCTTTCAAA GTCGAATCCGACGTCGTTTCGCTCGACGGCCATGATTTTCTTGAGACCGATGCAGAGATAGGTGACGACGGGAAGCTTC ACGTCACCGTGAGAAAATCCAACGCTTCACGTCGTTCGTTCTGCGGCCCGAACATGACTCCACGGCCGGATAATCTCAC CGGAGCTGAGATTTATAGTCTCAGCACCACTCCTAGAGGCGATAATTTCAACCACTCTGATTTTTACAACATGATGGGT TTCCCCGGTGGTCGTCTCTCCAATTTCGGTCCGGCGGATATGTACTCCGTTCAATCATCTAGAGGTCCAACTCCTCGAC CTGATAACTTCGAGGAGAATTGCGCCATGGCATCCTCCCCGAGATTCGGGTATTACCCTGGAGGAGGAGCCGGGTCTTA TCCGGCTCCGAATCCAGAGTTCTCTTCAACCACCACATCTACCGCCAATAAAAGCGTCAATAAAAACCCGAAAGACGTT AATACGAATCAGCAGACGACTCTTCCAACGGGCGGCAAGTCAAACAGCCATGACGCCAAGGAGCTTCACATGTTCGTCT GGAGCTCAAACGGGTCACCCGTTTCAGACCGGGCGGGTCTTAACGTTTTCGGCGGAGCACCTGACAACGATCAAGGCGG AAGATCTGACCAAGGTGCTAAAGAGATCCGTATGTTAGTCCCAGATCAATCTCACAACGGCGAGACCAAAGGGGGTGGC ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACA AGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGCTGATCTGCACCACCGGCAA GCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATG AAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCA ACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAA GGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAG AAGAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGC AGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGA CCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTG TACAAGGGAGGTGGACCCGGTTAGCAATCTTTAGATAACTTTCTTGATAAGTCACTGCAAATTTTCTCAATTCTGTTTT GTGTGGTGAATATAGCTGTAGCTCATCCAGCAAGTGGAGATTTCGGAGGAGAACAACAATTTAGTTTCGCCGGAAAAGA AGAAGAAGCAGAGAGACCAAAAGACGCCGAGAATGGTCTAAACAAACTTGCTCCAAATTCCACGGCGGCGCTACAATCC AAGACAGGTCTAGGAGGAGCCGAAGCAAGTCAACGAAAAAATATGCCTCCGGCGAGTGTGATGACAAGGCTGATACTGA TAATGGTTTGGAGGAAACTCATCAGAAACCCAAACACTTACTCTAGTCTCATTGGACTTATTTGGGCTCTCGTCGCTTT CCGGTTAGTAAATCAAAATTAATTGTTTTCTTAACTCGAACCAACCGGTT

