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Regulatory mechanisms for vacuolar trafficking of PIN auxin transporters

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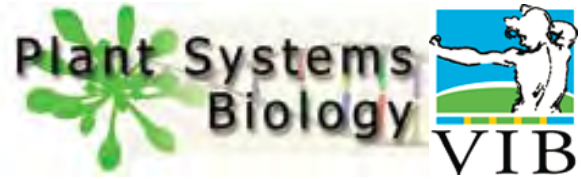

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List of Abbreviations

2,4-D	2,4-DICHLOROPHENOXY ACETIC ACID
2,4,5-T	2,4,5-TRICHLOROPHENOXY ACETIC ACID
5-BR-IAA	5-BROMO-INDOLE-3 ACETIC ACID
5-CL-IAA	5-CHLORO-INDOLE-3 ACETIC ACID
ABA	ABSCISIC ACID
ABCB	ATP BINDING CASSETTE OF B-TYPE
ABP	AUXIN BINDING PROTEIN
ACC	1-AMINOCYCLOPROPANE CARBOXYLIC ACID
AFB	AUXIN SIGNALING F-BOX
ALA	ALANINE
ALX	ALTERED EXPRESSION OF APX2
amiRNA	ARITIFICIAL MICRORNA
AMSH3	ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM
AP	ADAPTOR PROTEIN
ARF	AUXIN RESPONSE FACTOR
ARF	ADP-RIBOSYLATION FACTOR
ASP	ASPARTIC ACID
At	ARABIDOPSIS THALIANA
ATP	ADENOSINE-5'-TRIPHOSPHATE
AUX	AUXIN RESISTANT
AXR	AUXIN RESISTANT
BA	BENZOIC ACID
BAP	6-BENZYLAMINOPURINE
BEN	BFA-VISUALIZED ENDOCYTIC TRAFFICKING DEFECTIVE
BFA	BREFELDIN A
BL	BRASSINOLIDE
BRI	BRASSINOSTEROID INSENSITIVE
BRX	BREVIX RADIX
CAN	CANTHARIDIN
CCD	CAROTENOID CLEAVAGE DIOXYGENASE
CDK	CYCLIN-DEPENDENT KINASE
cDNA	COMPLEMENTARY DNA
CES	CELLULOSE SYNTHASE
CEV	CONSTITUTIVE EXPRESSION OF VSP1
CHMP	CHARGED MULTIVESICULAR BODY PROTEIN/CHROMATIN MODIFYING PROTEIN
CLSM	CONFOCAL SCANNING LASER MICROSCOPY
CME	CLATHRIN MEDIATED ENDOCYTOSIS
CoA	COENZYME A
Col	COLUMBIA
ConA	CONCANAMYCIN A
CPI	CYCLOPROPYLSTEROL ISOMERASE
CY	CARBOCYANINE

D	DWARF
DA	DIHYDROLIPOYL ACETYLTRANSFERASE
DAG	DAYS AFTER GERMINATION
DD	DIHYDROLIPOYL DEHYDROGENASE
DMSO	DIMETHYL SULFOXIDE
DNA	DEOXYRIBO NUCLEIC ACID
DRP	DYNAMIN RELATED PROTEIN
EC	EXOCYST COMPLEX
ECL	ELECTRO CHEMILUMINESCENCE
EE	EARLY ENDOSOME
EEF	EUKARYOTIC ELONGATION FACTOR
EIR	ETHYLENE INSENSITIVE ROOT
ELI	ECTOPIC LIGNIN
EMS	ETHYL METHANESULFONATE
ENP	ENHANCER OF PINOID
EPI	EPIDERMIS
ER	ENDOPLASMATIC RETICULUM
ERP	EXTRACELLULAR RECEPTOR-BASED POLARIZATION
ESCRT	ENDOSOMAL-SORTING COMPLEXES REQUIRED FOR TRANSPORT
FRY	FIERY
GA	GIBBERELIC ACID
GA	GOLGI APPARATUS
GAP	GTPASE ACTIVATING PROTEIN
GBF	GOLGI-ASSOCIATED BREFELDINA-RESISTANT GUANINE NUCLEOTIDE EXCHANGE FACTOR
GEF	GUANINE NUCLEOTIDE EXCHANGE FACTOR
GFP	GREEN FLUORESCENT PROTEIN
GLU	GLUTAMIC ACID
GLV	GOLVEN
GNL	GNOM LIKE
GUS	β-GLUCURONIDASE
HA	HUMAN INFLUENZA HEMAGGLUTININ
HL	HYDROPHILIC LOOP
HOS	HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES
HS	HEAT SHOCK
I3CA	INDOLE-3 CARBOXYLIC ACID
IAA	INDOLE-3 ACETIC ACID
IAAM	INDOLE ACETIC ACID-TRYPTOPHAN MONOOXYGENASE
IAA-L-ALA	INDOLE-3 ACETYL-L-ALANINE
IAR	IAA-CONJUGATE RESISTANT
IBA	INDOLE-3 BUTYRIC ACID
ICR	INTERACTOR OF CONSTITUTIVE ACTIVE ROP
ILA	INDOLE-3 LACTIC ACID
IND	INDEHISCENT
INSP	INOSITOL PHOSPHATE
IXR	ISOXABEN RESISTANT
KYN	L-KYNURENINE
LAX	LIKE AUX1

LE	LATE ENDOSOME
MAB	MACCHI-BOU
MAX	MORE AXILLARY GROWTH
MDR	MULTIDRUG RESISTANT
MEL	MAB4/ENP/NPY1 LIKE
MIN	HOPM INTERACTOR
mRNA	MESSENGER RIBONUCLEIC ACID
MS	MURASHIGE AND SKOOG
MT	MITOCHONDRIAL
MTPS	MITOCHONDRIAL TARGETING PEPTIDE
MVB	MULTIVESICULAR BODY
NAA	NAPHTHALENE-ACETIC ACID
NADH	NICOTINAMIDE ADENINE DINUCLEOTIDE PLUS HYDROGEN
NPH	NON-PHOTOTROPIC HYPOCOTYL
PAGE	POLY ACRYLAMIDE GEL ELECTROPHORESIS
PAR	PARTITIONING DEFECTIVE
PAS	PASSTICINO
PCR	POLYMERASE CHAIN REACTION
PD	PYRUVATE DEHYDROGENASE
PDC	PYRUVATE DEHYDROGENASE COMPLEX
PEO-IAA	α-(PHENYL ETHYL-2-ONE)-INDOLE-3 ACETIC ACID
PGP	PHOSPHOGLYCOPROTEIN
PHOT	PHOTOTROPIN
PI	PROPIDIUM IODIDE
PID	PINOID
PIG	PIN INSENSITIVE TO GR24
PIN	PIN-PINFORMED
PIP	PLASMA MEMBRANE INTRINSIC PROTEIN
PL	PLASTID
PLC	PHOSPHOLIPASE C
PM	PLASMA MEMBRANE
PP	PROTEIN PHOSPHATASE
PVC	PREVACUOLAR COMPARTMENT
QC	QUIESCENT CENTER
RAM	ROOT APICAL MERISTEM
RC	RETROMER COMPLEX
RCN	ROOTS CURL IN NPA
RE	RECYCLING ENDOSOME
REPP	REGULATOR OF PIN POLARITY
RGF	ROOT GROWTH FACTOR
RON	ROTUNDA
ROP	RHO OF PLANTS
RT	REAL-TIME
SA	SALICYLIC ACID
SCF	SKP1/CUL1/F-BOX
SDS	SODIUM DODECYL SULFATE
SEM	STANDARD ERROR OF THE MEAN
SER	SERINE

SL	STRIGOLACTON
SMT	STEROL METHYL TRANSFERASE
SNX	SORTIN NEXIN
STA	STAUROSPORINE
SUPO	SUPPRESSOR OF PIN1 OVEREXPRESSION
TGN	TRANS GOLGI NETWORK
THR	THREONINE
TIR	TRANSPORT INHIBITOR RESPONSE
TUB	TUBULIN
TWD	TWISTED DWARF
UAS	UPSTREAM ACTIVATION SEQUENCE
VAN	VASCULAR NETWORK DEFECTIVE
VLCFA	VERY-LONG-CHAIN FATTY ACID
VPS	VACUOLAR PROTEIN SORTING
VSR	VACUOLAR SORTING RECEPTOR
WAG	WAVY ROOT GROWTH
WXR	WEAK AUXIN RESPONSE

Research Objectives

The phytohormone auxin, through coordinated generation of local accumulations within plant tissues, acts as a central regulator of developmental and environmental responses for plants. The establishment of auxin gradients is facilitated, most prominently, by the activity of efflux carriers of the PIN family, most of which exhibit plasma membrane localization restricted to the specific polar domain. The polar localization of PIN proteins is tightly controlled by multiple mechanisms, including regulated PIN transcription, subcellular trafficking, posttranslational protein modifications and last but certainly not least, regulated protein turn-over. Increasing number of cellular requirements and molecular players, involved in the generation of PIN polarity is known. The knowledge about signals regulating maintenance of PIN polarity is still, largely incomplete. Therefore, the primary research objective of this work was to investigate novel molecular mechanisms involved in the regulation of conditional endocytosis and vacuolar targeting of PIN proteins for their subsequent degradation. This objective included the characterization of signals of both hormonal and non-hormonal nature. Additionall goal of this study was, by implementation of forward genetics approach, to identify and describe novel molecular players involved in the generation of intrinsic PIN polarity.

Chapter 1

Auxin on the road navigated by cellular PIN polarity

Pawel Baster and Jiří Friml

Auxin on the road navigated by cellular PIN polarity

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Abstract

The generation of asymmetry, at both cellular and tissue level, is one of the most essential capabilities of all eukaryotic organisms. It mediates basically all multicellular development ranging from embryogenesis and *de novo* organ formation till responses to various environmental stimuli. In plants, the awe-inspiring number of such processes is regulated by the phytohormone auxin and its directional, cell-to-cell transport. The mediators of this transport, PIN auxin transporters, are asymmetrically localized at the plasma membrane, and this polar localization determines the directionality of intercellular auxin flow. Thus, auxin transport contributes crucially to the generation of local auxin gradients or maxima, which instruct given cell to change its developmental program. Here, we introduce and discuss the molecular components and cellular mechanisms regulating the generation and maintenance of cellular PIN polarity, as the general hallmarks of cell polarity in plants.

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INTRODUCTION

The emergence of multicellularity during the evolution of species had its inevitable repercussions. Efficient intercellular communication was one of such. In other words, to achieve a mutual goal, neighboring cells (single elements of the system) needed to perceive and transduce externally or internally generated signals. As a result, the multicellular organism, as a whole, should be able to translate these signals into a developmental response. This basic necessity for efficient internal communication underlies the origin of the small signaling molecules, termed hormones, present in both plants and animals (Alberts et al., 2007). While the essential role of hormones in cell-to-cell communication is evident in both cases, the response of the organism into which the hormonal signal is translated, diverge between two kingdoms. In animal species solutions based on a modulation of behavior were primarily promoted (Davies, 2004). In contrast, plants, due to the sessile nature of their lifestyle, developed a remarkable repertoire of mechanisms which allow them, through fine-tuning of metabolism or body shaping, to adjust and survive in ever changing and often adverse environments (Tanaka et al., 2006). These mechanisms are facilitated not only by intercellular communication but also by tightly regulated cell division, morphogenesis and differentiation. Importantly, most, if not all of them, are based on cell polarization and repolarization which guide tissue and organ patterning and thus underpin basic shape and functionality of an organism. The phenomenon of cell polarity can be reflected in various aspects like disproportional growth or asymmetrical distribution of the cellular components. The term itself, however, is much broader and in largest sense applies to the generation of any asymmetry in relation to an axis (Sauer and Friml, 2004; Geldner, 2009).

Despite its complexity, plant development, with its remarkable polarization-based flexibility, is coordinated most prominently by a single phytohormone - auxin - which serves itself as a polarizing cue (Berleth and Sachs, 2001; Sauer et al., 2006; Leyser, 2011). Moreover, generation of local auxin accumulations (gradients) plays an essential role in plethora of crucial events during plant development, like embryogenesis (Friml et al., 2003; Schlereth et al., 2010), organogenesis (Benková et al., 2003), phyllotaxis (Reinhard et al., 2003), root meristem organization (Sabatini et al., 1999; Friml et al., 2002a), root stem cell differentiation (Ding and Friml, 2010) or vascular tissue patterning (Scarpella et al., 2006). Although essentially all plant tissues possess the capacity for auxin biosynthesis (Mano and Nemoto, 2012; Ljung 2013) and metabolism (Ludwig-Müller, 2011; Ruiz Rosquete et al., 2012), it is the directional auxin transport that most significantly contributes to establishing of auxin gradients across plant tissues (Tanaka et al., 2006). The existence of such a cell-to-cell transport of auxin, which is the unique feature among other phytohormones, was predicted in mid 1970s by so called 'chemiosmotic model' (Rubery and Sheldrake, 1974; Raven, 1975). This model postulated an auxin efflux from the cell, facilitated by polarly localized exporters, as a critical step during intercellular auxin translocation. These predictions were spectacularly confirmed by characterization of the broad spectrum of developmental phenotypes caused by mutations in the *PIN-FORMED* (*PIN*) genes (Gälweiler et al., 1998; Luschnig et al., 1998; Okada et al., 1991). Basically all different phenotypes found in various *pin* mutants could be mimicked by treatments with auxin transport inhibitors (Vieten et al., 2007). Transport assays from both plant and heterologous systems, provided later, showed that PIN proteins indeed mediate auxin export from the cells (Petrasek et al., 2006). The intercellular auxin transport, beside of PINs, rely on the coordinated activity of two other transporter families. These are: AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) and

MULTIDRUG RESISTANCE/PHOSPHOGLYCOPROTEIN/ATP BINDING CASSETTE OF B-TYPE (MDR/PGP/ABCB) proteins, facilitating influx and efflux of auxin from the cell, respectively (Bennett et al., 1996; Noh et al., 2001; Kramer, 2004; Yang et al., 2006; Mravec et al., 2008; Swarup et al., 2008; Verrier et al., 2008). Despite the fact that multiple components are involved, a critical control of the directionality of auxin flux is attributed to the efflux activity of the PIN transporters at their highly defined, polar, subcellular domains (Wiśniewska et al., 2006; Blakeslee et al., 2007; Titapiwatanakun et al., 2009). PIN family consist of eight members, most of which (PIN1, 2, 3, 4, 6, and 7) dependent on the tissue or developmental context exhibit plasma membrane (PM) localization restricted mainly to the apical (shootward; shoot-apex-facing) or basal (rootward; root-apex-facing) side of the cell (Zazímalová et al., 2007). Notably, also AUX/LAXs and ABCBs, which serve as an additional source of auxin for PIN-mediated transport (Geisler et al., 2005; Mravec et al., 2008; Christie et al., 2011; Kubeš et al., 2012), in some cases display asymmetric distribution (Swarup et al., 2001; Panikashvili et al., 2007; McFarlane et al., 2010).

Although some similarities can be found (Geldner, 2009), in general, the mechanisms underlying cell polarity in plants differ from those, characterised in animals (Tepass et al., 2001; Humbert et al., 2006; Wells et al., 2006; Chen et al., 2010). It seems that in both animal and plant systems the delivery of protein to the place of action by subcellular trafficking is equally important (Dudu et al., 2004; Altschuler et al., 2008; Geldner, 2009; Shivas et al., 2010). On the other hand, the most prominent trafficking-based determinants of polarity found in animals, like CRUMBS, SCRIBBLE and PAR complexes are missing in plant genomes (Geldner, 2009). Additionally, counterparts of so called 'tight junctions' which in animals serve as diffusion barriers, dividing PM of epithelial cells into apical and baso-lateral, polar domains (Giepmans and van Ijzendoorn, 2009), are missing in majority of plant cell types. A similar structure is present in plants in form of 'Casparian Strip', belts of specialized cell wall material generating an extracellular diffusion barrier, found exclusively in endodermis (Roppolo et al., 2011). Polar trafficking pathways described in plants cells appear to be more complex than those found in animals. Besides apical and basal PM domains, characteristic for animal epidermal cells, also outer- and inner-lateral domains, with corresponding polar cargos, can be found in similar cell types in plants (Miwa et al., 2007; Langowski et al., 2010; Takano et al., 2010). What is more, the differences are reflected not only on the cellular but also on the tissue level. Plants in contrast to animals cannot use the mechanism of invasive, cell-migration-based tissue patterning due to the presence of the rigid extracellular matrix, cell wall, encapsulating plant cells and making them immobile (Dettmer and Friml, 2011).

Considering the aforementioned differences it is not surprising that alternative, to animal, solutions were promoted by evolution in plant kingdom. In this chapter these plant-specific mechanisms for cellular polarization will be discussed. The polarity of cellular components will be mainly considered in context of PIN proteins, due to their essential role in auxin-mediated plant development. What is more, in case of PIN family enough molecular components and polarity generating/maintaining signals is known for drawing a comprehensive and interesting overview. First, subcellular trafficking machinery relevant for PIN polarity will be described. Next, cargo-based followed by cell-structural determinants for targeting and maintaining of PINs at their polar domain will be presented. Finally, the feedback mechanisms for PIN polarization will be discussed. It should be taken into account that such a categorization, due to frequently redundant nature of the biological processes, is largely subjective and serves mainly for presentation purposes.

LONG JOURNEY WITH UNSURE DESTINATION - TRAFFICKING FOR PIN POLARIZATION

Polar localization of PIN auxin transporters, restricted to the specific side of the cell relies, among other mechanisms, on the function of complicated network, encompassing multiple bypassing and interconnected pathways collectively referred to as 'intracellular trafficking' (Paul and Frigerio, 2007; Bassham et al., 2008; Robinson et al., 2008; Irani and Russinova, 2009; Zárský et al., 2009). Auxin transporters, subjected to intracellular trafficking, originate from *de novo* synthesis and are secreted to the PM during so called anterograde transport. This mode of intracellular transport generally involves sequential steps including protein folding in Endoplasmatic Reticulum (ER), translocation through the cis- and trans-cisternae of the Golgi Apparatus (GA) with final arrival to the cell surface (Vitale and Denecke, 1999; Matheson et al., 2006).

CONSTITUTIVE PIN CYCLING FOR RAPID REPOLARIZATIONS

The polar localization of PIN proteins, contrary to usually presented, static snapshot pictures, in reality is very dynamic. Once delivered to the cell surface, PINs undergo continuous shuttling between PM and intracellular compartments by rounds of internalization (endocytosis) and polar recycling (exocytosis). These processes are jointly referred to as 'Constitutive Endocytic Cycling' (Geldner et al., 2001; Dhonukshe et al., 2007; Kleine-Vehn et al., 2011). It is not entirely clear to which extent the initial secretion of *de novo* synthesized PINs to the PM occurs in polar fashion (Langowski and Friml, unpublished) or whether the endocytic cycling is generating the polar distribution following apolar secretion (Dhonukshe et al., 2008a).

Nevertheless, internalized, vesicle-encapsulated PINs on endocytic trafficking route from the PM reach their first station, namely the Early Endosome (EE). This subcellular compartment is critical as it is the intersection between secretory and endocytic routes and, in plants, originates from and associates with Trans Golgi Network (TGN) (Dettmer et al., 2006; Lam et al., 2007; Viotti et al., 2010). Thus, sorting of vesicular cargo for various subcellular destinations, occurs at EE/TGN (Liu et al., 2002; Dettmer et al., 2006). Once destined for recycling, PINs are trans-located from EE/TGN to the hypothetical compartment called Recycling Endosome (RE), where they fall under the control of ADP-Ribosylation Factor GTPase (ARF-GTPase) machinery. ARF-GTPases, by recruitment of vesicle coat proteins and organizing cytoskeleton at membrane surfaces, control vesicle trafficking. Their spatio-temporal activity is determined by the antagonistic activity of ARF-Guanine Nucleotide Exchange Factors (ARF-GEFs) and ARF-GTPase-Activating Proteins (ARF-GAPs), which are activating and deactivating ARF-GTPase complex, respectively (D'Souza-Schorey and Chavrier, 2006). The GNOM protein, belonging to the Golgi-Associated, BrefeldinA-Resistant Guanine Nucleotide Exchange Factor (GBF) class of ARF-GEFs most prominently controls the polar recycling of PINs to the PM (Geldner et al., 2003). This protein, and more specifically its Sec7 domain, is a target of fungal toxin BrefeldinA (BFA), which by inhibiting GNOM-mediated exocytosis, causes reversible intracellular accumulation of constitutively endocytosed proteins and aggregation of TGN into so called 'BFA-compartments' or 'BFA-bodies'. This effect serves as a tool to visualize the constitutive cycling of PM proteins (Geldner et al., 2001). Interestingly, GNOM controls preferentially PIN recycling to the basal side of the cell whereas pathway by which PINs are targeted to the apical domain is, most likely, additionally controlled by BFA-insensitive ARF-GEF (Kleine-Vehn 2008a;

Kleine-Vehn et al., 2008b).

Besides ARF-GTPases, also RabA1B, a member of small Rab-GTPase family and INTERACTOR OF CONSTITUTIVE ACTIVE ROP1 (ICR1), an effector of RHO OF PLANTS1 (ROP1) RAC-GTPase, were recently associated with defective PIN recycling (Hazak et al., 2010; Feraru et al., 2012). Importantly, genetic interference with ICR1 results in severely disturbed polarity of PIN1 and PIN2, as well as various developmental defects (Hazak et al., 2010). Moreover, ICR1 was shown to interact with Sec3A (Lavy et al., 2007), one of the Exocyst Complex (EC) components. EC is known to participate in the extensive fusion of exocytic vesicles at specific sites of PM during so called Polarized Exocytosis. Polarized Exocytosis is controlled, among the others, by Rho GTPases (Zárský et al., 2009). Interestingly, another subunit of the EC, Exo70, influences polar auxin transport through the regulation PIN1 and PIN2 recycling (Drdová et al., 2013).

The fundamental role of the cellular scaffolding and cytoskeleton for intracellular PIN trafficking should be also underlined here. PIN constitutive cycling seems to depend mainly on actin filaments since pharmacological interference with the integrity of this component abolishes internalization and recycling of PINs. On the other hand, microtubules are essential for both PIN trafficking in dividing cells as well as for maintenance of the general polarity of the cell (Geldner et al., 2001; Friml et al., 2002b; Petrasek and Schwarzerova, 2009; Dhonukshe et al., 2008b; Boutté et al., 2006; Kleine-Vehn et al., 2006; Kleine-Vehn and Friml, 2008; Klein-Vehn et al., 2008b; Kleine-Vehn et al., 2008c). The components of cytoskeleton not only serve as an orientation cues, according to which PINs polarize (Heisler et al., 2010), but also provide guidance for vesicle trafficking (Voigt et al., 2005).

The functional significance of an energy-demanding process such as constitutive cycling still remains unclear. Evidently, this process provides the means for fast and *de novo* synthesis-independent repositioning of PIN auxin transporters in response to internal and external cues. It, thus, allows developmental flexibility conditioned by rapid redirection of auxin fluxes within tissues (Michniewicz et al., 2007a; Vanneste and Friml, 2009). Auxin transporters were proposed to have an additional function, similar to known, dual-function receptor/transporters (Hertel, 1983; Foti et al., 2004; Holler and Dikic, 2004). In this scenario, endocytic cycling, analogously to the situation in animals, would serve as a way to transduce the signal and to regenerate receptors during ligand-dependent endocytosis. Another, attractive but also unproven, scenario highlights a possible analogy between auxin efflux and neurotransmitter release. In this scenario, PIN transporters localized at the surface of constitutively cycling intracellular vesicles would mediate uptake of auxin from the cytosol into these vesicles and after their arrival and fusion with the PM, auxin would be released from the cell, similarly to the synaptic release of neurotransmitters (Friml and Palme, 2002; Baluška et al., 2003). This would also provide a connection between the effect of established inhibitors of auxin transport on both vesicle trafficking and on PIN-dependent auxin transport (Geldner et al., 2001; Dhonukshe et al., 2008b). It is important to note that all these scenarios for functionality of constitutive cycling in the process of auxin transport are not mutually exclusive and the latter two (transporter/receptor and “neurotransmitter”) are highly speculative.

On the other hand, changes in PIN polarity have been observed in courses of many developmental processed including embryogenesis (Friml et al., 2003), organogenesis (Benkova et al., 2003; Reinhardt et al., 2003), fruit development (Sorefan et al., 2009), vascular tissue formation and regeneration (Scarpella et al., 2006; Balla et al., 2011), as well as in response to light (Ding et al., 2011)

or to gravity (Kleine-Vehn et al., 2010; Rakusova et al., 2011) during tropisms. Such a dynamic translocation of polar cargos from one cell side to another via recycling endosomes is called transcytosis (Tuma and Hubbard, 2003; Kleine-Vehn et al., 2008a; Kleine-Vehn et al., 2008b) and it appears that plants evolved to utilize this mechanism to adjust their development in response to different cues via redirecting of PIN-dependent auxin fluxes.