PIN3 -P2A	CTCGAGTGGAGCATCACAATCTTTTCCCTCTCCACACTTCCCAACACTCTTGTTATGGGGATTCCTCTCTTGATCGCCA TGTATGGCGAATACTCTGGTTCCCTCATGGTCCAAATCGTCGTCCTCCAGTGTATCATCTGGTACACGCTTCTCCTTTT TCTCTTCGAGTTTCGTGGCGCCAAGATGCTCATCATGGAGCAGTTCCCTGAGACGGCTGCTTCCATTGTTTCTTTCAAA GTCGAATCCGACGTCGTTTCGCTCGACGGCCATGATTTTCTTGAGACCGATGCAGAGATAGGTGACGACGGGAAGCTTC ACGTCACCGTGAGAAAATCCAACGCTTCACGTCGTTCGTTCTGCGGCCCGAACATGACTCCACGGCCGTCAAATCTCAC CGGAGCTGAGATTTATAGTCTCAGCACCACTCCTAGAGGCTCTAATTTCAACCACTCTGATTTTTACAACATGATGGGT TTCCCCGGTGGTCGTCTCTCCAATTTCGGTCCGGCGGATATGTACTCCGTTCAATCATCTAGAGGTCCAACTCCTCGAC CTTCAAACTTCGAGGAGAATTGCGCCATGGCATCCTCCCCGAGATTCGGGTATTACCCTGGAGGAGGAGCCGGGTCTTA TCCGGCTCCGAATCCAGAGTTCGCTGCAACCACCACAGCTACCGCCAATAAAAGCGTCAATAAAAACCCGAAAGACGTT AATACGAATCAGCAGACGACTCTTCCAACGGGCGGCAAGTCAAACAGCCATGACGCCAAGGAGCTTCACATGTTCGTCT GGAGCTCAAACGGGTCACCCGTTTCAGACCGGGCGGGTCTTAACGTTTTCGGCGGAGCACCTGACAACGATCAAGGCGG AAGATCTGACCAAGGTGCTAAAGAGATCCGTATGTTAGTCCCAGATCAATCTCACAACGGCGAGACCAAAGGGGGTGGC ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACA AGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGCTGATCTGCACCACCGGCAA GCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATG AAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCA ACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAA GGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAG AAGAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGC AGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGA CCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTG TACAAGGGAGGTGGACCCGGTTAGCAATCTTTAGATAACTTTCTTGATAAGTCACTGCAAATTTTCTCAATTCTGTTTT GTGTGGTGAATATAGCTGTAGCTCATCCAGCAAGTGGAGATTTCGGAGGAGAACAACAATTTAGTTTCGCCGGAAAAGA AGAAGAAGCAGAGAGACCAAAAGACGCCGAGAATGGTCTAAACAAACTTGCTCCAAATTCCACGGCGGCGCTACAATCC AAGACAGGTCTAGGAGGAGCCGAAGCAAGTCAACGAAAAAATATGCCTCCGGCGAGTGTGATGACAAGGCTGATACTGA TAATGGTTTGGAGGAAACTCATCAGAAACCCAAACACTTACTCTAGTCTCATTGGACTTATTTGGGCTCTCGTCGCTTT CCGGTTAGTAAATCAAAATTAATTGTTTTCTTAACTCGAACCAACCGGTT
PIN3 -P2D	CTCGAGTGGAGCATCACAATCTTTTCCCTCTCCACACTTCCCAACACTCTTGTTATGGGGATTCCTCTCTTGATCGCCA TGTATGGCGAATACTCTGGTTCCCTCATGGTCCAAATCGTCGTCCTCCAGTGTATCATCTGGTACACGCTTCTCCTTTT TCTCTTCGAGTTTCGTGGCGCCAAGATGCTCATCATGGAGCAGTTCCCTGAGACGGCTGCTTCCATTGTTTCTTTCAAA GTCGAATCCGACGTCGTTTCGCTCGACGGCCATGATTTTCTTGAGACCGATGCAGAGATAGGTGACGACGGGAAGCTTC ACGTCACCGTGAGAAAATCCAACGCTTCACGTCGTTCGTTCTGCGGCCCGAACATGACTCCACGGCCGTCAAATCTCAC CGGAGCTGAGATTTATAGTCTCAGCACCACTCCTAGAGGCTCTAATTTCAACCACTCTGATTTTTACAACATGATGGGT TTCCCCGGTGGTCGTCTCTCCAATTTCGGTCCGGCGGATATGTACTCCGTTCAATCATCTAGAGGTCCAACTCCTCGAC CTTCAAACTTCGAGGAGAATTGCGCCATGGCATCCTCCCCGAGATTCGGGTATTACCCTGGAGGAGGAGCCGGGTCTTA TCCGGCTCCGAATCCAGAGTTCGATGATACCACCACAGATACCGCCAATAAAAGCGTCAATAAAAACCCGAAAGACGTT AATACGAATCAGCAGACGACTCTTCCAACGGGCGGCAAGTCAAACAGCCATGACGCCAAGGAGCTTCACATGTTCGTCT GGAGCTCAAACGGGTCACCCGTTTCAGACCGGGCGGGTCTTAACGTTTTCGGCGGAGCACCTGACAACGATCAAGGCGG AAGATCTGACCAAGGTGCTAAAGAGATCCGTATGTTAGTCCCAGATCAATCTCACAACGGCGAGACCAAAGGGGGTGGC ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACA AGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGCTGATCTGCACCACCGGCAA GCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATG AAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCA ACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAA GGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAG AAGAACGGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGC AGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCTACCAGTCCGCCCTGAGCAAAGA CCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTG TACAAGGGAGGTGGACCCGGTTAGCAATCTTTAGATAACTTTCTTGATAAGTCACTGCAAATTTTCTCAATTCTGTTTT GTGTGGTGAATATAGCTGTAGCTCATCCAGCAAGTGGAGATTTCGGAGGAGAACAACAATTTAGTTTCGCCGGAAAAGA AGAAGAAGCAGAGAGACCAAAAGACGCCGAGAATGGTCTAAACAAACTTGCTCCAAATTCCACGGCGGCGCTACAATCC AAGACAGGTCTAGGAGGAGCCGAAGCAAGTCAACGAAAAAATATGCCTCCGGCGAGTGTGATGACAAGGCTGATACTGA TAATGGTTTGGAGGAAACTCATCAGAAACCCAAACACTTACTCTAGTCTCATTGGACTTATTTGGGCTCTCGTCGCTTT CCGGTTAGTAAATCAAAATTAATTGTTTTCTTAACTCGAACCAACCGGTT

Table S1. Sequences of synthesized fragments containing the PIN3 phosphhorylation mutations.

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Chapter 5.

Conclusions and Perspectives

CONCLUSIONS AND PERSPECTIVES

Already for a long time auxin has been recognized as a crucial signaling molecule that controls plant growth and development. Since then, auxin has been shown to be essential for plant development in mediating diverse responses, such as senescence regulation (Ellis et al., 2005), fruit formation (De Jong et al., 2009), leaf abscission (Rubinstein, 1963), and response to pathogens (Kazan and Manners, 2009; Fu and Wang, 2011) and abiotic stresses (Wang et al., 2010). Auxin governs the establishment and maintenance of polarity, apical dominance, and tropic responses to light and gravity (Woodward and Bartel, 2005; Vanneste and Friml, 2009). At the cellular level, the presence of auxin is recognized by auxin receptors at the cell surface (ABP1) or in the nucleus ($SCF^{TIR/AFB}$) that trigger different auxin responses. Fast, nontranscriptional reactions triggered by the auxin-binding protein 1 (ABP1) include control of cell division and cell elongation by altering cell wall plasticity (Steffens et al., 2001; David et al., 2007; Braun et al., 2008), cell morphogenesis, cytoskeleton rearrangement (Xu et al., 2010, Chen et al., 2012, Nagawa et al., 2012; Chen et al., 2014), and clathrin-mediated endocytosis (Robert et al., 2010). Despite all the about ABP1, there are still plenty of unsolved questions in its signaling pathway, prompting us to unravel some of them.

We revealed that the auxin binding to ABP1 is essential for its function, supporting the role of ABP1 as an auxin receptor at the cell surface. We identified the interacting partners of *Arabidopsis* ABP1 from the SKU5/SKS protein family that are involved in the binding of ABP1 at the endoplasmic reticulum (ER) and facilitate its secretion to the apoplast. We also characterized potential phosphorylation sites in the PIN-FORMED3 (PIN3) protein structure that can be crucial for proper polar localization of PIN3 during various environmental responses.

Auxin binding to ABP1 is crucial for its cellular functions and developmental roles

The structure of the ABP1-binding pocket had been identified a long time ago (Woo et al., 2002). However, the question why the auxin binding to ABP1 is important for its functions has still not been addressed. We showed that the auxin-binding pocket of ABP1 is essential for its known cellular functions and developmental roles. Twelve different *abp1* mutant variants with substitutions in the metal core or hydrophobic amino acids of the auxin-binding pocket indicated that the intact auxin-binding pocket is a crucial prerequisite for proper ABP1

function. Unfortunately an important part that is missing is the binding assay. Despite our great effort we failed to heterologously overexpressed and purified ABP1 protein from three different expression systems (*E. coli*, *Nicotiana bentamiana* and insect cells line). ABP1 protein is forming the multimeric structure therefore using endogenous expression system may distort our biochemical measurements. Nevertheless, mutagenized ABP1 variants could not facilitate many of its biological roles, such as activation of the downstream components Rho of Plants (ROPs) of its signaling pathway that mediate the clathrin association with membranes to regulate endocytosis or promote ABP1 secretion to the cell surface. Auxin binding is also important for secretion of ABP1 protein to the cell surface. To unveil whether signal for secretion comes from auxin sensing at the cell surface, performing transmission electron microscopy with *tmk1/tmk2/tmk3/tmk4* quadruple mutant (Dai et al., 2013; Xu et al., 2014) would significantly help.

Also a number of various developmental processes are controlled by ABP1 in an auxin-dependent manner, including morphology of leaf epidermal cells, vascular tissue differentiation, or root growth and root meristem activity. Hence, these findings revealed that auxin binding to ABP1 is crucial for its function, supporting the role of ABP1 as auxin receptor.