EARLY ENDOCYTIC PROCESSES FOR PIN POLARIZATION

The first step of endocytic recycling is the internalization from the PM. The most prominent route of PIN internalization occurs through the creation of the membrane curvature from which upon scission the intracellular vesicle is formed, during 'Clathrin Mediated Endocytosis' (CME). Clathrin is a self-assembling protein, recruited to the membranes where it contributes to membrane deformation and serves as a vesicular coat constituent (Chen et al., 2011). The fundamental role of CME in PIN trafficking and thus its essential contribution to generation of intrinsic polar localization of PINs is well characterized. It was demonstrated by pharmacological and genetic interference with the clathrin function (Dhonukshe et al., 2007; Kitakura et al., 2011). The notion that auxin efflux carriers from the PIN family are cargos of CME was further supported by identification of DYNAMIN RELATED PROTEIN 1 (DRP1) as associated with PIN1 and important for its endocytosis (Mravec et al., 2011). In plants the precise function of the dynamin superfamily, represented, among others, by DRP1 is not well characterized. It is speculated that these proteins might regulate membrane dynamics by modulation of its scission and tubulation (Praefcke and McMahon, 2004; Heymann and Hinshaw, 2009). Importantly, proteins belonging to this family were previously implicated in plant CME (Konopka et al., 2008; Fujimoto et al., 2010) and interference with DRP1 function results in altered distribution of PIN1 in dividing cells which eventually leads to a range of developmental phenotypes related to defective auxin transport (Mravec et al., 2011).

Interestingly, ARF-GEF GNOM, function of which is typically associated with recycling to the PM, was also implicated in the regulation of endocytosis, based on its partial localization at the PM and PIN endocytosis defects observed in *gnom* knock-down mutants (Naramoto et al., 2010). In addition, another GBF subfamily member GNOM LIKE1 (GNL1) and ARF-GAP - VASCULAR NETWORK DEFECTIVE 3 (VAN3) have been functionally associated with PM and endocytic processes, corroborating the notion that ARF GTPase machinery is involved in endocytosis (Teh and Moore, 2007; Naramoto et al., 2010).

Following internalization from the PM, the early endocytic trafficking of PINs has been shown to rely on the BFA-VISUALIZED ENDOCYTIC TRAFFICKING DEFECTIVE1/ HOPM INTERACTOR7 (BEN1/MIN7/BIG5) ARF-GEF, belonging to BFA-Inhibited Guanine Nucleotide-Exchange Protein (BIG) subfamily (Tanaka et al., 2009). PIN internalization is regulated also by Rab5/ARA7, a member of RabGTPase family (Dhonukshe et al., 2008a) and an universal component of membrane fusion in eukaryotes – VACUOLAR PROTEIN SORTING45 (VPS45/BEN2) (Tanaka et al., 2013). Genetic interference with all aforementioned components regulating endocytosis or early endocytic trafficking leads, besides perturbations in PIN trafficking and polarity, to significant developmental aberrations like embryogenesis and organogenesis defects, reduced growth and apical dominance, leaf venation pattern discontinuity and root meristem disorganization. These observation links early endocytic processes to PIN polarity and auxin-mediated development.

LATE ENDOCYTIC TRAFFICKING FOR PIN ABUNDANCE AT THE CELL SURFACE

Certain proportion of internalized PINs, based on signals which are not fully understood, is targeted for the late endocytic pathway. This mode of transport originates generally at EE/TGN, where proteins are sorted, proceeds through Late Endosomes (LE), Prevacuolar Compartments/Multivesicular Bodies (PVC/MVB) and terminates at the final destination of membrane proteins – the lytic vacuole. This subcellular route eventually results in protein degradation. Vacuolar targeting defines additional mechanism, by which polar localization of PINs and specifically the aspect of their membrane abundance can be controlled (Müller et al., 2007; Scheuring et al., 2011). As mentioned above, plant trafficking machinery encompasses multiple bypassing, often uni-directional transport routes. Accordingly, PINs can be retrieved from late endocytic pathway and thus avoid degradation. This is accomplished by the Retromer Complex (RC) capacity for retrieval of certain trafficking components like VACUOLAR SORTING RECEPTORS (VSR) from PVC to EE/TGN (Arighi et al., 2004; Seaman, 2005). Both the core component of this complex – VACUOLAR PROTEIN SORTING29 (VPS29) and its prominent interactor – SORTIN NEXIN1 (SNX1) were shown to control the rate of PINs progression towards the vacuole (Kleine-Vehn et al., 2008c; Nodzyński et al., 2013). Such a mechanism is in agreement with widely accepted and evolutionary conserved function of RC (Arighi et al., 2004; Seaman, 2005; Shimada et al., 2006). Notably, a more unorthodox function, related more directly to PIN polarity has been also proposed for RC at the level of early endocytic recycling (Jaillais et al., 2006; Jaillais et al., 2007).

The significance of the final steps during late endocytic trafficking of PIN proteins for their polar localization and abundance is not to be underestimated. The Endosomal-Sorting Complexes Required For Transport (ESCRT) machinery, controlling the formation of internal vesicles within PVC/MVB, which upon fusion are released into vacuolar lumen, appears to play important role for PIN polarization (Winter and Hauser, 2006; Wollert et al. 2009; Scheuring et al. 2011). Interfering with the function of CHARGED MULTIVESICULAR BODY PROTEIN/CHROMATIN MODIFYING PROTEIN 1A and 1B (CHMP1A/CHMP1B), components of ESCRT machinery, leads to severe developmental defects including seedling lethality. These phenotypes were associated with inaccurately generated auxin distribution correlated with the ectopic (PVC/MVB and vacuolar membranes) PIN localization (Spitzer et al., 2009). Also an alternative, PVC-bypassing, late endocytic pathway, dependent on the ADAPTOR PROTEIN (AP) Complex 3 subunits β and δ, generally regulates vacuolar function and thus PIN degradation rate, although it does not affect PIN polarity or abundance at the PM (Feraru et al., 2010; Zwiewka et al., 2011).

WHERE TO GO AND WHY TO STAY? – CUES AND CELLULAR REQUIREMENTS FOR PIN POLARITY

CARGO-BASED DETERMINANTS FOR POLAR PIN LOCALIZATION

One of the most important initial findings concerning the determination of PIN polarity was derived

from the ectopic PIN expression in particular cell types. The PIN2 promoter-driven expression of PIN1 targeted this protein predominantly to the basal side of root epidermal cells contrasting to the native apical PIN2 localization in the same cells. Consecutive introduction of the fluorescent tag into certain place within central hydrophilic loop of PIN1 was sufficient to cause basal-to-apical switch in PIN1 localization.

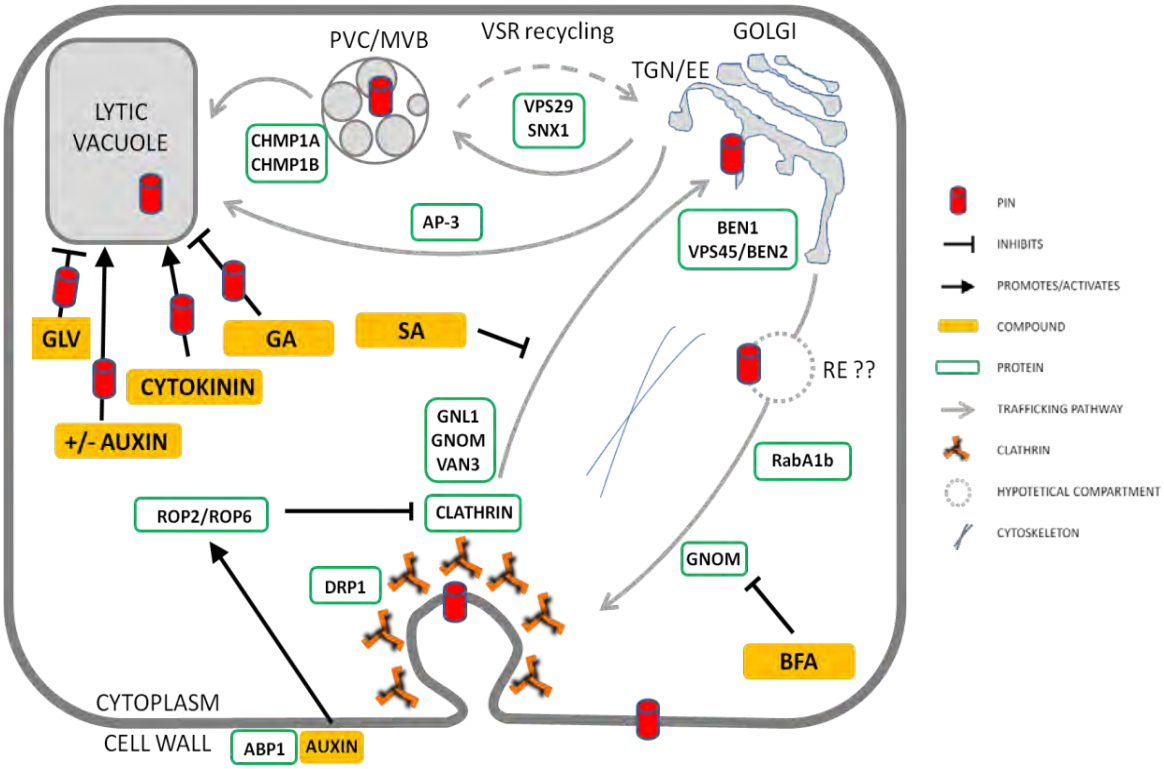

Figure 1. Intracellular trafficking for polarization of PINs. Auxin, when extracellularly bound to ABP1, rapidly inhibits clathrin-mediated PIN endocytosis through mutually exclusive ROP2/ROP6 signaling. Formation of the PIN-containing clathrin-coated vesicles requires the function of DRP1 and is inhibited by SA. Subsequent internalization of cargo vesicles from the plasma membrane is mediated by Rab5 GTPase ARA7, ARF-GEFs GNOM, GNL1 and ARF-GAP VAN3 and proceeds along cytoskeletal cell scaffold. Early endocytic trafficking of PINs requires function of VPS45 BEN2 and ARF-GEF BEN1. PIN recycling depends on RabA1b GTPase and GNOM, a target of BFA-mediated inhibition of exocytosis. PIN vacuolar targeting route passing through PVC/MVB includes the regulation by Retromer Complex components VPS29 and SNX1, as well as ESCRT components CHMP1A and CHMP1B. PVC/MVB-bypassing route for regulating vacuolar function is mediated by AP-3 complex. Cytokinin and long-term, above- or below-optimal auxin levels reduce the membrane abundance of PINs by promoting their turn-over. In contrast, vacuolar targeting of PINs is inhibited by GA and GLV peptides.

This was a clear indication that some determinants of polar PIN localization are encoded intrinsically in PIN amino-acid sequence (Wiśniewska et al., 2006). This sequence-based instruction turned out to be the phosphorylation status of specific serine residues located within PIN central hydrophilic loop (Huang et al., 2010; Zhang et al., 2010). Current model postulates that de-phosphorylated PINs are preferentially recruited to the basal, GNOM-dependent and BFA-sensitive pathway, whereas

phosphorylation targets PINs into the apical cell side, independently of GNOM function (Friml et al., 2004; Michniewicz et al., 2007b; Kleine-Vehn et al., 2009).

Readjustments of PIN phosphorylation status rely on the antagonistic activity of protein kinases and phosphatases. AGC3 protein kinases PINOID (PID) and its homologs WAVY ROOT GROWTH1/2 (WAG1/WAG2) (Benjamins et al., 2001; Friml et al., 2004; Santner and Watson, 2006; Dhonukshe et al., 2010) phosphorylate, while PROTEIN PHOSPHATASE2A (PP2A) (Muday and DeLong, 2001; Michniewicz et al., 2007b; Ballesteros et al., 2013) dephosphorylate PINs. Along with PID and WAG proteins, also other kinases such as D6 protein kinase (Zourelidou et al., 2009) or CDPK-RELATED KINASE5 (Rigó et al., 2013) can phosphorylate PIN proteins and regulate their function but their exact role is less clear. On the other hand, phosphatase subunit PP2AA interacts with another Ser/Thr protein phosphatase, FyPP1, to form functional holoenzyme. FyPP1 and its close homolog FyPP3 were reported to interact with and directly de-phosphorylate PINs (Dai et al., 2012). Importantly, fluctuations of PIN phosphorylation status both above and below certain native threshold lead eventually to severe developmental aberrations like defective embryogenesis and patterning of shoot apical meristem and root (Christensen et al., 2000; Benjamins et al., 2001; Friml et al., 2004; Michniewicz et al., 2007b; Dhonukshe et al., 2010; Li et al., 2011; Dai et al., 2012).

Given the crucial role of PID-mediated PIN phosphorylation in PIN polarity it is important to highlight that the regulation of PID activity, on various levels, have also impact on polar PIN localization and thus on auxin fluxes. Calcium (Ca^{2+}), which is one of the most ubiquitous secondary messengers in eukaryotes, appears to be the prominent part of such a system for the regulation of PID kinase activity. Early experiments in animal, and yeast systems have associated the appearance of cytosolic Ca^{2+} with the phospholipase C (PLC)-generated inositol trisphosphate ($InsP_3$). PLC signaling is known to be important for various biological processes including cell division and differentiation (Michell et al., 2008). The plant field has followed this paradigm upon an observation that $InsP_3$ is able to trigger the release of Ca^{2+} from the cellular storage compartments (Blatt et al., 1990; Gilroy et al., 1990; Krinke et al., 2007; Tang et al., 2007). Controversially, up to date no unambiguous $InsP_3$- activated Ca^{2+} channel could be identified in plants (Testerink and Munnik, 2011). Interestingly, there are indications that $InsP_6$ can function as a signaling molecule, triggering Ca^{2+} release with a much higher potency than $InsP_3$ (Lemtiri-Chlieh et al., 2003). Moreover, $InsP_3$ when microinjected into plant, can be rapidly converted into $InsP_6$ (Munnik and Testerink, 2009), explaining the earlier observations of $InsP_3$ being able to release Ca^{2+}. PID kinase was shown to be regulated by both phospholipid and Ca^{2+} signaling. Some PID interactors bind Ca^{2+} (Benjamins et al., 2003; Zegzouti et al., 2006). Moreover, *suppressor of PIN1 overexpression*-1 (*supo-1*) mutant, encoding ALTERED EXPRESSION OF APX2 8/FIERY1/HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES2/ROTUNDA1 (ALX8/FRY1/HOS2/RON1/SAL1) inositol polyphosphate 1-phosphatase also with 3'(2'),5'-bisphosphate nucleotidase activity exhibits aberrant PIN polarization presumably as a result of changed PID activity (Zhang et al., 2011a). It was proposed that the above effect is a result of altered content of cytosolic Ca^{2+} in a mutant due to disturbed $InsP_3$ metabolism. Although in light of recent findings, an alternative explanation, in which $InsP_6$ would be the signal activating Ca^{2+} cascade, seems more plausible (Munnik and Nielsen, 2011).

Another mode of PID activity regulation applies to its transcription. INDEHISCENT (IND), a basic helix–loop–helix transcription factor through negative regulation of PID and WAG2 expression influences polar localization of PIN1 and PIN3 proteins. Interestingly, in this case local auxin minimum, which is

required for valve margin formation in *Arabidopsis* fruit, is not properly established. Consequently, abnormal fruits which fail to open and thus do not disperse seeds are observed in *ind* mutant (Sorefan et al., 2009). Another regulator of PID activity is ENHANCER OF PINOID/MACCHI-BOU4 (ENP/MAB4) (Treml et al., 2005; Furutani et al., 2007) and its close homologs MAB4/ENP/NPY1-LIKEs (MEL1, MEL2, MEL3, MEL4) that are known to influence polar PIN localization (Furutani et al., 2011). MAB4 encodes NON-PHOTOTROPIC HYPOCOTYL3-like (NPH3)-like protein which associates with light activated kinase PHOTOTROPIN1 (PHOT1), a blue-light receptor (Motchoulski and Liscum, 1999). NPH3 has been shown to modulate PIN2 trafficking in root phototropism (Wan et al., 2012).

Regulation of PID activity provides also an entry point for various external signals, such as light or gravity, to which plants are able to dynamically respond by modulation of their growth. For example PIN3 and PIN7, during hypocotyl gravitropic response, polarize to the bottom side of gravity-sensing endodermal cells and mediate the differential auxin accumulation at the lower side of hypocotyl for asymmetric bending and growth (Rakusova et al., 2011). Accordingly, during phototropic response, blue-light-dependent signaling cascade causes polarization of PIN3 away from the light, in hypocotyl endodermal cells. This coincides with an establishment of auxin maximum at the shaded side of an organ (Ding et al., 2011). Importantly, in both cases PIN repolarization is initiated by differential recruitment into GNOM-mediated trafficking pathway and depends on the PID-mediated phosphorylation status of the PIN protein (Ding et al., 2011; Rakusova et al., 2011).

Beside PIN phosphorylation, also another post-translational protein modification appears to be instructive for PIN localization. The destabilization of PINs from the PM and their sorting for vacuolar delivery was associated with the linking of the polyubiquitin chains to specific lysine residues within PIN2 central hydrophilic loop (Leitner et al., 2012). Moreover, PIN2 degradation was shown to be dependent on the 26S proteasome, the universal proteolysis complex of eukaryotic organisms, which targets ubiquitinated, typically soluble proteins (Sieberer et al., 2000; Abas et al., 2006). Although recent data clearly shows that ubiquitination is a crucial part of PIN abundance control (Abas et al., 2006; Leitner et al., 2012). It remains unclear how proteasome activity can contribute to the degradation of PM proteins such as PINs, which were shown to be targeted to the lytic vacuoles (Kleine-Vehn et al., 2008c; Laxmi et al., 2008; Shirakawa et al., 2009; Marhavy et al., 2012; Baster et al., 2013).

CELL STRUCTURAL DETERMINANTS OF PIN POLARITY MAINTENANCE

The cues and mechanisms described in the previous paragraph dealt mainly with targeting of PINs to their respective polar domains but not with their maintenance there. As mentioned before, crucial polarity trafficking components as well as ‘tight junctions’, which in animals limit migration of the surface proteins between polar domains of epithelial cells, are absent in the plant kingdom. On the other hand plant cells are surrounded by cell wall, a structure absent in animal cells. It appears therefore that plants, in order to preserve an abundance and asymmetry of proteins within fluid PM, developed overlapping but also alternative, to animals, mechanisms. Lateral diffusion, process through which transmembrane proteins migrate within the lipid bilayer, can serve as an example of polarity regulation mechanism common for plants and animals. PINs, when compared with non-polarly localized PM proteins, display reduced rates of lateral diffusion (Dhonukshe et al., 2008a; Men et al., 2008; Kleine-Vehn et al., 2011). This exceptionally low lateral diffusion for auxin transporters was proposed to be

linked with processes that actually immobilize fraction of PINs within specific structures at the PM, called clusters, which have been detected by super-resolution microscopy approaches (Kleine Vehn et al., 2011). The nature of these highly immobile structures is not entirely clear, however their appearance might be related with specific sterol and lipid composition of the PM (Kleine Vehn et al., 2011; Men et al., 2008; Roudier et al., 2010; Carland et al., 2010; Martinière et al., 2012). In fact, the *sterol methyl transferase 1* (*smt1*) mutant, function of which is required for appropriate synthesis and composition of major membrane sterols (Diener et al., 2000) is characterized by defective polar auxin transport correlating with mislocalization of PIN1 and PIN3 proteins (Willemsen et al., 2003). Similarly, the improper reestablishment of PIN2 polarity following cytokinesis as a consequence of defective PIN2 endocytosis was reported for sterol biosynthesis, *cyclopropylsterol isomerase1-1 (cpi1-1)* mutant (Men et al., 2008). The involvement of sterols in polar distribution of PIN proteins is additionally supported by the fact that the internalized PIN2 co-localizes with the sterol marker filipin and a prolonged disruption of membrane sterols by filipin treatments reduces the heterogeneity and polar localization of PIN2 in the PM (Grebe et al., 2003; Kleine-Vehn et al., 2006; Kleine-Vehn et al., 2011).

Also other molecular components, aside of sterols, which physically scaffold structure of the membrane, appear very important for maintaining PIN polarity. Sphingolipids, membrane constituents and signaling molecules (Dickson et al., 2006), were shown to influence cell polarity (Hoekstra et al., 2003; Nyasae et al., 2003). The immunophilin-like protein PASSTICINO1 (PAS1) (Bach et al., 2008; Roudier et al., 2010) is involved in the biosynthesis and metabolism of Very-Long-Chain Fatty Acids (VLCFAs), one of the composites of sphingolipids. In case of *pas1* mutant, patterning defects at the cellular level were attributed to altered auxin distribution during key events in plant life. Disturbed formation of auxin gradients was associated with abnormal PM distribution of PIN1 protein due to defective VLCFA synthesis (Roudier et al., 2010). Additionally, PIN1 abundance in its polar domain was shown to be stabilized by interaction with PGP1/PGP19 ABCB transporters (Titapiwatanakun et al., 2009). Notably, the efflux activity of these ABCB transporters at the PM is positively regulated by PID, most likely through direct phosphorylation. In this case another immunophilin - TWISTED DWARF (TWD) by interaction with PID appears to decrease ABCB activity at the cell surface (Bouchard et al., 2006; Henrichs et al., 2012; Wang et al., 2012; Wang et al., 2013).

Finally, recent reports suggest that not only structure of the PM but also the integrity of the cell wall is required for maintenance of PIN polarity. Such a notion was suggested upon characterization of *regulator of PIN polarity3 (repp3)* mutant (Feraru et al., 2011), exhibiting defects in localization of ectopically expressed PIN1. The mutation responsible for *repp* mutant phenotype was found in the gene coding for CELLULOSE SYNTHASE CATALYTIC SUBUNIT3/CONSTITUTIVE EXPRESSION OF VSP1/ISOXABEN RESISTANT1/ECTOPIC LIGNIN1 (CESA3/CEV1/IXR1/ELI1). CESA3 is a part of the enzymatic complex required for synthesis of 1,4 glucans, molecules which are able to associate to form cellulose microfibrils and thus scaffold cell wall (Richmond and Somerville, 2000; Ellis and Turner, 2001; Scheible et al., 2001; Caño-Delgado et al., 2003; Desprez et al., 2007). Additionally, pharmacologically induced cell wall degradation or inhibition of the cell wall biosynthesis resulted in similar phenotypes as in case of *repp3* mutant. Interestingly, plasmolysis-based experiments forcing detachment of the polar domain from the cell wall suggested that the mechanisms immobilizing PIN-containing PM clusters might relate to cellulose-based connections between the polar domain and the cell wall (Feraru et al. 2011; Martinière et al., 2012).

TRANSPORT IN LOOPS - HORMONAL FEEDBACK REGULATIONS OF PIN POLARITY

AUXIN FEEDBACK ON PIN-DEPENDENT AUXIN TRANSPORT

Polar distribution of PIN auxin transporters can be regulated through plethora of controlling mechanisms with various molecular players involved. Intriguingly, another layer of complexity for auxin-driven plant development emerges from the self-organizing abilities of auxin transport. Already early experiments implied that auxin-induced changes could instruct capacity and directionality of auxin flow and thus auxin would have the ability to shape its own transport (Sachs, 1981; Sachs, 1991). Indeed, the effect of auxin on PIN amounts and PIN localization and thus existence of multiple feedback mechanisms at various levels has been validated experimentally. One of such mechanisms is a well-characterized nucleus-based $SCF^{TIR1/AFB}$-dependent auxin signaling (Dharmasiri and Estelle, 2004, Dharmasiri et al., 2005a and 2005b; Kepinski and Leyser 2005; Chapman and Estelle, 2009), involved in the regulation of PIN transcription (Peer et al., 2004; Vieten et al., 2005; Heisler et al., 2005; Scarpella et al., 2006). This auxin signaling is not only regulating *de novo* PINs synthesis but appears to control also rate of their turn-over in the vacuoles. Therefore, $SCF^{TIR1/AFB}$-dependent auxin signaling appears to have a double role in transport feedback, controlling both PIN transcription and abundance at the PM by promoting, upon prolonged auxin exposure, PIN vacuolar targeting for degradation (Baster et al., 2013). The gravitropic response of the roots serves as an example of the process facilitated by such a dual mechanism in which fluctuations of auxin above or below certain physiological threshold through ($SCF^{TIR1/AFB}$)-dependent signaling mediate PIN degradation (Abas et al., 2006; Kleine-Vehn et al., 2008c; Baster et al., 2013). In addition, this transcriptional signaling is also required to feed-back on PIN polarity and thus directionality of auxin transport in both root- and shoot-based model systems (Sauer et al., 2006; Balla et al., 2011).

The auxin feedback loop which has recently drawn considerable amount of attention, due to its proposed contribution to the polarization of auxin transporters, is the non-transcriptional auxin effect on PIN endocytosis. Indeed auxin, rapidly upon application, inhibits PIN internalization and promotes the retention of PINs at the PM correlating with increased auxin efflux capacity (Paciorek et al., 2005). Although the mechanism is still largely elusive, it was proposed that auxin, when extracellulary bound to AUXIN BINDING PROTEIN1 (ABP1), executes this inhibitory function through dynamic activation of mutually exclusive RHO-LIKE GTPASE2 and 6 (ROP2/ROP6) pathways downstream of ABP1. This mechanism is functionally important in developmental processes like patterning of the leaf epidermis or root gravitropism (Robert et al., 2010; Xu et al., 2010; Nagawa et al., 2012; Chen et al., 2012; Lin et al., 2012).