Unfortunately, we could not investigate the *abp1* mutant variants in the absence of the wild-type allele. We were not able to complement the so far only knock-out allele that has been characterized (Chen et al., 2001) with any prepared mutant variant construct. It is interesting that we were not able to complement this line even with the material (*35S::ABP1*) that is published in the original paper. This construct anyway rises doubts, as the 35S promoter in mostly inactive during the early embryogenesis (Custers et al., 1999) and thus cannot participte in rescuing the embryo lethal phenotype of *abp1* knock-out mutant. Therefore we decided to complement the mutant with the constructs possessing the native promotor, but our effort was not successful. The inability to complent this mutant with any phenotype we prepared brings question whether the embryo lethal phenotype is connected with *abp1* mutation. It is possible that there is another mutation in the gene close enough to *ABP1* gene that cannot be simply outcrossed. Our last attempt is to complement the mutation with the BAC clone possessing part of the 4th chromosome, but even positive result will not give us an answer about the presence of another mutation. To answer this problem, we decided to prepare new mutation in *ABP1* gene by using the novel methods for direct DNA editing, CRISPR-Cas9 (Ran et al., 2013). The approach may bring more light into this and help us to unravel the true nature of *abp1* mutant phenotypes.

One of our goals, to investigate some novel functions of ABP1 protein by using the mutant variants remains unfulfilled. Another open question that we wanted to reveal is the role of ABP1 in the ER, where it is not supposed to bind auxin (Bauly et al., 2000). By preparing the non-auxin binding mutant we wanted to simulate this status. Unsuccess in unravelling these questions is mostly due of the inability to complement the mutant allele, therefore they are still open for the future research.

Auxin mediates secretion of its ABP1 receptor through the SKU5-interacting partners

As an extracellular auxin receptor, ABP1 binds auxin with high affinity (Jones and Venis, 1989), but based on its structure (Woo et al., 2002), ABP1 can probably not associate directly with membrane fractions. Therefore, interacting partners that bridge ABP1 and the plasma membrane deserve our attentions.

Recently, the transmembrane receptor-like kinase 1 (TMK1) has been identified identified as an ABP1 pair, coupling extracellular auxin perception by ABP1 with intracellular downstream components (Xu et al., 2014). We showed that members of the SKS (SKU5-similar homologs) protein family can also interact with ABP1, but most probably do not directly facilitate transfer of the signal across the plasma membrane. The ER-localized and cell surface-docking glycosylphosphatidylinositol (GPI)-anchored SKEWED5 (SKU5) protein binds ABP1, both at the ER and on the cell surface in a auxin-dependent manner. The secretion of SKU5 to the cell surface is facilitated by the ABP1-dependent auxin signaling, while the ABP1 secretion to the apoplast is promoted by SKU5/SKSs.

These discoveries imply that there are two possible scenarios of auxin perception. Either auxin binds ABP1 at the cell surface, then signal is transmitted via TMK1 protein to the ER where ABP1 binds SKU5 and SKS4 and this complex is secreted to the apoplast. The second option is the auxin is sensed by ABP1 at the ER. After binding ABP1 molecule slightly change the conformation by exposing the C-terminal part, that is then available for binding with SKU5 protein that helps with the secretion to the apoplast. This scenario is in contrast with previous observations showing that ABP1 cannot bind auxin at pH 7 (Bauly et al., 2000) that is present in the ER (Martinière et al., 2013; Schen et al., 2013). Therefore further evaluation of binding abilities would need to be performed.

To distinguish which of these scenarios is correct, we would need to use the *tmk1/tmk2/tmk3/tmk4* quadruple mutant line (Dai et al., 2013; Xu et al., 2014) and observe the secretion of ABP1 protein after the auxin treatment. It would help us also to confirm that SKU5 secretion is mediated via ABP1 signaling pathway. Unfortunatelly this line was not

available for us. Another interesting experiment would be to test the effect of ABP1-M2X mutant variant in the process of secretion. If these proteins travel together from the ER to the cell surface, levels of ABP1-M2X in the apoplast should not be elevated after auxin even in the SKU5 overexpression line.

It would be also interesting to have a closer look on the C-terminus of ABP1 protein, as this was used as a bait for pulling down CBP1 protein, homolog of SKU5 from *Zea mays* (Shimomura, 2006). By direct mutagenesis we should evaluate which amino acid(s) are responsible for the binding with SKU5 and also how important role the KDEL retention signal is playing in the process of binding.