Additional complexity of the system, which depends on auxin-mediated feedback and regulates plant development, emerges from the fact that some of its elements are interconnected. For example CME, activity of which was shown to be non-uniform throughout the root meristem, directly influences auxin-responsive gene expression. Such a mechanism is based on the positive auto-regulatory feedback mediated by BREVIS RADIX (BRX). This plant-specific transcription factor, through auxin-regulated PM-to-nucleus transfer and subsequent transcriptional activation of certain auxin response factor targets,

controls the cell elongation and proliferation in the root tip. Therefore, the differential pattern of endocytosis splits the transcriptional auxin signaling within the root meristem and might thus provide additional positional information to interpret auxin gradients (Mouchel et al., 2004; Santuari et al., 2011).

Notably, the experimental approaches aiming to explain the principles of auxin feedback-mediated plant development are more and more prominently supported by computational models. For example, so called 'Extracellular Receptor-based Polarization' (ERP) model, proposed recently, integrates transcription-based intracellular feedback mechanisms with a competitive utilization of auxin receptors in the cell exterior for the spatial regulation of PIN internalization (Wabnik et al., 2010; Wabnik et al., 2011). Given the fact, that during various developmental processes PINs can behave differentially (polarize either towards or away from the auxin source) (Grieneisen et al., 2007; Blilou et al., 2005; Benjamins and Scheres, 2008; Kleine-Vehn et al., 2008b), it is worth mentioning, that ERP model proposes mechanistic principles explaining these contrasting self-organizing properties of auxin transport (Wabnik et al., 2010; Wabnik et al., 2011). It, however, remains to be seen whether this, still largely theoretical, model corresponds to the biological reality. This question will be solved only after the molecular mechanism underlying the polarization of PINs and auxin transport will be elucidated.

OTHER HORMONAL REGULATIONS OF PIN-DEPENDENT AUXIN TRANSPORT

It seems that not only auxin can shape the capacity and directionality of its transport. Other hormones, by influencing the PM stability of PIN auxin transporters, can be also integrated into the PIN-dependent auxin distribution network. Most of the plant hormones have been shown to regulate transcription of numerous genes downstream of their corresponding signaling pathways, thus many of them directly or indirectly influence also the transcription of *PIN* genes. Such an effect is well characterized in case of cytokinin and ethylene, both of which have also multiple developmental functions (Swarup et al., 2007; Ruzicka et al., 2007 and 2009; Dello Ioio et al., 2008; Zhang et al., 2011b; Bishopp et al., 2011; Liu et al., 2013).

Other hormones prominently modulate PIN activity by post-transcriptional regulation. For example, a stabilization of PINs at the PM by interference with their endocytosis was observed when plants were subjected to pharmacologically or genetically induced accumulation of the plant hormone salicylic acid (SA) (Du et al., 2013). In contrast, gibberelic acid (GA) deficiency observed in GA biosynthesis mutants promotes degradation of PIN proteins whereas treatment with GA increases PIN protein stability by inhibiting PIN vacuolar trafficking. This mechanism again appears to be important for correct gravitropic response of the root (Willige et al., 2011; Löfke et al., 2013). Similar effect, stabilization of PIN2 at the membrane, with analogous developmental outuput; perturbations in root gravitropism, could be observed in both *Arabidopsis* plants overexpressing *GOLVEN* (*GLV*) genes encoding for small secretory peptides of ROOT GROWTH FACTOR (RGF) family as well as upon exogenous applications of such compounds (Matsuzaki et al., 2010; Whitford et al., 2012). In contrast to these stabilizing effects, cytokinin promotes vacuolar trafficking of PINs thus destabilizing them from the PM. Functionality of this mechanism was demonstrated during lateral root organogenesis (Marhavy et al., 2010). These frequent observations on the effects of various signaling pathways converging at the regulation of PIN-dependent auxin distribution network are in line with the model that PIN-mediated asymmetric auxin

distribution functions as a versatile mechanism integrating multiple internal and external signals (Vanneste and Friml, 2009).

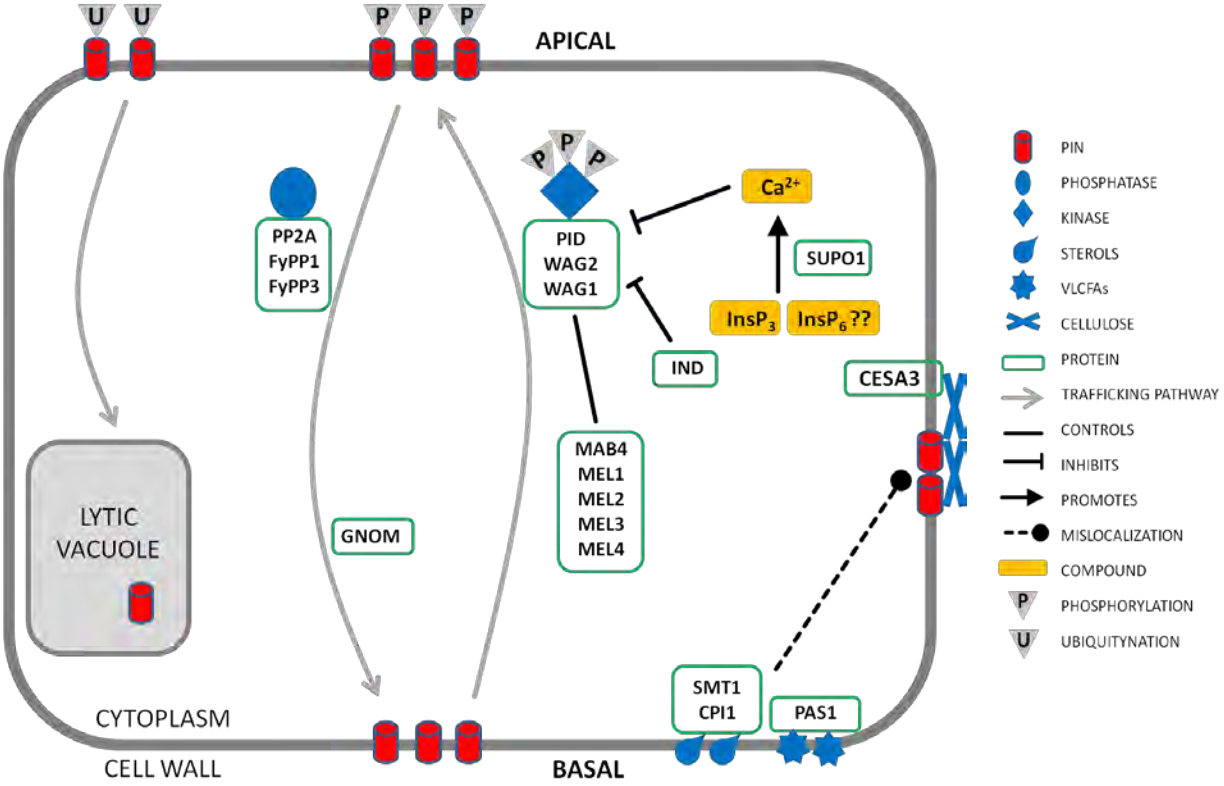

Figure 2. Post-translational modifications and cellular requirements for polarization of PINs. PINs de-phosphorylated by PP2A, FyPP1 or FyPP3 phosphatases are preferentially recruited to the basal, GNOM-dependent, BFA-sensitive pathway whereas phosphorylation by PID, WAG1 or WAG2 kinases targets PINs into apical cell side independently of GNOM function. The transcription of PID kinase is controlled by IND transcription factor. The activity of the kinases is influenced also by MAB4, its homologs - MELs and by $InsP_3$-mediated (alternatively $InsP_6$-mediated) cellular Ca^{2+} levels regulated by SUPO1. The polar localization of PINs depends on the sterol and VLCFA composition of the membrane controlled by SMT1, CPI1 and PAS1, respectively. Membrane abundance of PINs, regulated by their vacuolar targeting, depends on the ubiquitination status of the protein. Cellulose content of the cell wall regulated by CESA3 contributes to the maintenance of PIN polarity.

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Chapter 2

$SCF^{TIR1/AFB}$-auxin signaling regulates PIN vacuolar trafficking and auxin fluxes during root gravitropism

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$SCF^{TIR1/AFB}$-auxin signaling regulates PIN vacuolar trafficking and auxin fluxes during root gravitropism

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Abstract

The distribution of the phytohormone auxin regulates many aspects of plant development including growth response to gravity. Gravitropic root curvature involves coordinated and asymmetric cell elongation between the lower and upper side of the root, mediated by differential cellular auxin levels. The asymmetry in the auxin distribution is established and maintained by a spatio-temporal regulation of the PIN-FORMED (PIN) auxin transporter activity. We provide novel insights into the complex regulation of PIN abundance and activity during root gravitropism. We show that PIN2 turnover is differentially regulated on the upper and lower side of gravistimulated roots by distinct but partially overlapping auxin feed-back mechanisms. In addition to regulating transcription and clathrin-mediated internalization, auxin also controls PIN abundance at the plasma membrane by promoting their vacuolar targeting and degradation. This effect of elevated auxin levels requires the activity of SKP-Cullin-F-box$^{TIR1/AFB}$ (SCF$^{TIR1/AFB}$)-dependent pathway. Importantly, also suboptimal auxin levels mediate PIN degradation utilizing the same signaling pathway. These feed-back mechanisms are functionally important during gravitropic response and ensure fine-tuning of auxin fluxes for maintaining as well as terminating asymmetric growth.

Author Contributions: JKV, SR and JF designed the research; PB performed most of the experiments, WG, BDR performed the experiment visualized on Supplementary Figure 9; UK performed the experiment visualized on Supplementary Figure 4; PB analyzed the data; JF, SR, SV and PB wrote the manuscript.

INTRODUCTION

The phytohormone auxin is an important regulator of cell morphogenesis shaping and directing growth of organs within different developmental contexts and in response to environmental signals (Vanneste and Friml, 2009). To ensure optimal growth and development, plants have acquired elaborated mechanisms to control the local auxin homeostasis, including control of auxin metabolism (Cheng et al., 2006, 2007; Stepanova et al., 2008; Tao et al., 2008), subcellular compartmentalization (Mravec et al., 2009; Barbez et al., 2012; Ding et al., 2012) and directional auxin transport mediated by plasma membrane-resident transporters, such as ABCB, PIN-FORMED (PIN) and AUXIN-RESISTANT 1 (AUX1) (Bennett et al., 1996; Geisler et al., 2005; Petrášek et al, 2006; Cho et al., 2007; Swarup et al., 2008; Jones et al., 2009). One of the prominent growth responses mediated by auxin transport is root gravitropism. Changes of the orientation relative to the gravity vector are perceived in the root tip, by the sedimentation of statoliths, defined as gravity-sensing organelles (Harrison and Masson, 2008; Leitz et al., 2009; Morita, 2010). This process appears to induce the relocation of the auxin efflux carriers (Petrášek et al., 2006) PIN3 and PIN7 to the lower side of the gravity-sensing cells, which presumably aligns auxin flux with gravity vector towards the lower side of the root tip (Friml et al., 2002; Harrisson and Masson, 2008; Kleine-Vehn et al., 2010). From there, another auxin efflux carrier, PIN2, which is apically (shootward, upper cell side) localized in the lateral root cap and epidermal cells, mediates the directional auxin flow from the root tip to the elongation zone where control of elongation occurs (Luschnig et al., 1998; Müller et al., 1998, Abas et al., 2006, Wiśniewska et al., 2006). Hence, the PIN-mediated establishment of the asymmetric auxin distribution leads to a differential growth between the lower and the upper side of the root. As a consequence, root bends and re-orients in respect to the gravity vector, allowing the efficient exploration of the soil (Firn et al., 2000; Swarup et al., 2005).

The mechanisms underlying the PIN3 and PIN7 polarization in gravity-sensing columella cells and control of the PIN2 abundance at the plasma membrane for defined gravitropic response remain largely elusive. Nevertheless, some of the molecular processes controlling the subcellular localization of PIN proteins have been characterized (Grunewald and Friml, 2010). PIN proteins internalize continuously via a clathrin-mediated endocytotic pathway (Dhonukshe et al., 2007; Kitakura et al., 2011) and cycle back to the plasma membrane as shown by pharmacological approaches with a vesicle-budding inhibitor, Brefeldin A (BFA) (Geldner et al., 2001). This permanent cycling leads to a dynamic control of their polar localization and abundance at the plasma membrane (Kleine-Vehn et al., 2008a), which, in turn, determines the rate and direction of the auxin flow (Paciorek et al., 2005; Wiśniewska et al., 2006). The constitutive endocytic recycling enables also rapid switches in PIN polarity and, consequently, directionality of auxin fluxes in response to environmental signals, including light and gravity (Friml et al., 2002; Kleine-Vehn et al, 2010; Ding et al., 2011; Rakusová et al., 2011).

Beside the control of the polar localization, PIN protein activity can be also regulated by degradation. Numerous studies reported the occurrence of PIN degradation in the vacuoles (Abas et al., 2006, Kleine-Vehn et al., 2008b, Laxmi et al., 2008; Shirakawa et al., 2009; Leitner et al., 2012, Marhavý et al., 2012), to which they are targeted via a BFA-sensitive trafficking pathway, involving the retromer complex (Kleine-Vehn et al., 2008b). Moreover, PIN2 turnover depends on the proteasomal activity (Sieberer et al., 2000; Abas et al., 2006) and sorting for vacuolar delivery was recently associated with the formation of the polyubiquitin chains linked to the specific lysine residues at the PIN2 hydrophylic

loop (Leitner et al., 2012). Together, this data highlights the importance of post-transcriptional regulations in auxin flux determination.

Notably, auxin itself modulates its own distribution by providing feed-back on PIN biosynthesis and trafficking (Benjamins and Scheres, 2008). Short auxin treatments (≤ 2 h) activate the transcription of different PIN genes (Peer et al., 2004; Heisler et al., 2005; Vieten et al., 2005; Scarpella et al., 2006) and can stabilize PIN at the plasma membrane by inhibiting clathrin-mediated internalization (Paciorek et al., 2005; Robert et al., 2010). Recently it was found that AUXIN-BINDING PROTEIN 1 (ABP1) is a positive regulator of clathrin-mediated endocytosis which is inhibited upon auxin binding (Robert et al 2010; Chen et al., 2012). In contrast, prolonged application of auxin also promotes the turnover of PIN proteins via an unknown mechanism (Sieberer et al., 2000; Vieten et al., 2005; Abas et al., 2006). How this duality of auxin action on endocytosis versus degradation is regulated is unknown.

The BFA fungal toxin is known to inhibit the activity of specific ADP-ribosylation factor GTP-exchange factors (ARF-GEFs) (Peyroche et al., 1999; Sata et al., 1999; Geldner et al., 2003). In plants, the secretory pathway is readily inhibited by BFA, resulting in the intracellular accumulation of endocytosed plasma membrane proteins such as PIN proteins (Geldner et al., 2001). Upon inhibition of endocytosis (at approx. 25 µM of BFA), PIN proteins no longer end up in such a BFA-compartments (Paciorek et al., 2005; Men et al., 2008; Kitakura et al., 2011). Interestingly, it has recently been discovered that at higher concentrations (approx. 50 µM), BFA also inhibits vacuolar targeting and degradation of PIN proteins (Kleine-Vehn et al., 2008 and 2008b; Robert et al., 2010). Thus, different concentrations of BFA allow discriminating between effects on endocytosis for recycling and targeting for degradation (Robert et al., 2010). Notably, the aforementioned BFA concentration cut-off should not be taken precisly as most likely specificity of the BFA towards specific ARF-GEF's changes gradually.

Here we show that PIN2 protein abundance is dynamically and differentially controlled at the upper and lower side of a gravistimulated root. Both increased and decreased auxin levels change PIN2 stability by a post-transcriptional regulation of its vacuolar targeting. Moreover, we provide additional data to clarify the involvement of $SCF^{TIR1/AFB}$-based signaling in auxin-mediated PIN turn-over. These findings link auxin-mediated regulation of vesicle transport and asymmetric growth control during gravitropic response.

RESULTS

Dynamic changes of auxin response and PIN2 abundance in gravistimulated roots

To better understand the regulation of auxin transport activity in response to gravity, we have investigated the dynamics of root bending, auxin redistribution and abundance of PIN2 in gravistimulated roots of *Arabidopsis thaliana*. We have indirectly visualized the auxin redistribution by monitoring the activity of the synthetic auxin-responsive promoter DR5rev (Ulmasov et al., 1997) driving expression of a nuclearly localized VENUS protein (*DR5rev::3xVENUS-N7*; Heisler et al., 2005). Consistent with previous observations, after 2 h of gravistimulation, *Arabidopsis* root bent visibly (Figure 1 A-F) and an asymmetric increase of DR5, expression was observed at the less elongated (lower) root side (Ottenschläger et al., 2003; Paciorek et al., 2005), whereas at the upper side of the bending root, the DR5 response was reduced (Figure 1 G-M).

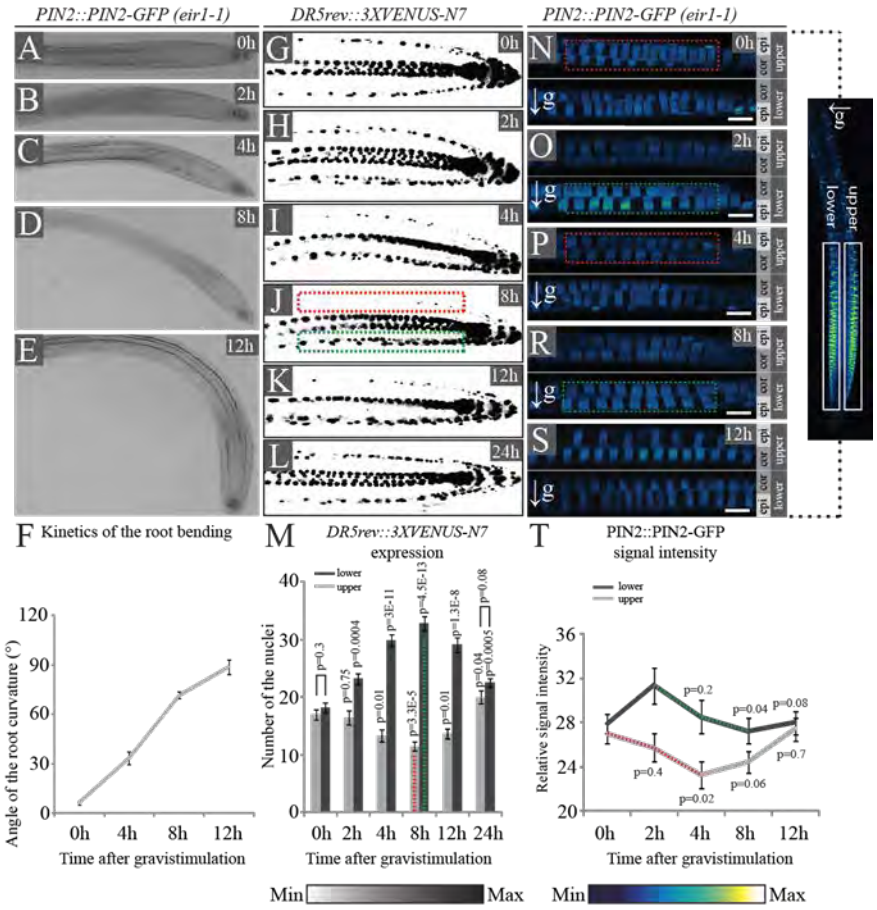

Figure 1. Localization of PIN2-GFP protein and auxin maxima during root gravitropic response. (A-E) Kinetics of the root bending in seedlings at 0 h (A), 2 h (B), 4 h (C), 8 h (D) and 12 h (E) after gravistimulation. (F) Angle of the root curvature in relation to horizon after gravistimulation. n=3 independent experiments with at least six roots analyzed for each assay. (G-L) Activity of *DR5rev::3XVENUS-N7* promoter in seedlings at 0 h (G), 2 h (H), 4 h (I), 8 h (J), 12 h (K) and 24 h (L) after gravistimulation. Pictures represent maximum intensity projection of median root sections (10 Z-sections spaced approximately 4.5 µm). (M) Quantification of the *DR5rev::3XVENUS-N7* expressing nuclei in the epidermal cells of the gravistimulated root. n=3 independent experiments with at least six roots analyzed for each assay. Note a minimum of *DR5rev::3XVENUS-N7* expression on the upper side as well as maximum on the lower side of the root 8 h after gravistimulation marked on panel J and graph M by red and green discontinuous lines, respectively. (N-S) PIN2-GFP protein localization in epidermal and cortical cells at 0 h (N), 2 h (O), 4 h (P), 8 h (R) and 12 h (S) after gravistimulation. Pictures represent maximum intensity projection of median root sections (10 Z-sections spaced approximately 1 µm apart). (T) PIN2-GFP signal intensity in gravistimulated roots. n=3 independent experiments with at least six roots analyzed for each assay. Note a decrease of the GFP signal intensity at the upper side of the root between 0 and 4 h after gravistimulation (discontinuous red line on panel N, P and graph T) as well as, at the lower side of the root, between 2 and 8 h after gravistimulation (discontinuous green line on panel O, R and graph T). Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Signal intensities are coded white to black and blue to yellow corresponding to increasing intensity levels (see color scale). cor – cortex, epi – epidermis, lower – lower side of gravistimulated root, upper – upper side of gravistimulated root. Scale bar = 10 µm.

This asymmetry in auxin response was maintained throughout gravity-induced root bending (Figure 1 A-F and 1 G-M). In time, the growth angle of the root became progressively parallel to the gravity vector

(Figure 1F) and with a delay, a balanced DR5 expression was re-establishing (Figure 1L and 1M). We have confirmed the formation of auxin lateral gradient in roots responding to the gravity with use of highly dynamic *DII-VENUS* reporter system (see Supplementary Figure 1; Brunoud et al., 2012). This system was previously used to precisely place the timing of auxin accumulation during root gravitropic response (Band et al., 2012). It is important to note that the timing of onset and disappearance of the *DR5rev::3XVENUS-N7* signal lags behind the real kinetics of the auxin distribution due to the time needed for VENUS maturation and turn-over. Nonetheless, in spite of the inherent short-comings of this reporter, we were able to demonstrate a clear spatio-temporal regulation of the auxin distribution during gravitropic bending.

To further characterize the regulation of the efflux carrier activity in response to gravity, we have investigated PIN2 abundance at the plasma membrane of gravistimulated roots. As previously suggested, following gravistimulation, PIN2 distribution became asymmetric between the upper and lower side of the root, in concordance with an asymmetrical auxin distribution (Paciorek et al., 2005; Abas et al., 2006; Kleine-Vehn et al., 2008b) (Figures 1 N-T). We have quantified the plasma membrane-localized PIN2 abundance at the lower and upper side of horizontally placed roots at different time points after gravistimulation. Within 2 h of gravistimulation, an increase of PIN2 at the plasma membrane of cells on the lower root side was detected, which spatially correlated with an increase in auxin response (Figure 1H, 1M, 1O and 1T). Following this temporal stabilization, the PIN2 level at the lower side of the root started to decrease to supposedly re-establish the pre-stimulation levels after 12 h of gravistimulation (Figure 1 O-T). Thus, at the lower root side, the PIN2 levels transiently increased before gradually decreasing to the pre-stimulation values.

In parallel, at the upper side of the bending root, where auxin response initially decreases (Figure 1 G-M), PIN2 protein levels at the plasma membrane steadily decreased in time (Figures 1 N-P and 1T), probably because of higher rates of protein degradation due to an increased targeting to the vacuole (Kleine-Vehn et al., 2008b; Figure 4 A-B). Notably, after 4 h of gravistimulation, PIN2 started to accumulate again at the plasma membrane, reaching levels close to the initial pre-stimulation levels at approximately 12 h after gravistimulation (Figures 1 P-T). Thus, at the upper root side, the PIN2 levels initially decrease, which is followed by an increase leading to re-establishment of the pre-stimulation values. The observed changes in signal intensity infer approximately 12 and 14 % change in PIN2 abundance on the lower and upper side of gravistimualted root, respectively. Notably, the recovery of symmetry in PIN2 protein levels at the plasma membrane after 12 h of gravistimulation at both lower and upper side of the root presumably reflects a re-established symmetric auxin flow, resulting in vertical root growth (Figure 1).

Overall, our data shows that a spatio-temporal regulation of the auxin distribution after gravistimulation correlates with complex and differential regulation of the PIN2 abundance at the lower and upper side of gravistimulated roots. Specifically, the increase of auxin response at the lower side of the root is accompanied with initial increase of PIN2 abundance followed by its gradual decrease. On the other hand, at the upper side of the root, we have detected decrease in auxin response that is accompanied with initial decrease in PIN2 abundance followed by its gradual increase. Importantly, the differential auxin accumulation in all observed cases preceeded changes in PIN2 abundance at the plasma membrane. The above findings also complement the observation of Luschnig et al. (1998) that a missense *pin2* allele fails to establish gravity-induced lateral auxin gradient in the root.