Another unsolved question is the conformation of the complex that is formed in the apoplast after auxin perception. It was shown that ABP1 is binding TMK1 protein in auxin-dependent manner (Xu et al., 2014). In our work we showed that ABP1 is binding SKS4 in auxin independent manner and that it can form complex with SKS4 and SKU5 in auxin-dependent manner. Further Split-GFP, FRET or co-immunoprecipitation experiments that would unravel which of the TMK1-ABP1-SKU5-SKS4 can interact together at the cell surface in the presence of auxin should be performed.

Mutagenesis of PIN3 phosphorylation sites

The PIN protein phosphorylation is an important mechanism to regulate and maintain proper PIN polarity as a response to different environmental stimuli (Vanneste and Friml, 2009). Up to date there were 2 kinases identified, which are capable of PIN phosphorylation: PINOID kinase (Michniewicz et al., 2007) and D6 protein kinase (Zourelidou et al., 2009).

In the last part of our work, we demonstrated the relevance of two phosphorylation sites (S1 and S2) in the process of PIN polar maintanance, both containing three phosphoryliable residues. We showed that the S2 site plays a certain role in the relocation of PIN3 during gravitropic responses in roots and hypocotyls and also during auxin-mediated relocation in hypocotyls. Phosphorylation of these sites is important also for the PIN3 vacuolar targeting in the PIN degradation process. However, the S1 site did not exhibit some defects in any of the abovementioned experiments, meaning that these residues most probably do not participate in the PIN polarity regulation.

This part of the thesis is still not finished and there are still some things that can be explored. Easy way for confirming some of our observed phenotypes would be to use *rcn1/rcnl1/rcnl2* triple mutant line. RCN proteins are members of PP6 heterotrimeric enzyme that is responsible for dephosphorylation of PIN proteins (Michniewitz et al., 2007). Crossing our

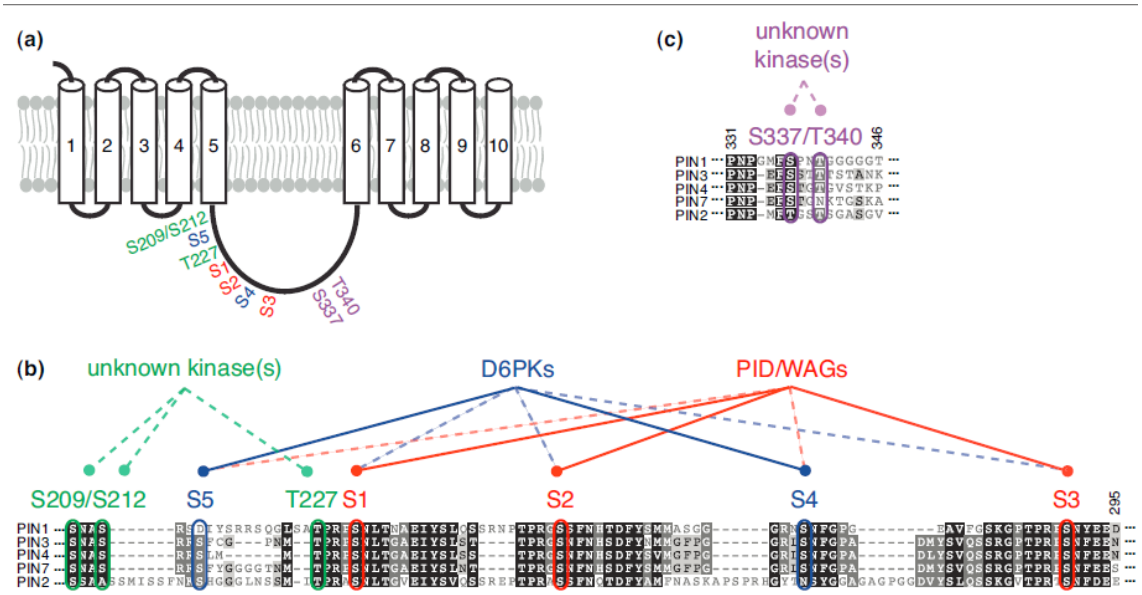

Figure 1. Topology and annotated partial sequence alignment of the 'long' PINs. (a) Schematic representation of the 'long' PINs with the predicted transmembrane-spanning domains and the cytoplasmic hydrophilic loop (CL) with identified phosphosites. (b) Sequence alignment of the N-terminal part of the CL with the five D6PK and PID/WAG phosphosites and their respective phosphosite preferences indicated by the blue/red color code and continuous or dashed lines Additional phosphosites that are unlikely targets of D6PK or PID are shown in green. (c) Alignment of the C-terminal part of the CL with two potential phosphosites that are likely not phosphorylated by PID or by D6PK. Amino acid references are for PIN1. Adapted from Barbosa and Schwechheimer, (2014).

PIN3 mutant lines into *rcn1/rcnl1/rcnl2* triple mutant should exhibit similar phenotypes as PID overexpression. Unfortunately the line was not available for us. It will be interesting to observe PIN3 relocation in the *pin3/pin4/pin7* triple mutant line lacking all three PIN proteins that are expressed in columella and are necessary for proper topic responsis.

It was shown that ABP1-dependent signaling is important for proper PIN3 relocation (Rakusová, unpublished), thus evaluation of relocation rate of PIN3 mutant variant in ABP1 overexpression line, ABP1 conditional knock-down lines or even ABP1-M2X line could help us to estime the importance of phosphorylation in this process.

We should also check whether our mutated proteins can be still phosphorylated. *In vitro* phosphorylation with so far known kinases (PID and D6PK) would reveal whether particular site was targeted. PID and D6PK prefer different phosphorylation sites, but can still partially phosphorylate the site of its concurrent. Therefore it would be interesting to observe the tropic responses and PIN3 relocation in PIN3 mutated lines in the *d6pk* background.

In 2012 another phosphorylation site in the PIN3 hydrophilic loop has been identified (Gangly et al., 2012). This M3 phosphorylation site is also targeted by PID kinase. This could explain mild phenotypes that we observed in our PIN3-P2A/D lines. Creating a PIN3 mutant variant

containing mutations in both P2 and M3 site should significantly increase the defects in tropic responses and in PIN3 relocation rate.

Recently, Barbosa and Schwechheimer, 2014 summarise the work aiming on PIN phosphorylation (Fig. 1). They present in total 10 different phosphorylation sites, from which 5 can be phosphorylated by so far unknown kinases. This is showing how complex mechanism is the PIN polarity regulation via phosphorylation and that there is still plenty of unsolved question in this attractive topic.

In conclusion, understanding of the ABP1 signalling pathway together with regulation and maintenance of the PIN polarity offer new perspectives for auxin engineering and, thus, plant improvement.

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SUMMARY

Auxin, one of the plant hormones, is a key regulator of plant growth and development. At the cellular level, it controls different processes, such as cell expansion, division, and differentiation. The polar auxin transport machinery that mediates the differential auxin distribution within tissues is one of the important features in the auxin action. Auxin transporters are dynamically regulated in response to external and internal stimuli. Changes in cellular auxin concentrations are transduced by auxin signaling systems to trigger different cellular responses. Until now, three proteins, or protein complexes, that can bind auxin have been identified of which two are localized in the nucleus, the SKP-Cullin-F box-Transport Inhibitor Response 1/Auxin-related F-Box ($SCF^{TIR1/AFB}$) (Ruegger et al., 1997; Gray et al., 2001; Dharmasiri et al., 2005; Kepinski and Leyser 2005) and the S-Phase Kinase-Associated Protein 2A (SKP2A) (Jurado et al., 2008; Jurado et al., 2010), and one, the Auxin-Binding Protein 1 (ABP1) occurs predominantly at the endoplasmic reticulum (ER) and cell surface (Jones and Venis, 1989; Rück et al., 1993). The ABP1 protein is involved in fast nontranscriptional auxin responses, such as regulation of the membrane potential and ion fluxes at the plasma membrane (Leblanc et al., 1999), auxin-induced cell swelling (Steffens et al., 2001), cell elongation and cell division (David et al., 2007; Braun et al., 2008), cell morphogenesis and cytoskeleton rearrangement (Xu et al., 2010, Chen et al., 2012, Nagawa et al., 2012; Chen et al., 2014), and clathrin-mediated endocytosis (Robert et al., 2010). Nevertheless, there are still many unsolved questions in the ABP1 signaling process.