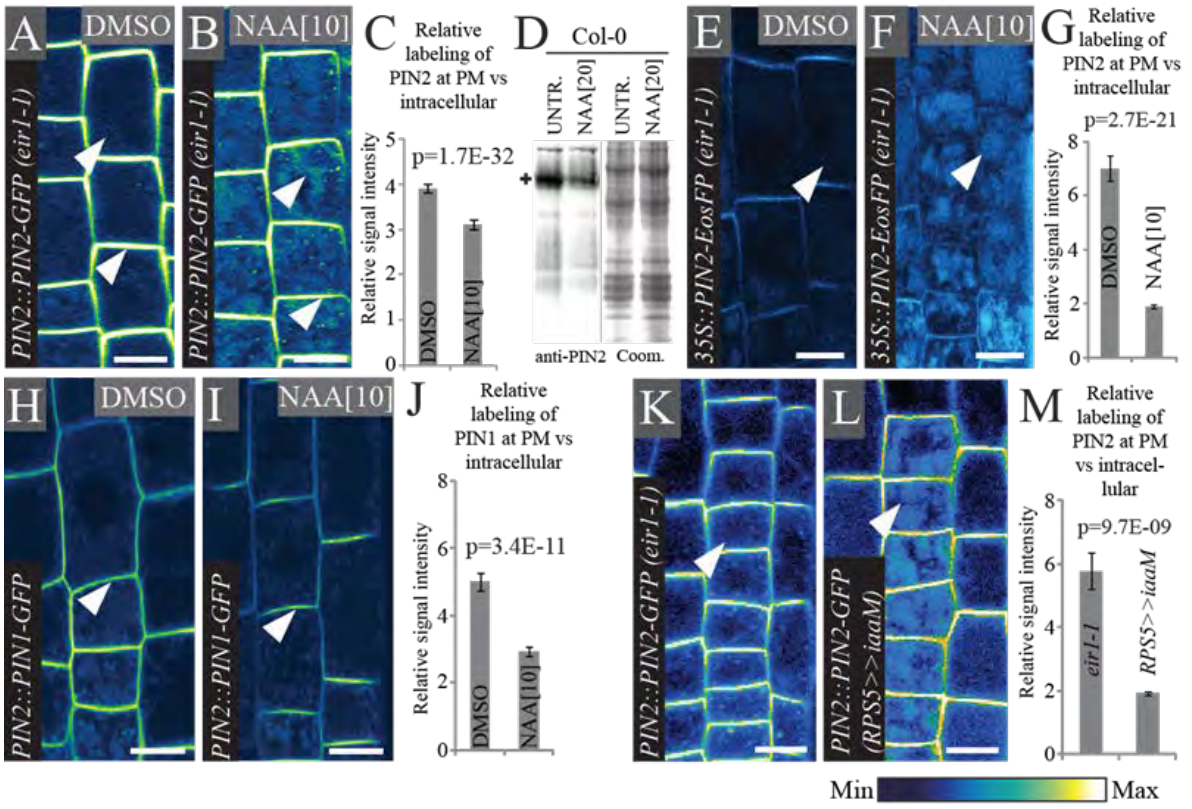

Figure 2. Auxin effect on PIN protein degradation. (A) and (B) Intracellular localization of PIN2-GFP (*eir1-1*) protein in seedlings incubated with DMSO (A) or with 10 µM NAA (B). (C) Relative PIN2-GFP abundance at the plasma membrane versus the intracellular signal in *PIN2::PIN2:GFP* (*eir1-1*) expressing line. n=3 independent experiments with at least six roots analysed for each assay and sixty cells counted in total. (D) Total membrane protein fractions probed with anti-PIN2 antibody. PIN2 protein level decreased when seedlings were treated 3 h with 20 µM NAA. PIN2 specific band at approximately 70 kD is marked with the cross. (E) and (F) Intracellular localization of 35S::PIN2-EosFP (*eir1-1*) protein in seedlings incubated with DMSO (E) or with auxin (10 µM/ 14 h) (F). The effect of auxin on 35S::PIN2-EosFP targeting to the vacuole was observed after an extended auxin treatment probably due to the stabilized expression under 35S promoter, similarly to what was observed with 35S::PIP2-GFP (see Supplementary Figure 4 G-I). (G) Relative PIN2-EosFP abundance at the plasma membrane versus intracellular signal in *35S::PIN2-EosFP* (*eir1-1*) expressing line. n=3 independent experiments with at least six roots analysed for each assay and ten cells counted for each root. (H) and (I) Intracellular localization of PIN2::PIN1-GFP protein in seedlings incubated with DMSO (H) or with 10 µM NAA (H). (J) Relative PIN1-GFP abundance at the plasma membrane versus intracellular signal in *PIN2::PIN1-GFP* expressing line. n=3 independent experiments with at least six roots analysed for each assay and ten cells counted for each root. (K) and (L) Intracellular localization of PIN2-GFP in *eir1-1* background (F_1 generation after cross with Col-0) (K) compared to *RPS5>>iaaM* background (F_1 generation after cross with PIN2::PIN2-GFP (*eir1-1*)) (L). (M) Relative PIN2-GFP abundance at the plasma membrane versus intracellular signal. n=1 with 60 cells analysed. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Arrowheads highlight differences in PIN protein retention at the plasma membrane and accumulation in the vacuoles. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

Auxin promotes PIN2 degradation in the vacuoles at the lower side of the root

First we have addressed the mechanisms underlying the regulation of PIN2 abundance at the lower side of the gravistimulated root. The initial, transient stabilization of PIN2 at the plasma membrane is presumably a result of a documented transient (≤ 2 h) inhibitory effect of higher auxin levels on PIN internalization (Paciorek et al., 2005; Robert et al., 2010; Chen et al., 2012; Lin et al., 2012). On the other hand, the following decrease in PIN2 levels that still coincides with a DR5-visualized local increase in auxin response (Figure 1) might be result of the long-term effect of auxin on PIN stability (Sieberer et al., 2000; Vieten et al., 2005). Therefore, we have tested the effect of prolonged (≥ 3 h) exogenous auxin application on PIN2 abundance at the plasma membrane. Following NAA treatment, we have observed a reduction of PIN2-GFP levels (in *PIN2::PIN2-GFP (eir1-1)* transgenic seedlings) at the plasma membrane concomitantly with an increase of a diffused vacuolar GFP signal (Figure 2 A-C; see Supplementary Figure 2). This observation was confirmed by a significant reduction of PIN2 abundance in membrane protein extracts from NAA-treated seedlings as detected by western blots (Figure 2D).

We have then addressed the cellular mechanism of the auxin effect on PIN2 abundance. In general, protein abundance at the plasma membrane is expected to reflect a sum of transcription, translation, targeting, and proteolysis. It has been shown previously that *PIN2* transcription does not change dramatically in response to auxin (Sieberer et al., 2000; Shin et al., 2005). Consistently, *PIN2* mRNA levels were shown to be induced by auxin with low amplitude and much slower kinetics than other *PIN* genes or other auxin inducible genes (Vieten et al., 2005; Lee et al., 2009). In agreement with those findings, in our experimental conditions, auxin treatment only mildly affected *PIN2* transcription (see Supplementary Figure 3), suggesting that auxin regulates PIN2 levels via a post-transcriptional mechanism. Moreover, auxin-mediated decrease of PIN2 from the plasma membrane occurred regardless of whether *PIN2* was expressed under its endogenous (Figure 2 A-C) or constitutive, heterologous 35S promoter (Figure 2 E-G), suggesting that the increased down-regulation of PIN2 is not an indirect effect of an excess of PIN2 protein in the cell's endomembrane system.

It is proposed that PIN proteins are degraded in the vacuoles (Laxmi et al., 2008; Kleine-Vehn et al., 2008b; Shirakawa et al., 2009; Marhavý et al., 2011), where GFP-tagged proteins can be visualized after an incubation in the dark (Tamura et al., 2003). In these conditions, we have found a decrease of PIN2-GFP at the plasma membrane and concomitant increase of fluorescence signal in the vacuoles in response to auxin treatment (Figure 2 A-C; see Supplementary Figure 2 K-M). This strongly suggests that auxin downregulates PIN2 abundance at the plasma membrane by enhancing PIN trafficking to the vacuole. Moreover, the auxin application destabilized both apical and basal PIN1 and PIN2 cargos from the plasma membrane (Figure 2 H-J; see Supplementary Figure 2 A-C, 4 A-C) and to lesser extent also non-polar integral plasma membrane proteins such as BRASSINOSTEROID INSENSITIVE1 (BRI1)-GFP and PLASMA MEMBRANE INTRINSIC PROTEIN2 (PIP2)-GFP (see Supplementary Figure 4 D-I). In addition, we could demonstrate that auxin reduces PIN2 protein levels (Figure 2D), thereby strongly suggesting that the observed vacuolar targetting of PINs is associated with protein degradation.

To further confirm the auxin effect on the degradation of plasma membrane proteins, we have genetically manipulated the endogenous auxin concentrations in *Arabidopsis* seedlings. We have constitutively over-expressed the *Agrobacterium tumefacens* indoleacetic acid-tryptophan monooxygenase (*iaaM*) under the strong ribosomal promoter RPS5. The iaaM enzyme converts

tryptophan into indole-3-acetamide, which is then hydrolyzed to indole-3-acetic acid (IAA) in plant cells (Klee et al., 1987; Romano et al., 1995; Weijers et al., 2001; Weijers et al., 2005). The transcription of *PIN2* was not altered in the *RPS5>>iaaM* transactivated line (See Supplementary Figure 5). We have then analyzed the abundance and intracellular distribution of PIN2-GFP marker crossed into the *iaaM* background. Similarly to exogenously applied, endogenously produced auxin promoted an increased PIN2 degradation as manifested by higher vacuolar GFP signal (Figure 2 K-M). The *iaaM* expression was shown to elevate cellular auxin concentration 2- to 10-fold (Klee et al., 1987; Romano et al., 1991 and 1995), Therefore, considering that we have not used additional media supplementation, neither with tryptofan nor with auxin, it can be expected, that the physiological threshold of auxin effect on increased PIN degradation is placed in the aformentioned range of auxin concentration change above normal/physiological level.

Taken together, these data shows that exogenously applied or endogenously produced auxin mediates the PIN targeting to the vacuole and promotes PIN2 degradation. This auxin effect presumably accounts for the decrease in PIN2 level at the lower side of the gravistimulated root after 4 h.

Auxin promotes PIN2 degradation by $SCF^{TIR1/AFB}$**-mediated signaling**

Next, we have assessed by which signaling pathway auxin promotes PIN2 degradation. We have previously shown that the inhibitory effect of auxin on PIN endocytosis is mediated by an ABP1-dependent signaling. Whereas auxin inhibits endocytosis instantaneously without *de novo* protein biosynthesis and nuclear auxin signaling (Paciorek et al., 2005; Robert et al., 2010; Chen et al., 2012: Lin et al, 2012), the auxin-induced PIN2 translocation to the vacuole for degradation required prolonged (≥ 3 h) auxin treatments (Figures 2 A-C, see Supplementary Figure 4 A-C). Given the fact that the earliest auxin-induced response proteins are detectable after approximately 10-15 min of auxin application (Badescu and Napier, 2006), the auxin effect on the vacuolar targeting might require transcriptional regulation and *de novo* protein synthesis mediated by the $SCF^{TIR1/AFB}$ pathway (Kepinski and Leyser, 2005; Dharmasiri et al., 2005a, 2005b; Badescu and Napier, 2006; Tan et al., 2007). To test this hypothesis we have used structural auxin analogues which can discriminate between ABP1- and $SCF^{TIR1/AFB}$-mediated signaling (Robert et al 2010). We have observed that treatment with IAA and all the synthetic auxin analogs, which induced transcriptional auxin response (as monitored by *DR5rev::GFP*), also promoted the degradation of PIN proteins. Moreover, the compounds, which did not induce *DR5rev::GFP* expression, did not cause a decrease in PIN abundance at the plasma membrane (Figure 3 A-J; see Supplementary Figure 6 and 7). These data suggests that the same auxin perception mechanism and downstream effectors mediates regulation of gene transcription and control the PIN stability at the plasma membrane. Indeed, in the quadruple *tir1afb1afb2afb3* mutant, auxin did not down-regulate the PIN protein levels, showing a resistance to the auxin effect on PIN degradation (Figure 4A, 4B, 4D, 4E and 4G). Importantly, resistance could also be observed in double *tir1afb1*, *tir1afb2*, *tir1afb3* and partially in the single *tir1-1* mutant background, which all show comparable PIN protein levels to the wild type in control (untreated) conditions (see Supplementary Figure 8). We have also tested the *abp1-5* allele that contained a point mutation in the auxin-binding domain of ABP1 (Napier et al., 2002) and thus, exhibited reduced auxin sensitivity (Robert et al., 2010; Xu et al., 2010). The auxin effect on PIN

degradation in the *abp1-5* mutant was comparable to the one observed in the wild type (Figure 4C, 4F and 4G).

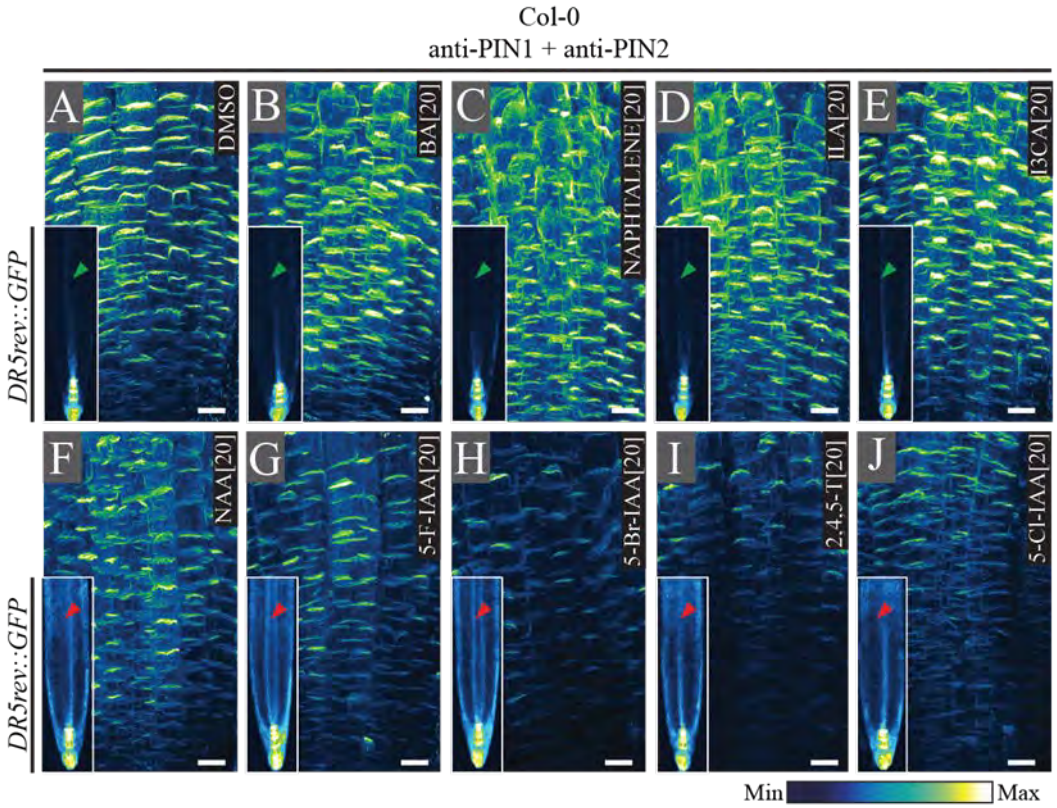

Figure 3. Auxin analogs affect the TIR1-mediated signaling pathway and induce PIN protein turnover. (A-E) Activity of auxin-responsive promoter *DR5rev::GFP* (note the absence of induction in the elongation zone of the root marked in the internal panels by green arrowheads) and PIN protein turnover is not induced by DMSO (A), BA (B), naphthalene (C), ILA (D), or I3CA (E). (F-J) Structural auxin analogs, such as NAA (F), 5-F-IAA (G), 5-Br-IAA (H), 2,4,5-T (I) and 5-Cl-IAA (J) are effective in both inducing auxin-responsive promoter *DR5rev::GFP* (note the induction in the elongation zone of the root marked in the internal panels by red arrowheads) and promoting degradation of PIN proteins. Immunolocalization pictures represent maximum intensity projection of 20 Z-sections spaced approximately 3.5 µm apart through the whole root. For quantitative analysis see Supplementary Figure 7. Green and red arrowheads highlight the absence and presence of the induction in the elongation zone, respectively. Effect of IAA on PIN degradation and induction of *DR5rev::GFP* expression is presented on Supplementary Figure 6. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

Next we have attempted to identify downstream molecular components of the $SCF^{TIR1/AFB}$ patway that are involved in the control of PIN2 degradation process. We have analyzed the promoter expression of 23 *ARF* genes in the root meristem using transcriptional nuclear GFP fusions (Rademacher et al., 2011). We have identified *ARF'*s 1, 2, 6, 9, 10, 16, and 19 as prominently expressed in epidermis of the root meristematic region where PIN2 is also specifically expressed (see Supplementary Figure 9). Subsequently, we have employed the 50 µM BFA and 20 µM NAA co-treatment on the *arf2*, *arf6*,

arf10arf16, *arf19* and *arf7arf19* mutant lines. We were able to observe an increased PIN2 accumulation in BFA induced agglomeration in the *arf2* mutant when compared to the wild type control.

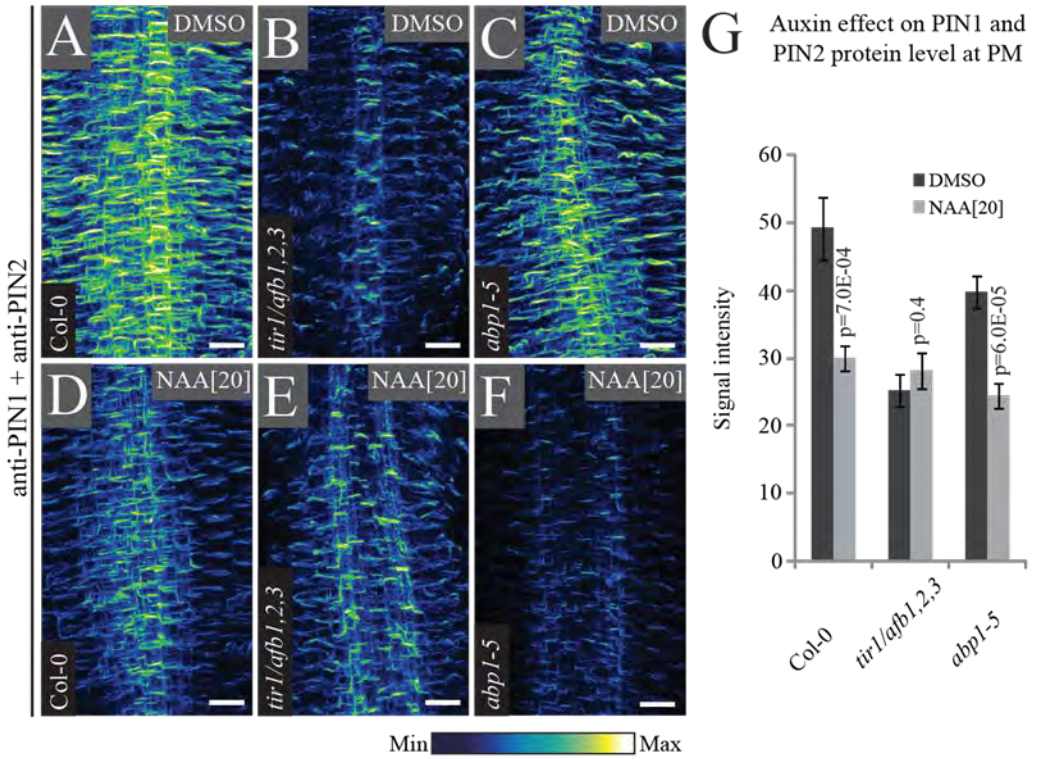

Figure 4. PIN protein degradation induced by auxin via the TIR1-mediated signaling pathway. (A-F) Immunolocalizations of PIN1 and PIN2 proteins after 14h treatment with 20 µM NAA. Auxin induced PIN protein degradation in the wild type (compare A to D) whereas *tir1/afb1afb2afb3* mutant is resistant to the auxin effect on PIN degradation (compare B to E). Auxin induced PIN protein degradation in the *abp1-5* mutant (compare C to F). Immunolocalization pictures represent maximum intensity projection of the sections through the whole root (20 Z-sections spaced approximately 3.5 µm). (G) Quantification of PIN1 and PIN2 signals at the plasma membrane. n=3 independent experiments with at least six roots analyzed for each assay. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. For the analysis of auxin-induced degradation in *tir1-1* single and double *tir1afb1*, *tir1afb2*, *tir1afb3* receptor mutant backgrounds see Supplementary Figure 8. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

This effect was not observed after treatment with lower concentration of BFA (see Supplementary Figure 10). This suggests that the mutation in the *ARF2* gene disturbs vacuolar trafficking of PIN2 protein. We therefore propose that this transcription factor could be more specifically involved in the control of PIN2 vacuolar targeting.

Overall, these data suggest that $SCF^{TIR1/AFB}$-dependent signaling is required for auxin-induced PIN2 degradation. Thus, at the lower side of the gravistimulated root, overlapping auxin effects on PIN2 endocytosis (ABP1-mediated) and PIN2 vacuolar targeting ($SCF^{TIR1/AFB}$-mediated) presumably account for

a transient increase in a PIN2-mediated auxin flow as well as for its subsequent decrease to the pre-stimulation levels.

Auxin depletion promotes PIN2 degradation at the upper side of the root

Next we have examined the mechanisms underlying the regulation of PIN2 abundance at the upper side of the gravistimulated root. Here, PIN2 levels steadily decreased coinciding with reduced DR5-visualized auxin response (Figure 1). As seen for endogenous PIN2 (Figure 1), a similar asymmetric distribution with decreased levels at the upper epidermal cell file was observed also for PIN2-EosFP expressed under control of constitutive 35S promoter (see Supplementary Figure 11) suggesting that this decrease occurs independently of *PIN* transcriptional regulation. Furthermore, this decrease correlated with the increased PIN2 vacuolar targeting (Kleine-Vehn et al., 2008b Figure 4 A-B) also consistent with post-transcriptional regulation.

We have tested whether decrease of PIN2 abundance at the plasma membrane and increased vacuolar targeting might be possibly a consequence of prolonged reduction in auxin levels. In *Arabidopsis* seedlings, not only the young leaves but also the cotyledons have a high capacity for auxin biosynthesis (Ljung et al., 2001). We have therefore reduced auxin biosynthetic capacity of the seedlings by removal of the cotyledons and shoot apical meristem (decapitation). We have observed that 14 h after such a decapitation, the growth rate of the roots was decreased but roots were still graviresponsive (see Supplementary Figure 12; Rashotte et al., 2000). By using *DR5rev::3XVENUS-N7* we have detected a significant decrease in DR5-monitored auxin response in the PIN2 expression domain 14 h after decapitation (Figure 5 A-C). These results are in line with previously reported findings showing that the auxin maximum in the root tip is highly stable and a decrease in auxin levels in the elongation zone can be detected only when auxin depletion by decapitation is prolonged (Grieneisen et al., 2007). Importantly, as a consequence of decapitation, we have observed a decreased PIN2 abundance at the plasma membrane and enhanced targeting to the vacuole (Figure 5 D-F). This effect was independent of transcriptional control (see Supplementary Figure 13) and could be reversed by exogenous auxin application (see Supplementary Figure 14). What is more, we have confirmed the reduction of PIN2 level by western blot analysis of membrane fractions isolated 14 h after decapitation (Figure 5G). To further simulate auxin depletion we have used two independent chemical biology based approaches. First, we have used the auxin-antagonist α-(phenyl ethyl-2-one)-indole-3-acetic acid (PEO-IAA) (Hayashi et al., 2008) that counteracts the auxin effect on transcription presumably by binding to the SCF^{TIR1} receptor (Nishimura et al., 2009). After 3 h of treatment, PEO-IAA caused a drop in auxin signaling as reflected by reduced expression of *DR5rev::3XVENUS-N7* reporter in epidermal and lateral root cap cells of the root apical meristem (Figure 6 A-C). Similar treatment increased vacuolar targeting of PIN2 protein (Figures 6 D-F) and, to lesser extent also non-polar integral plasma membrane proteins BRI1-GFP and PIP2-GFP (see Supplementary Figure 15). We have additionally observed that PEO-IAA disturbed the formation of the lateral gradient of PIN2 and consequently gravitropic response of the roots (see Supplementary Figure 16). Importantly, the PEO-IAA-induced destabilisation of PIN2 from the plasma membrane could be counteracted by exogenous auxin application (see Supplementary Figure 17). Western blot analysis of membrane protein fractions confirmed reduced PIN2 levels after PEO treatment (Figure 6G). This suggests that a lower throughput of $SCF^{TIR1/AFB}$-mediated transcriptional

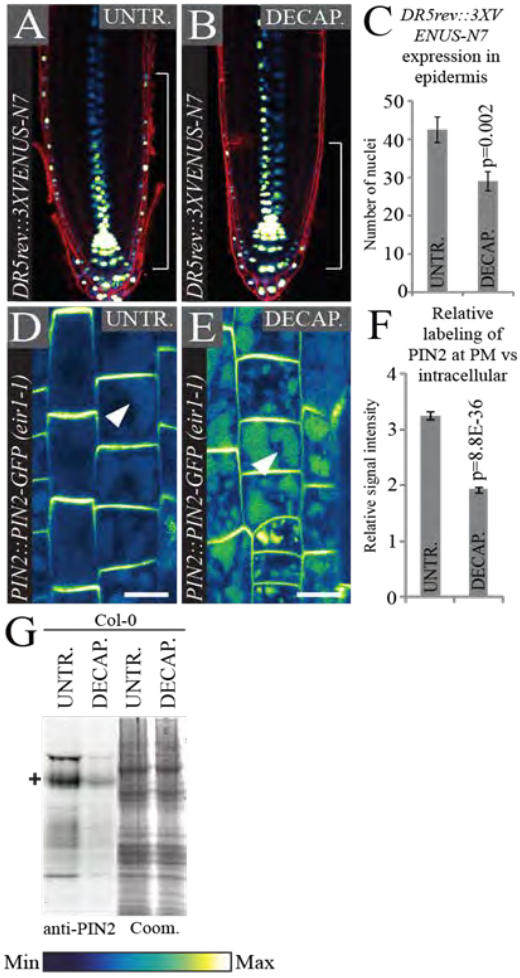

Figure 5. The effect of auxin depletion by decapitation on PIN2 protein turnover. (A) and (B) Activity of the auxin-responsive promoter *DR5rev::3XVENUS-N7* 14 h after the decapitation (B) compared to the untreated control (A). Note a decreased number of nuclei positive for *DR5rev::3XVENUS-N7* expression in epidermis and lateral root cap tissue, marked by the white line. (C) Quantification of *DR5rev::3XVENUS-N7* expression in epidermal tissue of seedlings 14 h after decapitation. n=3 independent experiments with at least ten roots analyzed for each assay. (D) and (E) Auxin depletion after decapitation in *PIN2::PIN2-GFP* (*eir1-1*) expressing seedlings resulted in increased vacuolar accumulation of PIN2 protein (E) than that of the untreated control (D). (F) Relative PIN2-GFP abundance at the plasma membrane versus intracellular signal in decapitated *PIN2::PIN2-GFP (eir1-1)* expressing seedlings. n=3 independent experiments with at least six roots analyzed for each assay and eight cells counted for each root. (G) Total membrane protein fractions were probed with anti-PIN2 antibody. PIN2 protein levels were decreased 14 h after decapitation. PIN2 specific band at approximately 70 kD is marked with the cross. Error bars represent standard error of the mean (SEM), P value calculated according to Student's t-test. Arrowheads highlight differences in vacuolar accumulation of the PIN proteins. White line highlights differences in *DR5rev::3XVENUS-N7* expression in epidermis and lateral root cap tissues. Red fluorescence represents propidium iodide staining. decap – decapitated, untr – untreated. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

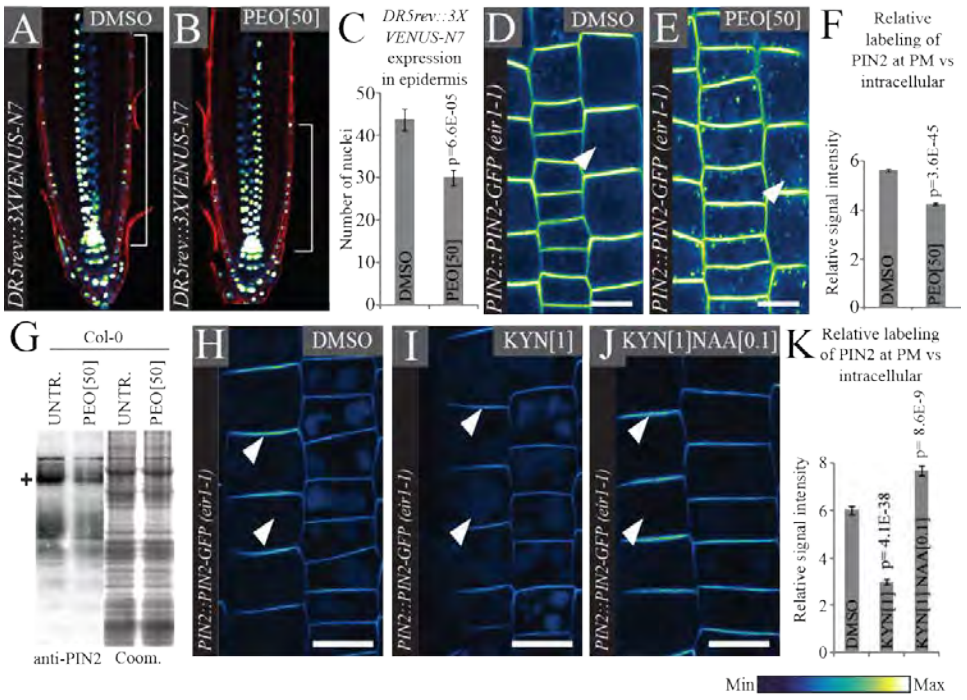

Figure 6. The effect of chemically-induced auxin depletion on PIN2 protein turnover. (A) and (B) Activity of the *DR5rev::3XVENUS-N7* promoter after 3-h treatment with 50 µM PEO-IAA (B) compared to the DMSO-treated control (A). Note a decreased number of nuclei positive for *DR5rev::3XVENUS-N7* expression in epidermis and lateral root cap tissue, marked by the white line. (C) Quantification of *DR5rev::3XVENUS-N7* expression level in epidermal tissue of seedlings after 3-h treatment with 50 µM PEO-IAA. n=3 independent experiments with at least six roots analyzed for each assay. (D) and (E) Chemical auxin depletion by treatment with 50 µM of PEO-IAA for 3 h resulted in higher vacuolar accumulation of PIN2 protein (E) when compared to DMSO-treated control (D). (F) Relative PIN2-GFP abundance at the plasma membrane versus intracellular signal in *PIN2::PIN2-GFP (eir1-1)* expressing seedlings treated with 50 µM PEO-IAA. n=3 independent experiments with at least six roots analyzed for each assay and ten cells counted for each root. (G) Total membrane protein fractions were probed with anti-PIN2 antibody. PIN2 protein levels were decreased after 3-h treatment with 50 µM PEO-IAA. PIN2 specific band at approximately 70 kD is marked with the cross. (H) and (I) Increased vacuolar accumulation and decreased plasma membrane abundance of PIN2-GFP after treatment with 1 µM L-Kynurenine (24 h/ dark) (I) compared to DMSO-treated control (H). (J) Destabilisation from the plasma membrane and vacuolar targeting of PIN2-GFP upon L-Kynurenin (see panel H and I) is reversed when co-treated with 0.1 µM NAA. (K) Relative PIN2-GFP abundance at the plasma membrane versus intracellular signal in *PIN2::PIN2-GFP (eir1-1)* expressing seedlings treated with L-Kynurenine. n=3 independent experiments with at least six roots analyzed for each assay and ten cells counted for each root. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Arrowheads highlight differences in vacuolar accumulation and plasma membrane abundance of PIN2 protein. White line highlights differences in *DR5rev::3XVENUS-N7* expression in epidermis and lateral root cap tissues. Red fluorescence represents propidium iodide staining. decap – decapitated, untr – untreated. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

auxin pathway decreases PIN stability. Second, we have interfered with Trp-dependent auxin biosynthesis by compromising the activity of key enzymatic components of this pathway. We have used L-Kynurenine, a competitive and specific inhibitor of TAA1/TAR enzymatic activity, which was shown to reduce *DR5-GUS* expression in the *Arabidopsis* roots (He et al., 2011). We have observed increased vacuolar accumulation coinciding with decreased plasma membrane abundance of PIN2 derived GFP signal after 24-h treatment with L-Kynurenine. Importantly this effect could be reversed by co-incubation with auxin (Figure 6 H-K).

As a complementary approach, we have genetically reduced the transcriptional auxin signaling. We have used a *HS::axr3*-1 which expresses a stabilised allele of IAA17 after heat shock, resulting in a strong dominant repression of $SCF^{TIR1/AFB}$ regulated transcripts (Knox et al., 2003). Importantly, while *PIN2* transcript levels were unaffected (Figure 7A), heat-shock diminished PIN2 levels in membrane protein fractions (Figure 7B) as revealed by western blot analysis. Consistently, heat shock caused an increase in vacuolar PIN2-GFP fluorescence signal along with decrease of the fluorescence levels at the plasma membrane and caused root agravitropism (Figure 7 C-E; Robert et al., 2010). This data implies that the genetic interference with $SCF^{TIR1/AFB}$ auxin signaling promotes PIN protein degradation. We have also analyzed the stability of PIN2 protein in decapitated *HS::axr3-1* seedlings. We could not observe an additive effect of decapitation on vacuolar targeting of PIN2 protein in this genetic background (see Supplementary Figure 18). This suggest that increased vacuolar targeting (and degradation as shown by western blot analysis) of PIN2 efflux carrier triggered by the removal of cotyledons and shoot apical meristem is most likely caused by the changes in auxin levels rather than by possible secondary effects of tissue wounding like changes in cytokinin or jasmonate activity (Crane and Ross, 1986; Wasternack, 2007; Sun et al., 2009; Marhavý et al., 2011).

Thus decreasing auxin levels or interfering with $SCF^{TIR1/AFB}$ auxin signaling leads to destabilization of PIN2 from the plasma membrane and higher rate of its vacuolar targeting. Overall our data suggest that both the auxin decrease below optimal as well as increase above optimal levels can destabilize PIN proteins at the plasma membrane and, subsequently, induce PIN trafficking to the vacuole for degradation. Hence, "optimal" auxin levels are required to stabilize PIN2 proteins for their action in gravitropic response.

DISCUSSION

Dual regulation of PIN vacuolar targeting and degradation by auxin levels

The data presented in this study indicates that both, a prolonged increase or decrease in cellular auxin levels induce targeting of PIN auxin transporters (Petrášek et al., 2006) to the vacuole, thereby regulating the abundance of the auxin carriers at the plasma membrane. It appears that an "optimal" auxin concentration is required to maintain PIN protein levels and thus auxin transport capacity at the plasma membrane. These effects of opposite auxin concentrations on PIN trafficking to the vacuole apparently depend on the canonical auxin signaling pathway, involving auxin-dependent degradation of Aux/IAA transcriptional repressor proteins (Dharmashiri et al., 2005a; Kepinski and Leyser, 2005). Given the known outlines of the PIN subcellular trafficking (Kleine-Vehn and Friml, 2008), auxin acts most likely

in the regulation of the balance between recycling of PIN proteins back to the plasma membrane versus trafficking to the vacuole, possibly by influencing these trafficking pathways or PIN sorting between them. The WEAK AUXIN RESPONSE1 WXR1/RUS2 protein might play a role in the auxin-mediated decision between PIN recycling and vacuolar targeting since the corresponding mutant shows defects in both transcriptional auxin response and PIN turnover (Ge et al., 2010). How the same outcome is achieved by two seemingly opposite signals is unclear. Different sets of proteins transcriptionally regulated by different auxin levels might possibly target different subcellular trafficking processes. Such a notion can be supported by the results of microarray experiment in which transcription profiling was analyzed in response to exogenous auxin and in conditional *axr3* auxin signaling mutant (http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-3283). These experimental conditions can be considered as increased and decreased auxin signaling environment, respectively.

Alternatively, different AFB auxin receptors might respond to different auxin levels in various cells and might have opposite effects on the downstream signaling, as recently suggested for AFB4 (Greenham et al., 2011). It is possible that the $SCF^{TIR1/AFB}$ signaling pathway induces downstream effectors that post-transcriptionally modify PIN proteins. Similarly to the PIN phosphorylation by the Ser/Thr protein kinase PINOID that directly affects the PIN polar targeting (Friml et al., 2004; Michniewicz et al., 2007; Kleine-Vehn et al., 2009; Huang et al., 2010; Zhang et al., 2010), other post-transcriptional modifications, such as PIN ubiquitination (Abas et al., 2006; Leitner et al., 2012), might change the subcellular sorting and trafficking of PIN proteins, leading to their preferential targeting to and degradation in vacuoles. Finally, $SCF^{TIR1/AFB}$ signaling can potentially affect a more general trafficking regulator since not only PIN proteins but also other plasma membrane proteins (although less effectively) are rerouted to the vacuole upon fluctuations in cellular auxin levels. Such a master regulator of vacuolar targeting could be subject to proteasome modifications and in turn direct post-translational modifications of PINs and other proteins. Such a hypothesis would integrate the involvement of both proteasomal and vacuolar lytic degradation in the regulation of PIN abundance. It would also clarify why PIN degradation is impaired in presence of proteasome inhibitor (Abas et al., 2006) given the fact that proteasome complex targets mainly soluble and not membrane proteins (Vierstra, 2009). Future work will address which trafficking pathways are targeted by this processes and whether an increase or a decrease in cellular auxin levels would activate a common or distinct pathways.

Auxin differentially regulates PIN2-mediated fluxes during root gravitropic response

Auxin can modify its own transport by regulating PIN transcription (Peer et al., 2004; Vieten et al., 2005; Heisler et al., 2005; Scarpella et al., 2006) and inhibiting PIN internalization from the plasma membrane (Paciorek et al., 2005; Robert et al., 2010; Chen et al., 2012; Lin et al., 2012). Here, we propose an integration of another auxin-regulated trafficking process, namely PIN turnover as a substantial element of the multilevel control mechanisms by which auxin orchestrates root reorientation in response to gravity stimulus. Our observations indicate that protein degradation is a significant part of the PIN regulatory network, particularly important during later phases of root gravitropic response. It has been previously shown that root reorientation to horizontal position results in auxin transport along the gravity vector leading to an establishment of temporal lateral auxin gradient across the organ

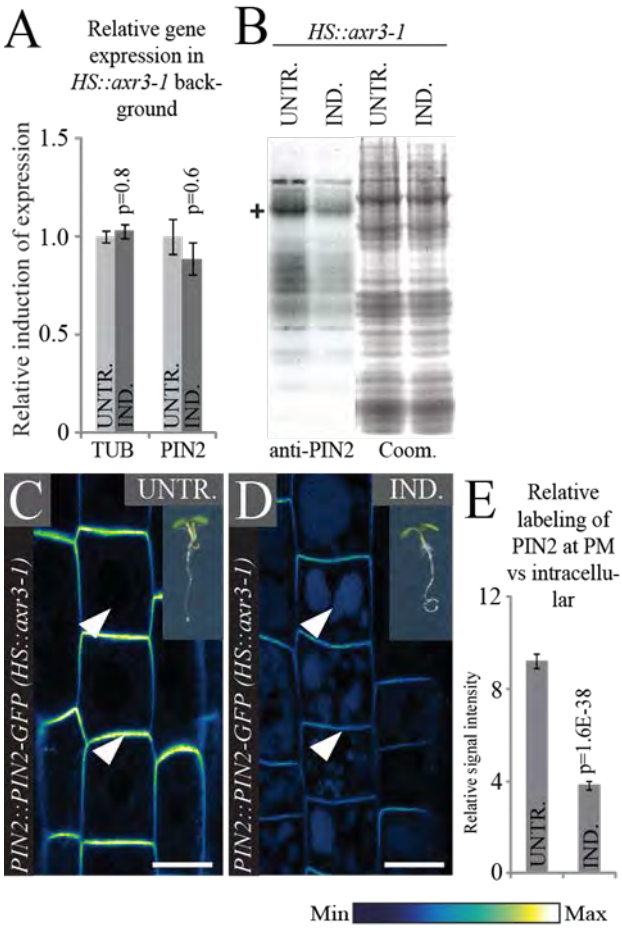

Figure 7. The effect of genetically reduced transcriptional auxin signaling on PIN2 protein turnover. (A) The effect of heat shock induction on *PIN2* expression in the root apical meristem. n=4 biological replicas with 3 technical repetitions for each. (B) Total membrane protein fractions isolated from *HS::axr3-1* genetic background were probed with anti-PIN2 antibody. PIN2 protein levels were decreased 5 h after heat shock induction. PIN2 specific band at approximately 70 kD is marked with the cross. (C) and (D) Higher vacuolar PIN2-GFP accumulation in TIR1-mediated auxin signaling-deficient background of the stabilized *IAA17* mutation (induced for 2 h at 37°C) (D) than in the same line without an induction (C). Internal panels illustrate the phenotype of *HS::axr3-1* seedlings without and after induction.(E) Relative labelling of PIN2-GFP signal at the plasma membrane versus intracellular in *HS::axr3-1* background. n=3 independent experiments with at least five roots analyzed for each assay and 200 cells counted in total. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Arrowheads highlight differences in the vacuolar accumulation of PIN proteins. ind – induced, untr – untreated. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

(Luschnig et al., 1998; Swarup et al., 2005). Our study suggests that this gradient does not only involve an increase in the auxin response at the lower side but also its decrease at the upper side of the gravistimulated root. This apparent auxin depletion at the upper side coincides with PIN destabilization at the plasma membrane most likely due to enhanced trafficking to the vacuole for degradation (Abas et

al., 2006; Kleine-Vehn et al., 2008b). As a consequence of this feedback regulation, lowered auxin transport capacity along the upper side of the root leads to decreased cellular auxin levels to "below optimal". Fluctuations in auxin level would then trigger changes in rates of cellular elongation (Barbier-Brygoo et al., 1991; Ishikawa and Evans, 1993; Evans et al., 1994, reviewed in Perrot-Rechenmann, 2010) eventually leading to a differential growth between two sides of the bending root (Zieschang and Sievers, 1991; Ishikawa and Evans, 1993) according to the classical Cholodny-Went hypothesis (Firn et al., 2000, Blancaflor et al., 2003). Interestingly, in the same developmental context similar cellular output (PIN2 degradation) although separated spatially and shifted temporarily is achieved by elevated auxin levels at lower side of the root. We are speculating that the transient stabilization of PIN2 observed there is the result of inhibitory auxin effect on clathrin-mediated PIN internalization (Paciorek et al., 2005; Robert et al., 2010; Chen et al., 2012; Lin et al., 2012). Elevated auxin levels also inhibit expansion of epidermal cells in the elongation zone at the lower side of the root. The subsequent decrease in PIN2 levels could be the result of the promoting effect of prolonged increased auxin levels on PIN2 degradation proceeding with slower kinetics than that of endocytosis (Robert et al., 2010). The interplay between these two auxin-mediated effects running with different kinetics would ultimately lead to reestablishment of the evenly distributed auxin flux on both sides and consequently vertical growth of the root.

The studies presented in this work address specifically a part of events following gravistimulation, namely how auxin influences the turnover of PIN2 thus regulating auxin flow from the place of gravity perception (root tip) to the responsive tissues in the elongation zone. These events follow the initial establishment of auxin asymmetry in the root tip presumably mediated by the gravity-induced relocation of PIN3 and PIN7 in the root columella cells (Friml et al., 2002; Harrisson and Masson, 2008; Kleine-Vehn et al., 2010). Beside PIN action in auxin transport, gravity-induced auxin translocation requires a crucial involvement of auxin influx machinery (Bennett et al., 1996; Marchant et al., 1999) and ATP-energized auxin transport utilizing ABCB transporters (Geisler et al., 2005; Blakeslee et al., 2007; Lewis et al., 2007; Mravec et al., 2008). The model of auxin action on auxin transport activity must be also integrated with other gravity-induced cellular signaling processes; many of which involve signals other than auxin (Evans and Ishikawa, 1997; Moulia and Fournier, 2009). Finally, it is tempting to speculate that the auxin effect on PIN protein degradation besides regulating root gravitropism might contribute to other processes, such as the auxin transport-mediated auxin maxima establishment during *de novo* organ formation (Benková et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Vernoux et al., 2010) where PIN degradation has been recently shown to play an important role (Marhavý et al., 2011).

Regulation of PIN activity at the plasma membrane by different auxin signaling pathways

Auxin has been demonstrated to influence its own efflux in a dual manner by either increasing or decreasing the incidence of PIN auxin transporters at the plasma membrane. These effects are achieved by the inhibition of PIN endocytosis (Paciorek et al., 2005; Robert et al., 2010) or promotion of PIN degradation (Sieberer et al., 2000; Vieten et al 2005; Abas et al., 2006; present work), respectively. The auxin inhibitory effect on PIN endocytosis was attributed to the nuclear auxin signaling pathway that depends on the $SCF^{TIR1/AFB}$ auxin receptors (Pan et al., 2009). This was, however, most likely an erroneous

interpretation as it did not account for the fact that trafficking inhibitor BFA (that was used to indirectly visualize rate of PIN internalization) targets besides PIN recycling to the plasma membrane also its trafficking to the vacuole (Peyroche et al., 1999; Sata et al., 1999; Geldner et al., 2003; Kleine-Vehn et al., 2008b; Robert et al., 2010). It seems, therefore, that authors unintentionally addressed a process of vacuolar trafficking rather than the effect on endocytosis. Several recent reports strongly support the idea that the auxin effect on endocytosis does not depend on $SCF^{TIR1/AFB}$ machinery but utilizes a direct, non-transcriptional ABP1-mediated signaling pathway that targets a general process of clathrin-mediated endocytosis (Robert et al., 2010; Nagawa et al., 2012; Lin et al., 2012; Chen et al., 2012). It has been proposed that ABP1 might sense auxin in the extracellular space where a small portion of the protein was detected (Jones and Herman, 1993; Bauly et al., 2000) and where ABP1 is active in terms of auxin response (Barbier-Brygoo et al., 1996; Gehring et al., 1998; Steffens et al., 2001). Thus, cell surface active ABP1 could activate a rapid signaling pathways depending on ROP GTPases to inhibit clathrin-mediated endocytosis without involvement of nuclear auxin signalling (Robert et al., 2010; Chen et al., 2012; Lin et al., 2012; Nagawa et al., 2012).

In this work we provide additional data to clarify the involvement of $SCF^{TIR1/AFB}$ pathway in PIN endocytosis versus vacuolar trafficking. We show by independent approaches that targeting of PINs to the vacuole for degradation is controlled by $SCF^{TIR1/AFB}$ mechanism explaining the results of Pan et al. (2009). Our results support a model, in which auxin regulates its own flux via distinct signaling pathways, which are controlling processes with different kinetics and specificities. This multi-level mechanism for the regulation of PIN-dependent, directional auxin flux presumably contributes to the adaptive plasticity of plant development.

MATERIAL AND METHODS

Plant material and growth conditions

All Arabidopsis thaliana mutants and transgenic lines employed in this study are in the Columbia (Col-0) background and have beed described previously: PIN2::PIN2-GFP (Xu and Scheres, 2005), DR5rev::3XVENUS-N7 (Heisler et al., 2005), DR5rev::GFP (Friml et al., 2003), DII-VENUS (Brunoud et al., 2012), BRI1::BRI1-GFP (Russinova et al., 2004), 35S::PIP2-GFP (Cutler et al., 2000), 35S::PIN2-EosFP (Dhonukshe et al., 2007), tir1afb1, tir1afb2, tir1afb3, tir1/afb1afb2afb3 (Dharmasiri et al., 2005b), HS::axr3-1 (Knox et al., 2003), RPS5>>iaaM (Weijers et al., 2005), abp1-5 (Xu et al., 2010) and tir1-1 (Ruegger et al., 1998), ARF promoter::GFP lines (Rademacher et al., 2011), arf2-8 (Ellis et al., 2005), arf6-2 (Nagpal et al., 2005), arf19-1 (Okushima et al., 2005), arf7arf19 (Wilmoth et al., 2005), arf10arf16 (Wang et al., 2005). Surface-sterilized seeds were sown on half-strength Murashige and Skoog (0.5 MS) agar plates and stratified for 2 days at 4°C. Plants were grown on vertically oriented plates under continuous light conditions at 22°C for 4-5 days.

Root gravitropism assay

Arabidopsis 4-day-old seedlings grown in continuous light conditions were covered with a layer of solid 0.5 MS medium and placed in Lab-Tek® II Chambered Coverglass (Nalge Nunc International). Chambers were gravistimulated by 90º rotation and transferred to darkness 2 h prior CLSM analysis. 10 Z-sections spaced approximately 1 and 4.5 µm apart for PIN2 and DR5 promoter analysis, respectively, were collected in the median root section. Single pictures were subsequently combined into the maximum intensity projection. For the specific quantification method used in each experiment please see Quantification Index.

Trans-activation experiment

RPS5::GAL4 and UAS::iaaM (both in wild type Col-0 background) were used for the cross. F1 progeny of RPS5::GAL4 x UAS::iaaM was crossed with a homozygous PIN2::PIN2-GFP (eir1-1). F1 generation was analyzed. F1 generation of the PIN2::PIN2-GFP (eir1-1) x Col-0 was used as a control.

Heat shock induction

All the CLSM analyses using HS::axr3-1 expressing line were performed after 2 h of heat shock induction at 37°C followed by 3 h incubation in continuous light at 22°C. In experiment visualized on Supplementary Figure 18, following decapitation, seedlings were subjected to 3 subsequent heat-shock inductions spaced over a total time of 16 h. Rationale was to maintain reasonable expression of mutated axr3-1 gene over this period of time.