In this PhD thesis, we reveal the importance of auxin binding for the proper ABP1 function in its known cellular functions and developmental roles. We demonstrated that mutagenized variants were not able to facilitate the activation of downstream components of the ABP1 signaling pathway, such as activation of RIC4, auxin-mediated inhibition of endocytosis, or ABP1 secretion to the cell surface. Thus, these cellular phenotypes launch several developmental defects in the morphology of leaf epidermal cells, cotyledon vasculature, root growth, and meristem activity. Moreover, our results indicate that auxin binding to ABP1 is crucial for its function as auxin receptor.

Furthermore, we identified another interacting partner for the ABP1 protein, the SKU5 protein localized in the ER and apoplast. Deficiency in the SKS protein family exhibits

phenotypes analogous to thos of the ABP1 immunomodulation lines. We showed that SKU5, together with SKS4, binds to ABP1 in the ER in an auxin-dependent manner and that these proteins facilitate ABP1 secretion from the ER to the cell surface. We propose a working model in which, after auxin binding at the cell surface, ABP1 binds to TMK1 that transmits the signal to the downstream intracellular components to, consequently, trigger SKU5 and SKS4 binding to ABP1 in the ER and to promote secretion of this complex to the cell surface.

Another aspect of our research assessed the relevance of the PIN3 phosphorylation in the PIN polarity regulation and maintenance. We present two potential phosphorylation sites in the PIN3 hydrophilic loop. We demonstrate the importance of the S2 site during gravitropic responses in roots and hypocotyls, vacuolar PIN3 targeting, or its relocation after auxin stimuli in hypocotyls. The S2 site is conserved among the long PIN proteins and plays apparently a certain role in the regulation and maintenance process of the PIN polarity. However, there are other phosphorylation sites that are more crucial to facilitate these porcesses.

Altogether, this PhD research unveils novel insights into the ABP1 structure,, processes in the ABP1 signaling pathway, and PIN polarity regulation.

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RESUME

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PUBLICATION ACTIVITY

1. **Grones P**, Grones J. 2010. Cloning, expression, purification and characterization of replication protein from plasmid pGP2 from *Acetobacter estunensis*. Advances in Bioscience and Biotechnology. 1(5), 417-425. DOI: 10.4236/abb.2010.15055 (ISI) $IF_{(2012)} – 0,4$
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1. Rakusova H., Wabnik K., Sauer M., **Grones P**. , Barbez E., De Rycke R., Chen X., Simon S., Robert H., Kleine-Vehn J., Friml J. *Coordination of cell and tissue polarities via ABP1-mediated extracellular auxin perception* => under review in PLOS ONE

2. Chen X., **Grones P.**, De Rycke R., De Rybel B., Wabnik K., Rakusova H., Cannoot B., Weijers D., Perrot-Rechenmann C., Friml J. *Secretion of ABP1 to Cell-surface is Assisted by SKU5 and SKS Homologues in Arabidopsis* => submitting to Cell

3. Grones P. and Friml J., *Auxin signaling – ABP1 and TIR1 connection* => under review in Journal of Cell Science

4. Grones P., Chen X , Simon S., Kaufman W.A., De Rycke R., Nodzynski T., Detry S., Savvides S., Zažímalová E., Friml J. *The importance of auxin binding for ABP1 function* => submitting to EMBO Reports

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12. Grones P., Cimova V., Bugala J., Grones J.: The Rep20 replication protein from plasmid of Acetobacter. XXIV. Biochemical Symposium SSBMB and ČSBMB, September 18th to 21th 2014, Bratislava Slovak republic.

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CONFERENCE TALK

1. Grones P., Friml J.**:** Characterization of ABP1 auxin binding pocket. 6th International PhD School Plant Development, Sept. 25. – Sept. 27., 2013, Retzbach, Germany

2. Grones P., Xu Chen, Friml J.**:** Importance of auxin binding for ABP1 function. Auxcentric, May 24. – May 25., Norwich, United Kingdom

3. Grones P., Friml J.**:** Importance of auxin binding for ABP1 function. Auxin and Cytokinin in Plant Development, June 29. – July 4. 2014, Prague, Czech Republic

LECTURES

Academic year 2013/2014: Participate in lectures „Methods in Molecular Biology“ at Comenius University, Bratislava, Slovak Republic

QUALIFICATION THESIS

Grones P.: Characterization of plasmid pGP2 from *Acetobacter estunensis* GP2. Diploma work, pp. 1-100, Comenius University, Bratislava 2010 (Diploma thesis).

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