Reagents

Compounds used in this study were: 5-chloro-indole-3-acetic acid (5-Cl-IAA), 5-bromo-indole-3-acetic acid (5-Br-IAA), indole-3-carboxylic acid (I3CA) (Ol-ChemIm Ltd, http://www.olchemim.cz/INDEX_e.HTM), indole-3-acetic acid (IAA), 5-fluoroindole-3-acetic acid (5-F-IAA), naphthalene-1-acetic acid (1-NAA), naphthalene, 2,4,5-trichloro-phenoxy acetic acid (2,4,5-T), benzoic acid (BA), indole-3-lactic acid (ILA), propidium iodide (PI), L-Kynurenine (KYN) (Sigma, http://www.sigmaaldrich.com), α-(phenyl ethyl-2-one)-indole-3-acetic acid (PEO-IAA) (Hayashi et al., 2008) synthesized as described (Robert et al., 2010), *N*-(3-Triethylammoniumpropyl)-4-(6-(4(Diethylamino)phenyl)hexatrienyl) Pyridinium Dibromide (FM4-64), Brefeldin A (BFA) (Invitrogen, http://www.invitrogen.com).

Drug Applications and Experimental Conditions

If not mention otherwise the live cell imaging experiments were performed using 10 µM NAA concentration. For vacuolar targeting analysis 4- to 5-day-old seedlings were incubated for 3 h (1 h light and 2 h darkness) in liquid or on solid 0.5 MS medium supplemented with NAA. In experiments visualized on Supplementary Figure 2 I-J, the concentration of NAA was reduced to 1 µM and time

extended to 5h. In experiments visualized on Figure 2 E-F, Supplementary Figure 4 A, B, G, H and Supplementary Figure 15 D-E time of treatment was extended to 14 h and 6 h, respectively. All immunolocalizations were performed using 4-day-old seedlings incubated in light for 4 h (auxin analogues) and 14 h (receptor mutants) in 0.5 MS medium supplemented with 20 µM of IAA, NAA, 5-Cl-IAA, 5-Br-IAA, I3CA, BA, ILA, I3CA, 2,4,5-T or naphthalene. The discrepancy in the time and concentration is the result of high subtlety of vacuolar targeting effect. Neverthless it can be observed already at the concentration of 1 µM NAA, while for the destabilization of the PINs at the plasma membrane longer treatment time and higher concentration (which still accounts for vacuolar targeting - see Supplementary Figure 2 D-G) is needed. Vacuolar targeting experiment with use of PEO-IAA was performed using 50 µM concentration and 3 h (1 h light and 2 h darkness) treatment time which is in agreement with findings described in Hayashi et al., (2008). Treatment of 14 h with concentration of 50 µM and 1 µM of PEO-IAA and NAA, respectively were used for the degradation rescue experiment. L-Kynurenine was used in 1 µM concentration in agreement with findings described in He et al., (2011). Time of treatment was arbitrarily set to 24 h. Rationale for this was the presence of redundant Trp-independent auxin biosynthetic pathways and time necessary for auxin level decrease. For BFA experiments pre-incubation with 20 µM NAA for 30 min followed by co-incubation with 20 µM NAA and 25 or 50 µM BFA for 90 min was applied. For FM4-64 staining, 5-min. treatment with 4 µM was performed and the tonoplast labelling was observed after 3 h incubation in liquid 0.5 MS medium supplemented with 10 µM NAA. For PI staining, 5-min. treatment with 1:250 dilution of original 1mg/ml solution was performed immediately before CLSM analysis.

Image processing, and statistical analysis

Signal intensities are coded blue to yellow corresponding to increasing intensity levels (visualized on the color scale under the figure) with exception of parts of Figure 1 and Supplementary Figure 9 where white to black and black to green color code is employed for convenient visualization. Red fluorescence represents propidium iodide or FM4-64 staining (specified in the figure legend). Quantitative results are visualized as means with error bars representing standard error of the mean (SEM), p value was calculated according to Student's t-test evaluation. All fluorescence signals were evaluated on the Zeiss LSM 710 or Olympus IX-81 confocal laser scanning microscope. For imaging GFP and VENUS, the 488- and 514-nm lines of the argon laser were used for excitation, and emission was detected at 510 and 530 nm, respectively. For semiquantitative measurement of fluorescence intensities, laser, pinhole, and gain settings of the confocal microscope were kept identical among treatments. The mean fluorescence intensity was measured with ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij). Images were processed with Adobe Photoshop 12.0 and Adobe Illustrator 15.0. Statistics were evaluated with Excel (Microsoft)

Quantification index

DR5 and *DII* promoter quantifications were performed as follows. In Figure 1 G-L the number of nuclei positive for *DR5rev::3XVENUS-N7* expression in epidermis above the QC plane was counted (as marked by green and red discontinuous line on Figure 1J). In Supplementary Figure 1 A-C the mean grey value of

DII-VENUS signal intensity was analyzed. The region of the root marked for quantification consisted of epidermal cells of median optical root sections above the QC plane and below the fixed point in the elongation zone (as marked by white line on Supplementary Figure 1A). The quantification index was used in order to visualize the differential distribution of auxin at the lower and the upper side of gravistimulated roots. In Figure 3 and Supplementary Figure 6 (internal panels) the quantification was performed by measuring the mean grey value of *DR5rev::GFP* signal intensity in the meristematic region (root apical meristem below QC plane) and non-meristematic region of the root (division zone above the QC plane and elongation zone below fixed point) and relating these two values (as marked by the white line on the internal panel of Supplementary Figure 6A). The quantification index was used in order to visualize induction in non-meristematic cells after treatment with auxin and its analogs. In Figure 5 A-B and 6 A-B the quantification was performed by measuring the amount of nuclei expressing *DR5rev::3XVENUS-N7* (rate of fluorescence exceeding fixed threshold) in the surface epidermal view. The quantification index was used to visualize the decreased auxin signaling in epidermal and lateral root cap tissues.
PIN, BRI1 and 35S promoter quantifications were performed as follows. All the analyses of PIN2 abundance at the plasma membrane (live cell imaging - Figure 1 N-S, Supplementary Figure 11 A-B, 14 E-G, 16 A-B, 17 A-D and all the immunolocalizations – Figures 3, 4, Supplementary Figures 2 A-B, 6 A-B, 8 A-D, 8 F-M) were performed by marking epidermal and cortical cells or cell files (live cell imaging) or whole area of the root (immunolocalizations). All the analyses of PIN2, BRI1 and PIP2 vacuolar targeting (Figures 2, 5, 6, 7 and Supplementary Figures 2 D-G, 4, 13 A-B, 14 A-C, 15 A, B, D, E) were performed by quantification of the signal intensity collected by marking the area of the apical plasma membrane of the cell and relating it to whole intracellular area of the same cell.

Immunodetection and microscopy

Whole-mount immunolocalization in Arabidopsis roots was done as described previously (Sauer et al., 2006). The rabbit anti-PIN1 (Paciorek et al., 2005) and rabbit anti-PIN2 (kindly provided by C. Luschnig) primary antibodies were used at a dilution of 1:1000 and fluorochrome-conjugated anti-rabbit-Cy3 secondary antibody (Dianova) was diluted 1:600.

Membrane protein extraction and gel blotting analysis

Approximately 15 mg of seeds were germinated vertically on solid 0.5 MS medium. 7 DAG seedlings were subjected to the treatment. Roots were cut at fixed distance from the root tip and collected for analysis. Microsomal membrane fraction was isolated as described previously (Abas and Luschnig, 2010). Equal amount of proteins were separated by 10% SDS-Urea PAGE as described (Abas et al., 2006) followed by either Coomassie Briliant Blue staining (for loading control) or blotting to ECL membranes (GE Healthcare). The membranes were subsequently treated with affinity-purified anti-rabbit PIN2 antibody (overnight at 4°C) and ECLTM-anti-rabbit IgG, horseradish peroxidase (GE Healthcare; 1:10000) (1 h at RT). Besides the specific band detected for PIN2 (around 70 kD), other peptides were detected on the western blot by PIN2 antibody. These are most likely conjugates or metabolites of PIN2 detected together with native protein, as reported and commented in Abas et al., (2006, Figure 2 legend). The

immunoreactive signals were detected using the ECL detection system (GE-Healthcare).

Quantitative RT–PCR

Total RNA was extracted with the RNeasy kit (Qiagen). Poly(dT) cDNA was prepared from total RNA with Superscript III (Invitrogen). Quantitative RT-PCR was done with LightCycler 480 SYBR Green I Master reagents (Roche Diagnostics) and a LightCycler 480 Real-Time PCR System (Roche Diagnostics). Targets were quantified with specific primer pairs designed with Beacon Designer 4.0 (Premier Biosoft International). Data was analyzed with qBASE v1.3.4 (Hellemans et al., 2007). Expression levels were normalized to the non-auxin-responsive genes CDKA (At3g48750), EEF (At5g60390) and TUB2 (At5g62690). For the presentation TUB2 reference gene was used. For primer sequences, see Supplementary Table 1.

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SUPPLEMENTARY INFORMATION

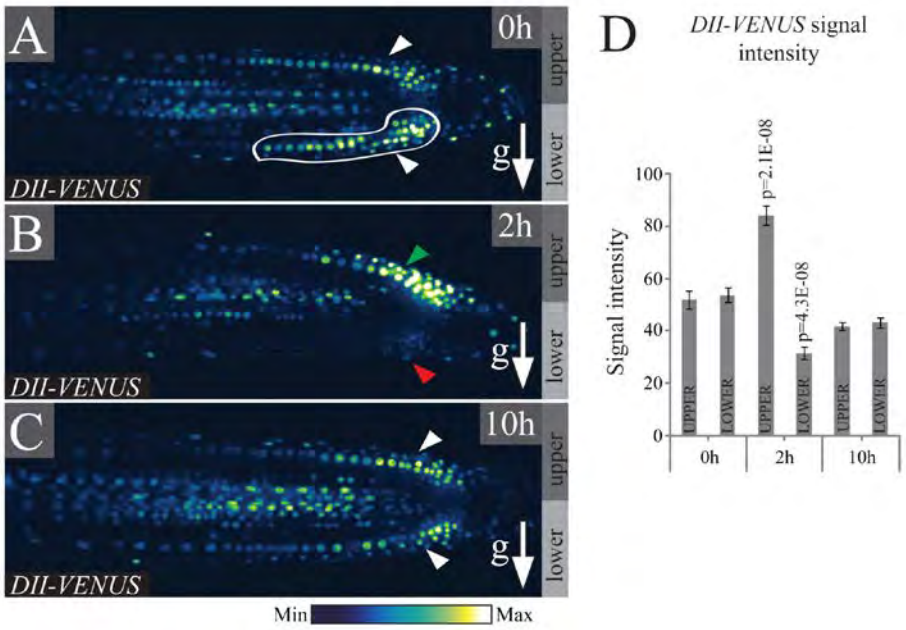

Figure S1. Auxin re-distribution during root gravitropic response in *DII-VENUS* line. (A-C) Activity of *DII-VENUS* promoter in seedlings at 0 h (A), 2 h (B) and 10 h (C) after gravistimulation. Note a presence of auxin maximum and minimum reflected by reduced and increased activity of *DII-VENUS* promoter, on the lower and the upper side of the root 2 h after gravistimulation, respectively. (D) Quantification of *DII-VENUS* signal intensity in the epidermal cells of the gravistimulated root. n=3 independent experiments with at least six roots analyzed for each assay. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. White, green and red arrowheads highlight a balanced, increased and decreased activity of *DII-VENUS* promoter, respectively. White line marks region acquired for quantitative analysis (see Material and methods section). Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale).

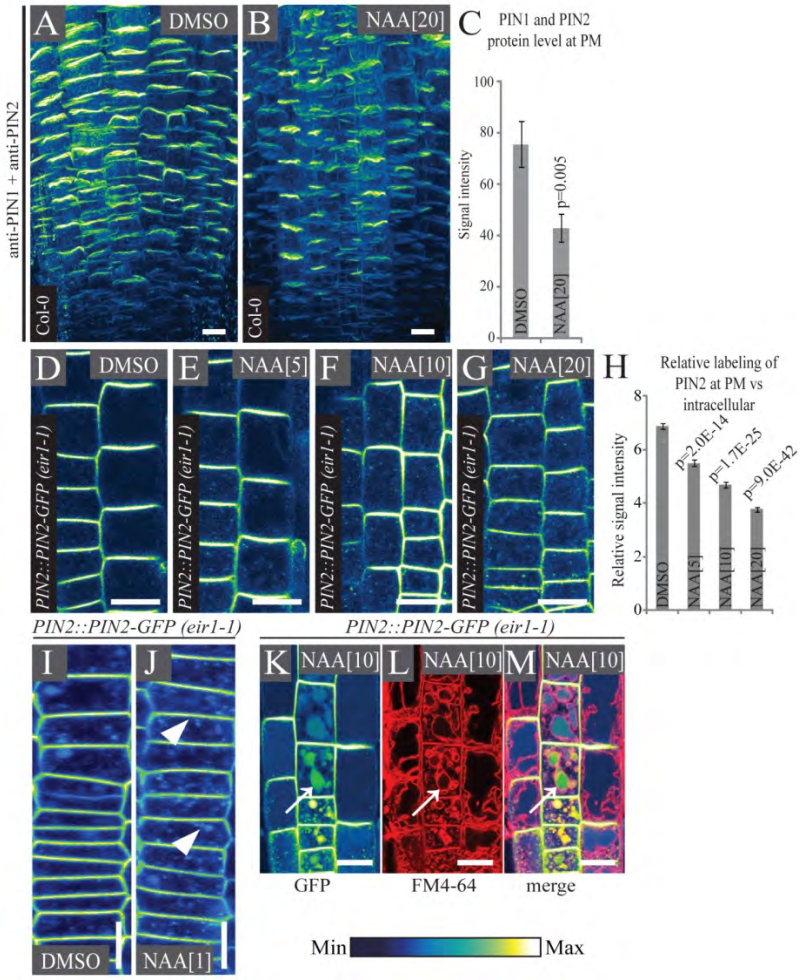

Figure S2. Auxin effect on the intracellular PIN protein localization. (A) and (B) PIN1 and PIN2 immunolocalization in DMSO-treated conditions (A) versus treatments with 20 µM NAA for 4 h (B). (C) Quantification of PIN1 and PIN2 signal intensity at the plasma membrane. n=3 independent experiments with at least four roots analysed for each assay. (D-G) PIN protein degradation promoted by 5 µM NAA (E) compared to DMSO-treated seedlings (D). This effect gradually increased with auxin concentrations, such as 10 µM (F) and 20 µM of NAA (G). (H) Quantification of the relative PIN2-GFP abundance at the plasma membrane versus the intracellular signal in *PIN2::PIN2-GFP (eir1-1)* line after treatment with 5 µM, 10 µM or 20 µM of NAA. n=1 with 50 cells analysed. (I) and (J) PIN protein degradation promoted by NAA (1 µM / 5 h) (J) compared to DMSO-treated seedlings (I). Note a decreased intensity of PIN2-GFP signal at the plasma membrane as well as increased number of vacuoles in the treated sample marked by the arrowheads. (K-M) 3 h exposure to 10 µM of NAA results in PIN2 protein targeting to the vacuoles. Note the presence of PIN2-GFP derived signal inside the tonoplast, stained by FM4-64, marked by the arrows. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Red fluorescence represents FM4-64 staining. Arrowheads highlight differences in the vacuolar accumulation and plasma membrane retention of PIN proteins. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

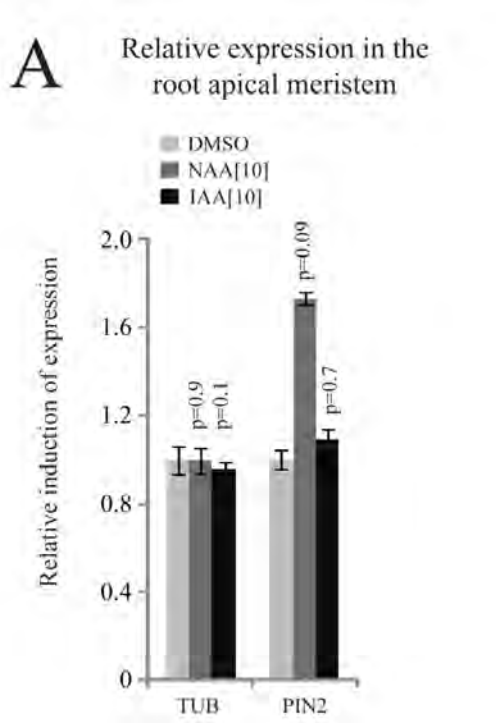

Figure S3. The effect of exogenous auxin application on *PIN2* transcription. (A) *PIN2* expression in the root apical meristem is not induced by 3-h treatment with 10 µM IAA (fc=1.1; p=0.7) and induced in low manner by 3-h treatment with 10 µM NAA (fc=1.7; p=0,09). n=2 biological replicas with 3 technical repetitions for each. Error bars represent standard deviation, p value calculated according to Student's t-test. fc – fold change, p – p value.

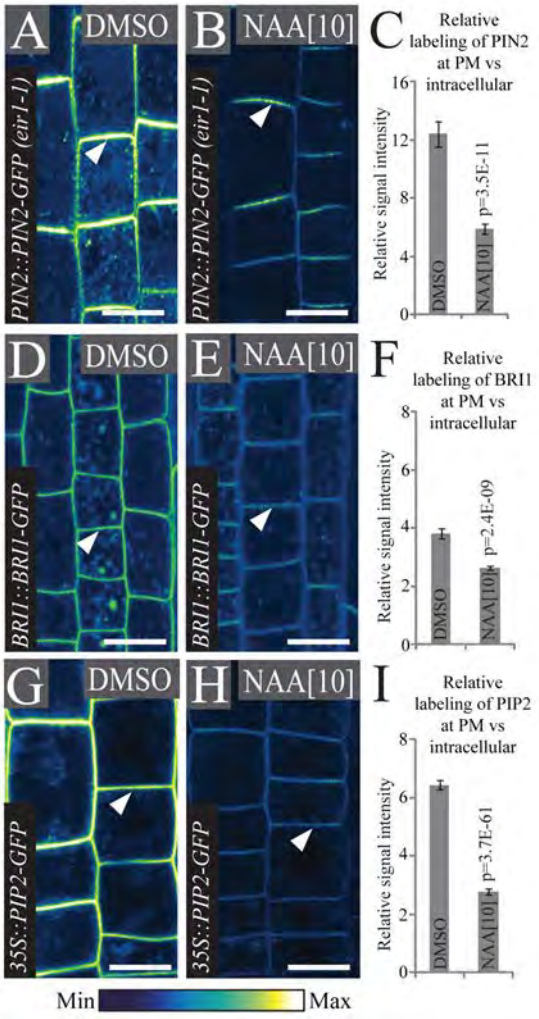

Figure S4. Auxin profusion affects protein level independently of its polar localization. (A) and (B) Increased degradation of PIN2 protein expressed under the native promoter upon auxin treatment (10 µM/ 14 h) (B) compared to the DMSO-treated control (A). (C) Relative PIN2-GFP abundance at the plasma membrane versus the intracellular signal. n=3 independent experiments with at least six roots analyzed for each assay and ten cells counted for each root. (D-E) and (G-H) Auxin treatments result in an elevated protein turnover in fluorescently tagged non-polar plasma membrane proteins, such as BRI1::BRI1-GFP (E) and 35S::PIP2-GFP (10 µM/14 h) (H) compared with the DMSO-treated controls (D) and (G). The effect of auxin on 35S::PIP2-GFP targeting to the vacuole was observed after extended auxin treatment probably due to the expression under the 35S promoter, similarly to what was observed with 35S::PIN2-EosFP (Figure 2E and 2F). (F) Relative BRI1-GFP abundance at the plasma membrane versus the intracellular signal. n=3 independent experiments with at least six roots analyzed for each assay and ten cells counted for each root. (I) Relative PIP2-GFP abundance at the plasma membrane versus the intracellular signal. n=3 independent experiments with at least six roots analyzed for each assay and ten cells counted for each root. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Arrowheads highlight differences in the protein retention at the plasma membrane. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

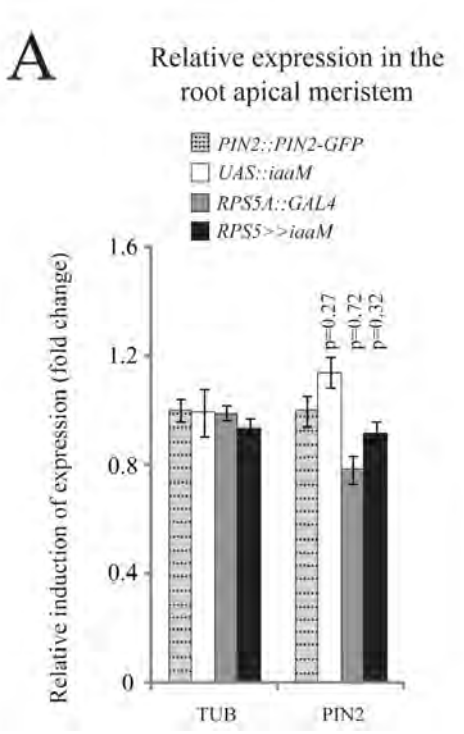

Figure S5. The effect of endogenous auxin over-production on *PIN2* transcription. (A) *PIN2* expression in the root apical meristem of *RPS5A>>iaaM* background is not affected (fc=0.9; p=0.32) in comparison to *PIN2::PIN2-GFP* line. n=2 biological replicas with 3 technical repetitions for each. Error bars represent standard deviation, p value calculated according to Student's t-test. fc – fold change, p – p value.

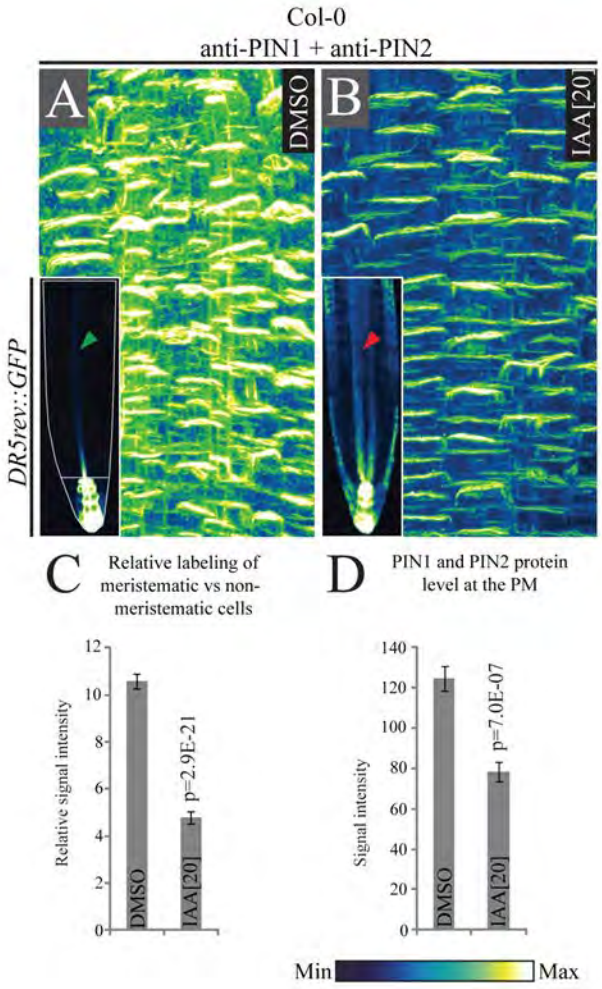

Figure S6. IAA affects the TIR1-mediated signaling pathway and induce PIN protein turnover. (A) and (B) Activity of *DR5rev::GFP* promoter and PIN protein turnover is induced by 4-h treatment with 20 µM IAA (B), when compared to DMSO treated control (A). Note the lack of induction in the elongation zone of the DMSO treated *DR5rev::GFP* seedlings marked in the internal panel by green arrowhead as well as the presence of induction in IAA treated *DR5rev::GFP* seedlings marked in the internal panel by red arrowhead. Immunolocalization pictures represent maximum intensity projection of 20 Z-sections representing the whole root. (C) Relative *DR5rev::GFP* signal of meristematic cells versus non-meristematic cells after treatment with 20 µM IAA for 4 h. n=3 independent experiments with at least six roots analyzed for each assay. (D) Quantification of PIN1 and PIN2 protein abundance at the plasma membrane after treatment with 20 µM IAA for 4 h. n=4 independent experiments with at least six roots analyzed for each assay. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Green and red arrowheads highlight the presence and the absence of the induction in the elongation zone, respectively. White line marks region acquired for quantitative analysis (see Material and Methods section). Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

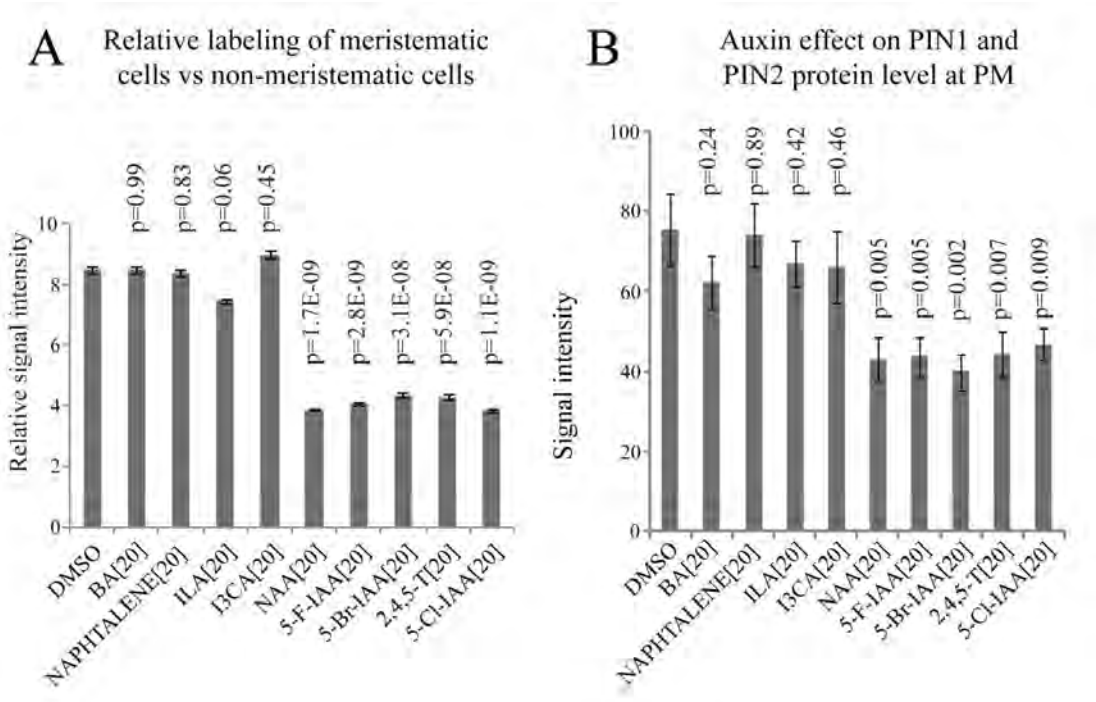

Figure S7. Effect of auxin and auxin structural analogs on the activity of the auxin-rensponsive *DR5rev::GFP* promoter and degradation of PIN1 and PIN2 proteins. (A) Relative *DR5rev::GFP* signal of meristematic versus non-meristematic cells after treatment with structural analogs of auxin. n=3 independent experiments with at least six roots analyzed for each assay. (B) PIN1 and PIN2 protein abundance at the plasma membrane after treatment with structural analogs of auxin. n=3 independent experiments with at least four roots analyzed for each assay. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test.

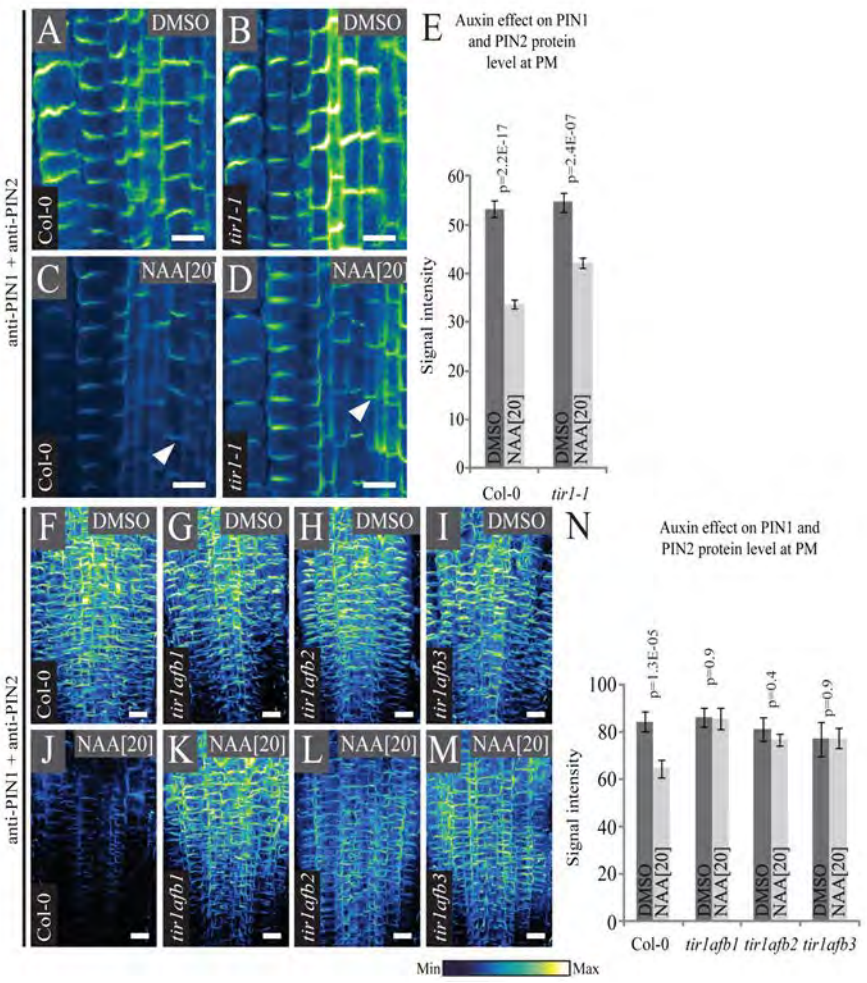

Figure S8. PIN protein turnover induced by auxin in the *tir1/afb* defective genetic background. (A-D) Immunolocalization of PIN1 and PIN2 proteins after a 14-h treatment with 20 µM NAA. Auxin induced PIN protein degradation in the wild type (compare A to C) whereas *tir1-1* mutant exhibits partial resistance to the auxin effect on PIN degradation (compare B to D). (E) Quantification of PIN1 and PIN2 signal intensity at the plasma membrane in the *tir1-1* genetic background. n=3 independent experiments with at least six roots analyzed for each assay. (F-M) Immunolocalization of PIN1 and PIN2 proteins after a 14 h treatment with 20 µM NAA. Auxin induced PIN protein degradation in the wild type (compare F to J) whereas *tir1afb1, tir1afb2* and *tir1afb3* mutants exhibits resistance to the auxin effect on PIN degradation (compare G to K; H to L and I to M). Immunolocalization pictures represent maximum intensity projection of 10 Z-sections representing the whole root. (N) Quantification of PIN1 and PIN2 signal intensity at the plasma membrane in the *tir1afb1, tir1afb2* and *tir1afb3* genetic background. n=3 independent experiments with at least six roots analyzed for each assay. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Arrowheads highlight differences in the PIN protein retention at the plasma membrane. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

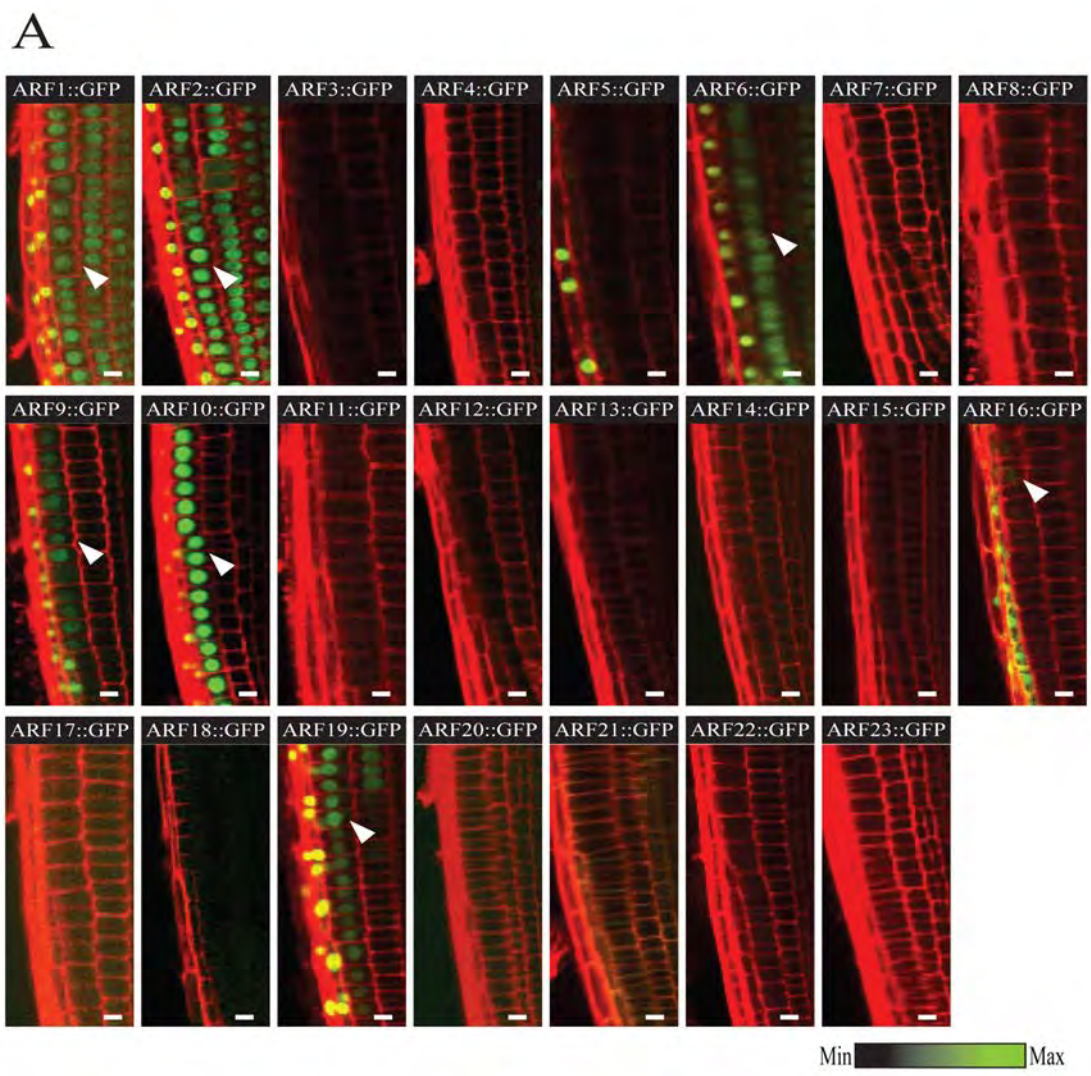

Figure S9. Expression pattern of *AUXIN RESPONSE FACTOR* (*ARF*) genes in epidermis of *Arabidopsis* root.
(A) Expression of n3GFP under control of ARF promoters in epidermis of *Arabidopsis* roots. Arrowheads highlight prominent epidermal expression of ARF1, 2, 6, 9, 10, 16 and 19. GFP signal intensities are coded black to green (see color scale). Red fluorescence represents propidium iodide staining. Scale bar = 10 µm.

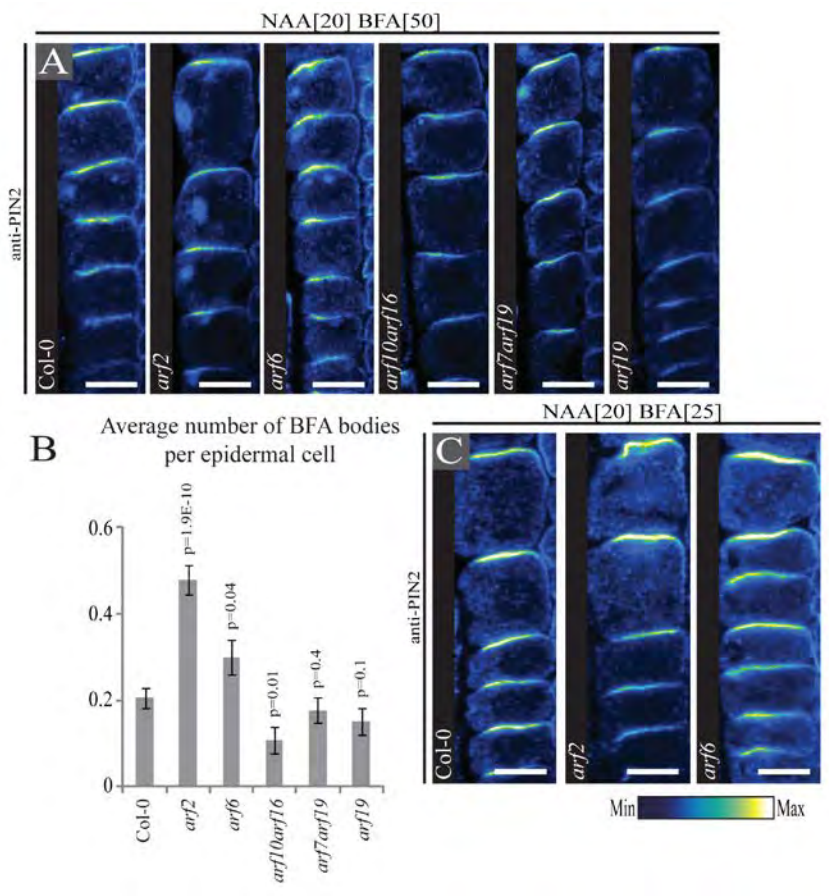

Figure S10. BFA-induced internalization of PIN2 protein in *arf* mutant genetic background. (A) BFA-induced (50 µM) internalization of PIN2 is completely inhibited by 20 µM NAA in wild type Col-0, *arf10arf16*, *arf7arf19*, *arf19*, partially in *arf6* (note a low statistical significance on graph B) but not in *arf2* genetic background. (B) Quantification of the average number of PIN2 containing BFA bodies (induced at 50 µM) per epidermal cell in *arf* genetic background. n=3 independent experiments with at least six roots analyzed for each assay. (C) BFA-induced (at 25 µM) internalization of PIN2 is inhibited in wild type Col-0, *arf2* and *arf6* genetic background. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

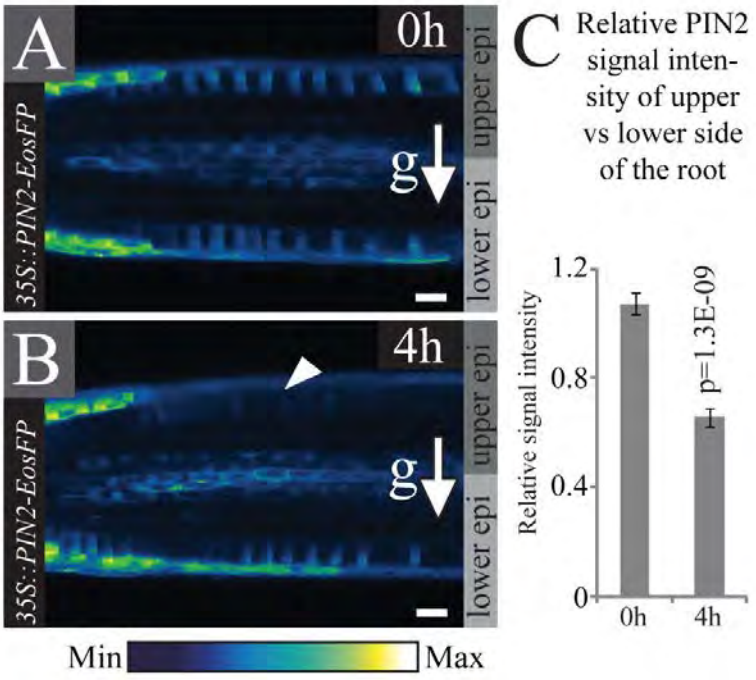

Figure S11. PIN2 protein turnover at the upper side of the gravistimulated root is independent of the transcriptional control. (A) and (B) Live cell imaging of PIN2 protein expressed under 35S promoter on the upper and the lower side of the root at 0 h (A), and 4 h (B) after gravistimulation. Note a decreased abundance of PIN2 protein on the upper side of the root 4 h after gravistimulation, highlighted by the arrowhead. Pictures represent maximum intensity projection of median optical root sections (10 Z-sections spaced approximately 4.5 µm). (C) Quantification of the relative PIN2-GFP signal intensity of the upper versus lower side of the gravistimulated transgenic *Arabidopsis* roots expressing PIN2 protein under 35S promoter. n=3 independent experiments with at least six roots analyzed for each assay. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Arrowheads highlight differences in PIN protein retention at the plasma membrane. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). decap – decapitated, epi-epidermis, untr – untreated. Scale bar = 10 µm.

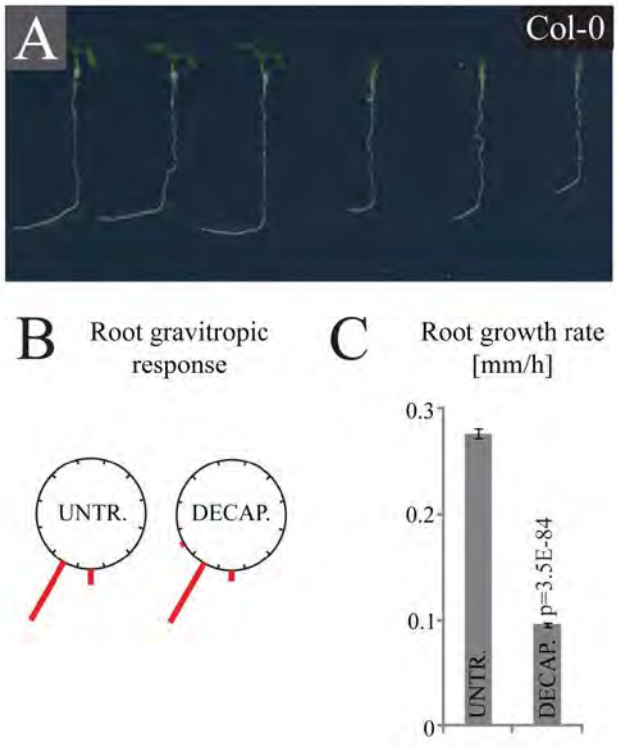

Figure S12. Root growth rate and gravitropic response of decapitated seedlings. (A) Gravitropic response of seedlings 38 h after decapitation and 24 h after gravistimulation. (B) Quantification of the root gravitropic response of decapitated seedlings. n=3 independent experiments with at least twenty roots analysed for each assay. (C) Quantification of the root growth rate of decapitated seedlings. n=3 independent experiments with at least twenty roots analysed for each assay. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. decap – decapitated, untr – untreated. Scale bar = 10 µm.

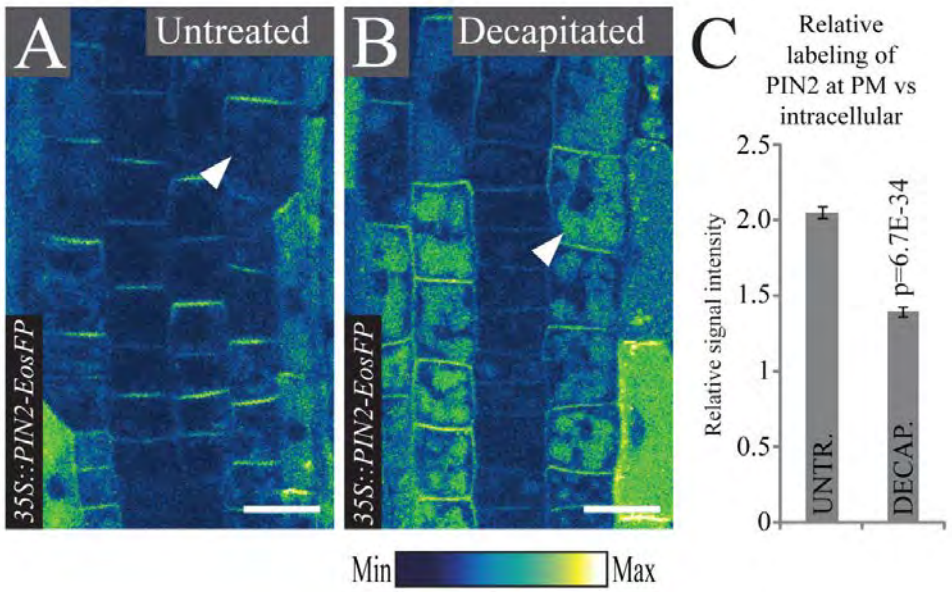

Figure S13. The effect of auxin depletion on PIN2 protein turnover is independent of the transcriptional control. (A) and (B) Elevated vacuolar accumulation of PIN2-EosFP protein expressed under the constitutive 35S promoter in decapitated plants (B) compared to untreated control (A). (C) Quantification of the relative PIN2-EosFP abundance at the plasma membrane versus the intracellular signal in roots expressing PIN2 protein under 35S promoter 14 h after the decapitation. n=3 independent experiments with at least six roots analyzed for each assay and ten cells counted for each root. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Arrowheads highlight differences in the vacuolar accumulation of PIN proteins. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). decap – decapitated, untr – untreated. Scale bar = 10 µm.

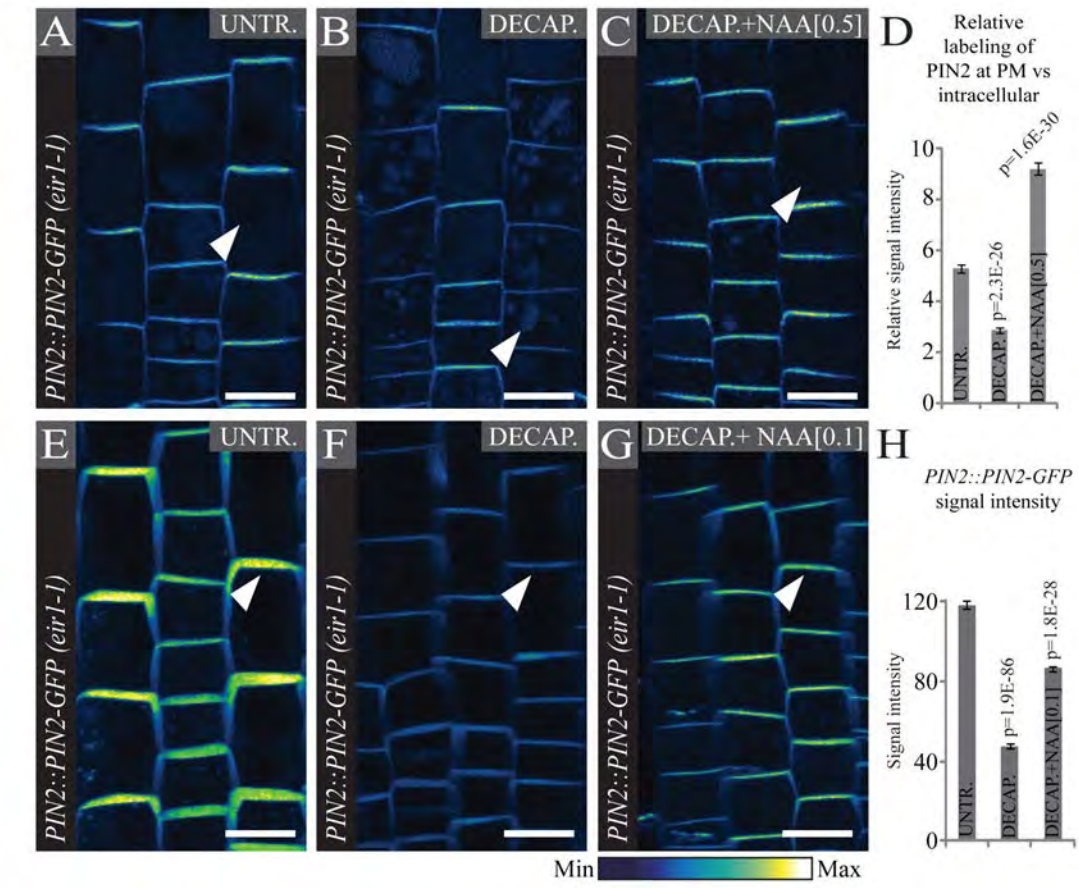

Figure S14. Auxin counteracts the effect of increased degradation of PIN2 protein induced by the removal of the cotyledons and shoot apical meristem. (A) and (B) Increased vacuolar accumulation of PIN2-GFP 14 h after decapitation (B) compared with un-decapitated control (A). (C) Exogenous auxin application (0.5 µM NAA/ 14 h) reverts increased vacuolar targeting of PIN2 protein induced by decapitation. (D) Quantification of the relative PIN2-GFP abundance at the plasma membrane versus the intracellular signal. n=3 independent experiments with at least six roots analyzed for each assay and ten cells counted for each root. (E) and (F) Decreased PIN2-GFP abundance at the plasma membrane 48 h after decapitation (F) compared with un-decapitated control (E). (G) Exogenous auxin application (0.1 µM NAA/ 48 h) reverts decreased plasma membrane abundance of PIN2 protein induced by decapitation. (H) Quantification of PIN2 signal intensity at the plasma membrane. n=3 independent experiments with at least six roots analyzed for each assay and ten cells counted for each root. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Arrowheads highlight differences in the protein retention at the plasma membrane and vacuolar accumulation. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

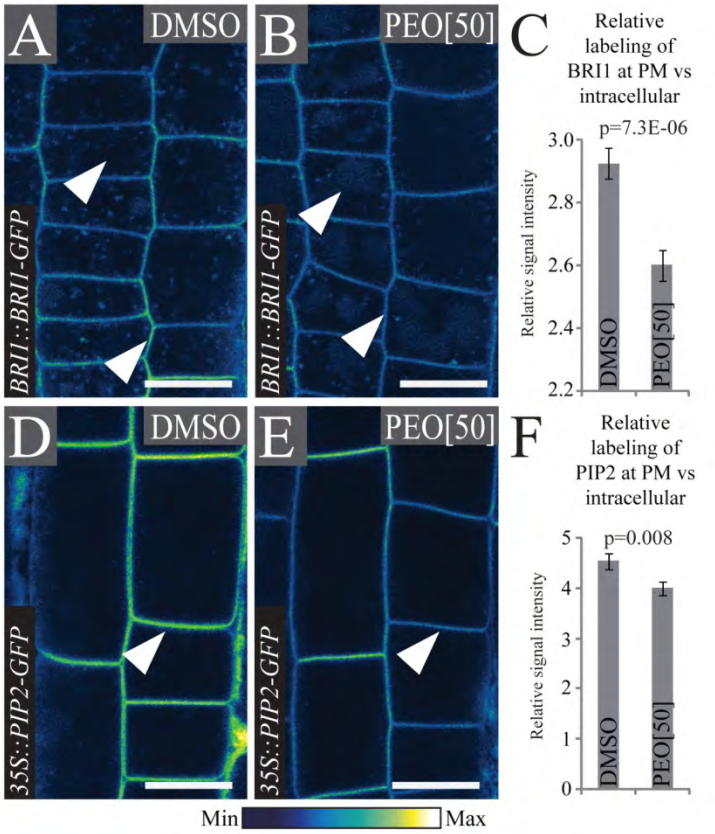

Figure S15. Auxin depletion induces destabilization of non-polar proteins from the plasma membrane. (A-B and D-E) Treatments with PEO-IAA resulted in an elevated protein turnover in fluorescently tagged non-polar plasma membrane proteins, BRI1::BRI1-GFP (50 µM/ 3 h) (B) and 35S::PIP2-GFP (50 µM/ 6 h) (E) compared with the DMSO-treated controls (A) and (D). The effect of auxin on 35S::PIP2-GFP targeting to the vacuole was observed after extended treatment time probably due to the expression under the 35S promoter resulting in high stability of the protein, similarly to what whas seen for auxin application (see Figure 2 E-F and Supplementary Figure 4 G-I). (C) Relative BRI1-GFP abundance at the plasma membrane versus the intracellular signal. n=3 independent experiments with at least six roots analyzed for each assay and eight cells counted for each root. (F) Relative PIP2-GFP abundance at the plasma membrane versus the intracellular signal. n=3 independent experiments with at least six roots analyzed for each assay and eight cells counted for each root. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Arrowheads highlight differences in the protein retention at the plasma membrane and vacuolar accumulation. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

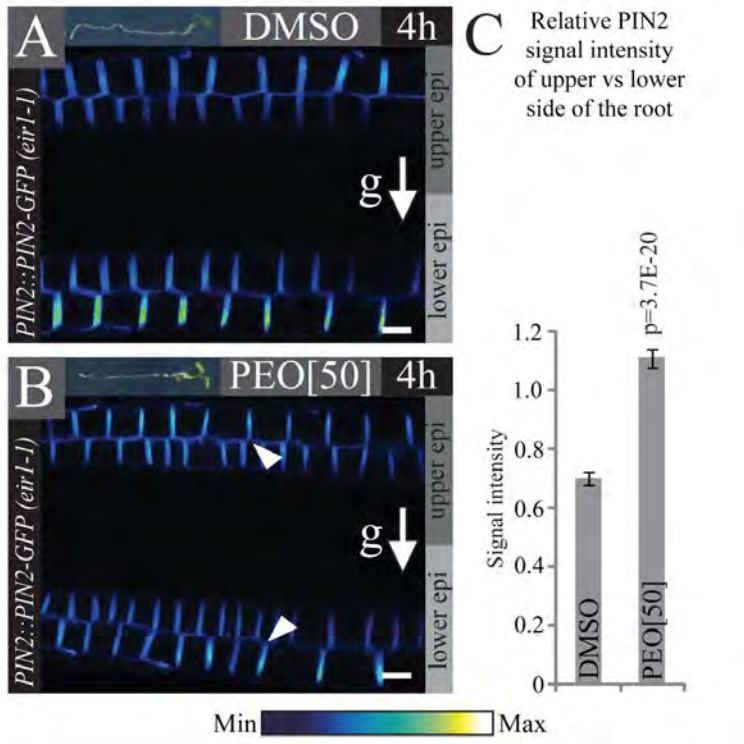

Figure S16. PEO-IAA disturbs gravitropic response and formation of gravity induced lateral PIN2 gradient in *Arabidopsis* roots. (A) and (B) Live cell imaging of the epidermal and cortical cells on the upper and the lower side of *PIN2::PIN2-GFP* expressing roots placed on DMSO (A) or 50 µM PEO-IAA (B) 4 h after gravistimulation. Note an equal distribution of PIN2 protein between upper and lower side of the roots placed on 50 µM PEO-IAA 4 h after gravistimulation, highlighted by the arrowheads. Pictures represent maximum intensity projection of median optical root sections (10 Z-sections spaced approximately 1 µm). Internal panels illustrate the phenotype of 4-h gravistimulated *PIN2::PIN2-GFP* seedlings without and after incubation with 50 µM PEO-IAA. (C) Quantification of the relative PIN2-GFP signal intensity of the upper versus lower side of the gravistimulated transgenic *Arabidopsis* roots. n=3 independent experiments with at least five roots analyzed for each assay. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Arrowheads highlight PIN2 protein retention at the plasma membrane. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). epi - epidermis. Scale bar = 10 µm.

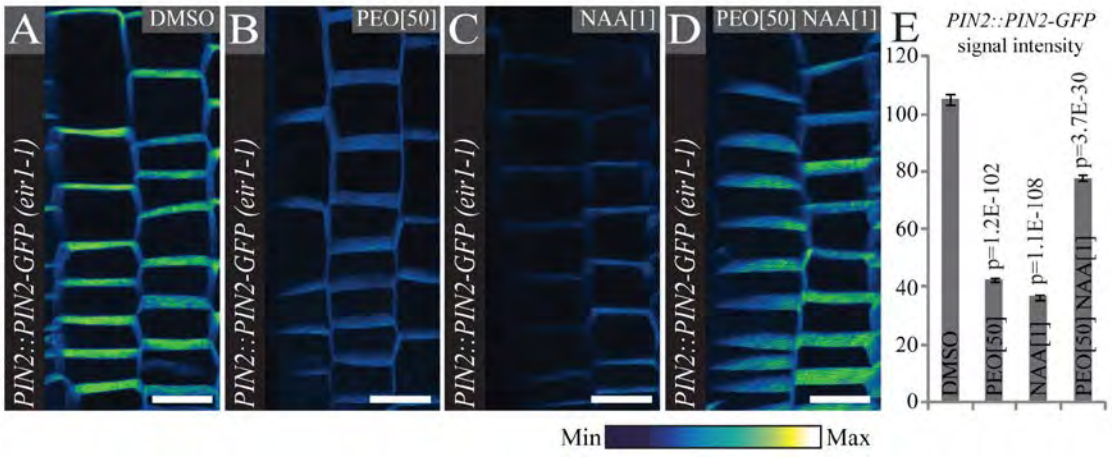

Figure S17. Auxin counteracts the effect of PEO-IAA on destabilization of PIN2 from the plasma membrane. (A-C) Decreased plasma membrane abundance of PIN2-GFP protein after 14-h treatment with 50 µM of PEO-IAA (B) or 1 µM NAA when compared with DMSO-treated control (A). (D) Simultaneous treatment with 50 µM of PEO-IAA and 1 µM NAA results in higher plasma membrane abundance of PIN2-GFP protein (D) when compared with treatment with only 50 µM of PEO-IAA (B) or only 1 µM NAA (C). (E) Quantification of PIN2 signal intensity at the plasma membrane. n=3 independent experiments with at least six roots analysed for each assay and ten cells counted for each root. Error bars represent standard error of the mean (SEM), p value calculated according to Student's t-test. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

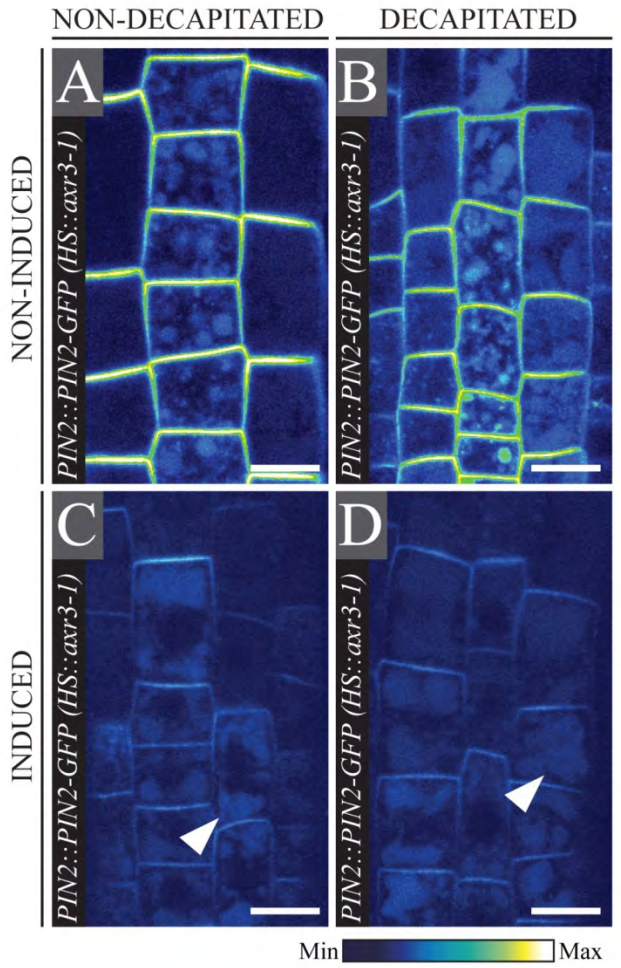

Figure S18. Vacuolar accumulation of PIN2-GFP protein in *HS::axr3-1* background after removal of the cotyledons and shoot apical meristem. (A-D) Live-cell imaging of PIN2-GFP protein in *HS::axr3-1* background. Seedlings non-induced and non-decapitated (A), non-induced and decapitated (B), induced and non-decapitated (C), induced and decapitated (D). Arrowheads highlighted a lack of additive effects in vacuolar accumulation of PIN2 protein after decapitation and induction of *axr3-1* mutation. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

Primer	
Q_CDKA_FW	ATTGCGTATTGCCACTCTCATAGG
Q_CDKA_REV	TCCTGACAGGGATACCGAATGC
Q_EEF_FW	CTGGAGGTTTTGAGGCTGGTAT
Q_EEF_REV	CCAAGGGTGAAAGCAAGAAGA
Q_PIN2_FW	ATTCCTCCTCACGACAACCTC
Q_PIN2_REV	GAGACAAGGGACCAAGCAA
Q_TUB_FW	ACTCGTTGGGAGGAGGAACT
Q_TUB_REV	ACACCAGACATAGTAGCAGAAATCAAG

FW, forward; REV, reverse

Table S1. qRT-PCR primer sequences

Chapter 3

The cell surface abundance and vacuolar targeting of PIN auxin carriers are regulated by their phosphorylation status

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Pawel Baster, Stéphanie Robert, Jűrgen Kleine-Vehn, Maciej Adamowski, Peter Grones and Jiří Friml

The cell surface abundance and vacuolar targeting of PIN auxin carriers are regulated by their phosphorylation status

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Abstract

The polar localization of PIN auxin carriers is crucial for proper establishment of auxin gradients, decisive for various developmental processes during plant life. Generation of PIN polarity requires, among others, regulated PIN transcription, subcellular trafficking and protein turn-over. The aspect of PIN polarity maintenance is strictly associated with the control of cell surface abundance and rates of auxin transporter vacuolar targeting for degradation. The conditional endocytosis of PINs for their subsequent proteolysis was recently demonstrated to depend on posttranslational protein modifications such as ubiquitination. PIN phosphorylation, other modification based on the addition of a chemical group to amino-acid sequence of the protein, was shown to instruct targeting of PINs to the specific polar domain within plasma membrane. In this study, we are exploring a hypothetical mechanism, based on PIN phosphorylation, for maintenance of once established PIN polar localization. We provide evidences that such a mechanism, operating in parallel to phosphorylation-based polar targeting of PINs, would preserve a cell surface abundance of PINs and trigger their targeting for vacuolar lysis.

Author Contributions: PB, JKV, SR and JF designed the research; PB performed most of the experiments, analyzed the data, prepared the figures and wrote the manuscript, MA was involved in generation of *pPIN1::PIN1-GFP* lines with mutated phosphorylation sites, PG performed the experiment visualized on Figure 7 H-J

INTRODUCTION

Interaction with the surrounding environment is an elementary part of every-day being for each living organism. In animal kingdom, evolution typically promoted behavioral-based solutions for adjusting to an ever-changing habitat (Davies, 2004). Plants, on the other hand, due to the stagnant nature of lifestyle, developed alternative mechanisms, allowing for fascinating plasticity of their development. Most, if not all, of these mechanisms are based on an asymmetrical growth or division of the cell and polar, in relation to an axis, distribution of the cellular components (Tejos and Friml, 2012). Latter of mentioned aspects finds a reflection in polar localization of PIN auxin carriers (Zazimalova et al., 2007). Majority of PIN family members function on the cell surface to regulate the rate and direction of an auxin flux (Petrasek et al., 2006; Wisniewska et al., 2006). This surface activity prominently contributes to the establishment of auxin gradients, crucial for numerous aspects of plant development (Tanaka et al., 2006). Conceptually, the polar localization of any protein can be considered as sum of processes which regulate a delivery, maintenance and removal of the protein from its polar domain. To date, various mechanisms for the generation of PIN polarity were described (see Chapter 1), while signals for conditional endocytosis and subsequent degradation of PINs remain more elusive.

Mechanistically, the cell surface abundance of PINs is a result of continuous cycles of endo- and exocytosis (see Chapter 1). Therefore, it is important to mention that clathrin, a regulator of the coated vesicles formation, plays a limiting role during PIN internalization (Dhonukshe et al., 2007; Kitakura et al., 2011). Moreover, a tyrosine motif identified within PIN2 sequence and required presumably for the interaction with the clathrin adaptor complex appears also important for the control of PIN endocytosis (Kleine-Vehn et al., 2011). PIN mobility is additionally restricted within PM by so called ‘membrane clusters’ and connections with the cell wall (Feraru et al., 2011; Kleine-Vehn et al., 2011).

What is more, various hormonal signals are known to influence the stability of PINs at the cell surface. Fluctuations of auxin concentration above or below certain native threshold were shown to target PINs for the late endocytic pathway (see Chapter 2). Pharmacologically or genetically induced cellular accumulation of the salicylic acid, by interference with endocytosis, causes stabilization of PINs at the PM (Du et al., 2013). Similar effect could be observed in *Arabidopsis* plants overexpressing *GLV* genes, encoding for small secretory peptides of RGF family, or upon exogenous applications of such compounds (Matsuzaki et al., 2010; Whitford et al., 2012). Gibberelic acid deficiency, observed in GA biosynthesis mutants, promotes degradation of auxin efflux carriers, whereas treatment with GA increases PIN PM abundance by inhibiting their vacuolar trafficking (Willige et al., 2011; Lofke et al., 2013). In contrast to these stabilizing effects, cytokinin triggers vacuolar trafficking of PINs (Marhavy et al., 2011). Not only hormonal, but also environmental signals, for example light, were shown to influence the stability of PINs at the PM. As demonstrated by experiments, prolonged dark treatments promoted PIN relocation to the vacuolar compartments (Laxmi et al., 2008).

Finally, a post-translational addition of a modifying chemical groups creates a highly-dynamic, due to reversibility, continuously fine-tuned regulatory system, in which the protein structure, activity, interactions, location, and half-life can be modulated (Deribe et al., 2010; Lofke et al., 2012). In yeast and animal cells, the formation of ubiquitin attachments within protein structure was shown to initiate their endocytosis and subsequent vacuolar targeting (Mukhopadhyay and Riezman, 2007). Also in plant systems, the protein turn-over mediated by 26S proteasome (ubiquitin recognizing machinery) serves as

a major mechanism to control the abundance of key regulatory proteins and enzymes, like auxin receptor complex SCF^{TIR1} (Vierstra, 2009 and 2012). Recently, the formation of the poly-ubiquitin chains, linked to the specific lysine residues within PIN2 hydrophilic loop, was associated with sorting of PIN2 for vacuolar delivery (Leitner et al., 2012). Interestingly, the ability of plant cells to properly process PIN proteins was previously shown to be impaired in presence of proteasome inhibitors pointing towards the involvement of proteasome in PIN breakdown (Sieberer et al., 2000; Abas et al., 2006). In addition, the lost activity of AMSH3 (ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM), a major de-ubiquitinating enzyme in *Arabidopsis*, impairs vacuolar biogenesis and late trafficking of endocytosed PIN2 (Isono et al., 2010).

Other post-translational protein modifications, like phosphorylation, are involved in the various regulatory networks. Activity and localization of receptor-like kinase BRI1 (BRASSINOSTEROID INSENSITIVE1) is modulated, at multiple levels, by phosphorylation-dependent processes. Upon de-phosphorylation by PP2A phosphatase complex, presumably at the PM, BRI1 gets inactivated and targeted for vacuolar degradation (Di Rubbo et al., 2011; Wu et al., 2011). Also PIN polar localization was shown to be regulated by a binary switch-like mechanism, based on reversible phosphorylation. The Ser/Thr kinase PID transfers phosphate moiety on the acceptor protein while PP2A antagonizes PID activity by hydrolyzing the phosphate groups (Friml et al., 2004; Michniewicz et al., 2007). As a consequence over-phosphorylated PINs are preferentially recruited for apical targeting pathway, independently of the GNOM function (Kleine-Vehn et al., 2009). Thus basal-to-apical shift in PIN polarity can be observed in gain-of-function PID or loss-of-function PP2A mutants (Friml et al., 2004; Michniewicz et al., 2007). Along with PID and WAG proteins, also other kinases such as D6 protein kinase (Zourelidou et al., 2009) or CDPK-RELATED KINASE5 (Rigo et al., 2013) can phosphorylate PIN proteins and regulate their function but their exact role is less clear. On the other hand, phosphatase subunit PP2AA interacts with another Ser/Thr protein phosphatase, FyPP1, to form functional holoenzyme. FyPP1 and its close homolog FyPP3 were reported to interact with and directly de-phosphorylate PINs (Dai et al., 2012). The PID-dependent phosphorylation was shown to occur on central serine residues (Ser231, Ser252 and Ser290) located within evolutionary conserved TPRXS (S/N) motif at PIN1 hydrophilic loop (Huang et al., 2010). Additionally, the significance for PIN polar distribution was demonstrated for the phosphorylation sites Ser337 and Thr340 although these, as shown by experiments, were not phosphorylated by PID directly (Zhang et al., 2010).

Here, we investigate the role of PIN protein phosphorylation as a possible mechanism for preserving cell surface abundance of PINs versus causing their vacuolar delivery for subsequent proteolysis. We show that the modulation of PIN phosphorylation status in the root apical meristem of *Arabidopsis* influences both the cell surface abundance and rates of PIN degradation. We also initiate the functional analysis of PIN phosphorylation sites, in context of auxin transporter membrane stability and vacuolar delivery.

RESULTS

In order to study the mechanism of PIN polarity maintenance, specifically in context of protein phosphorylation, we have employed a pharmacological approach. We have used a well-established, potent and specific inhibitor of protein phosphatases type 1 and 2A – cantharidin (CAN) (Li et al., 1993),

as well as staurosporine (STA), a broad range inhibitor of protein kinases, including Ser/Thr kinases (Ruegg and Burgess, 1989; Couldwell et al., 1994). Due to the high acidification of lytic vacuoles, maintained by the activity of H^+-ATPases and H^+-pyrophosphatases (Sze et al., 1999; Maeshima, 2000; Shen et al., 2013) GFP, when residing in these compartments, is rapidly degraded in presence of light (Tamura et al., 2003). Considering the above, in order to visualize a vacuolar occurrence of GFP-fused PINs, we have combined CAN or STA treatments with ConcanamycinA (ConA), a specific inhibitor of H^+-ATPase enzymatic activity (Drose et al., 1993; Matsuoka et al., 1997) and/or dark incubations (Kleine-Vehn et al., 2008a; Baster et al., 2013). We have analyzed the subcellular localization of PIN1-GFP and PIN2-GFP proteins in these experimental conditions (Figure 1 A-H; see Material and Methods). We were able to observe, that diffused vacuolar GFP signal, which is typically present for *pPIN2::PIN2-GFP* expressing *Arabidopsis* seedlings (Figure 1A and 1D), upon treatments with 10 µM CAN was decreased concomitantly with stabilization of the PIN2-derived GFP signal at the PM (Figure 1B and 1D). In contrary, when 1 µM STA was used, a reduction of PIN2-GFP levels at the PM accompanying an increased diffused GFP vacuolar signal was detected (Figure 1C and 1D). Differential distribution of the protein, as a result of pharmacologically induced changes in its phosphorylation status was not observed for PIN1 (Figure 1 E-H).

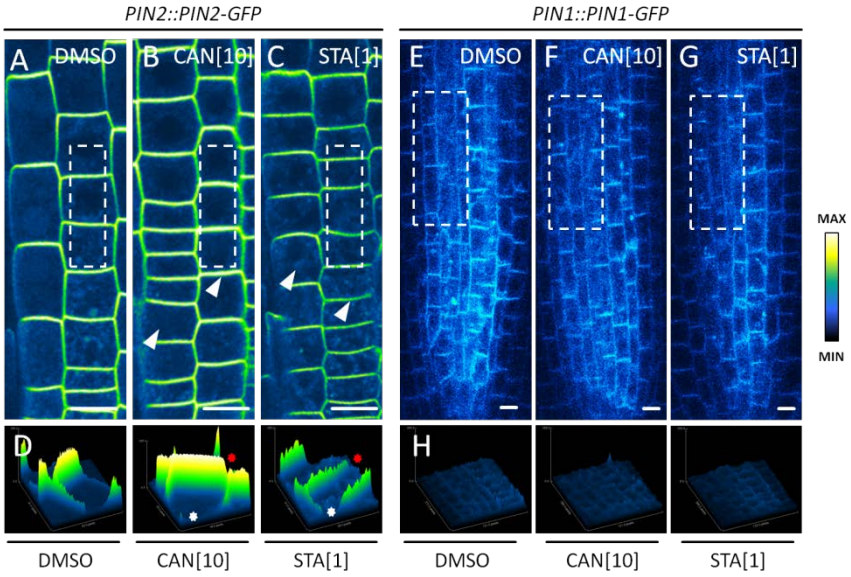

Figure 1. Pharmacological inhibition of kinase and phosphatase activity influences subcellular PIN2 but not PIN1 localization in *Arabidopsis* roots. (A-C) Subcellular localization of PIN2-GFP protein, when compared to DMSO treated control (A), is stabilized at the plasma membrane after 5h incubation in the dark on medium supplemented with 10 µM CAN (B). Treatment with 1 µM STA causes destabilization of the protein from the PM coinciding with increased GFP signal occurrence in vacuolar-like structures (C). (D) Signal intensity surface plot for images of *pPIN2::PIN2-GFP* after STA and CAN treatment. Note differences in PIN2 localization at PM (peaks) and intracellular (intervals), marked by red and white asterisks respectively. (E-G) Subcellular localization of PIN1-GFP protein is neither influenced by 10 µM CAN (F), nor by 1 µM STA (G) treatment, when compared to DMSO treated control (E). (H) Signal intensity surface plot for images of *pPIN1:PIN1-GFP* after STA and CAN treatment. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Arrowheads highlight differences in PIN protein retention at the plasma membrane and accumulation in the vacuoles. CAN – cantharidin, STA – staurosporine. Scale bar = 10 µm.

To further investigate how the pharmacological inhibition of kinase and phosphatase activity could influence the rates of vacuolar targeting and PM stability of PINs, we have used BrefeldinA (BFA). This fungal toxin, targets the Sec7 domain of ARF-GEF GNOM (Peyroche et al., 1999; Sata et al., 1999), causing the intracellular accumulation ('BFA-bodies' or 'BFA-compartments') of endocytosed PM cargo, such as PINs (Geldner et al., 2001). In plants, the secretory pathway is readily blocked by BFA. Upon additional inhibition of endocytosis (at approx. 25 µM of BFA), for example by auxin, PIN proteins no longer end up in such a BFA-compartments (Paciorek et al., 2005; Men et al., 2008; Kitakura et al., 2011). As shown recently, at higher concentrations (approx. 50 µM), BFA along with influencing exocytosis, also inhibits vacuolar targeting of PIN proteins. This effect, most probably, is due to BFA at higher concentrations acting on additional subset of ARF-GEFs (variable number of Sec7 domains in the protein structure) (Kleine-Vehn et al., 2008 and 2008a; Robert et al., 2010).

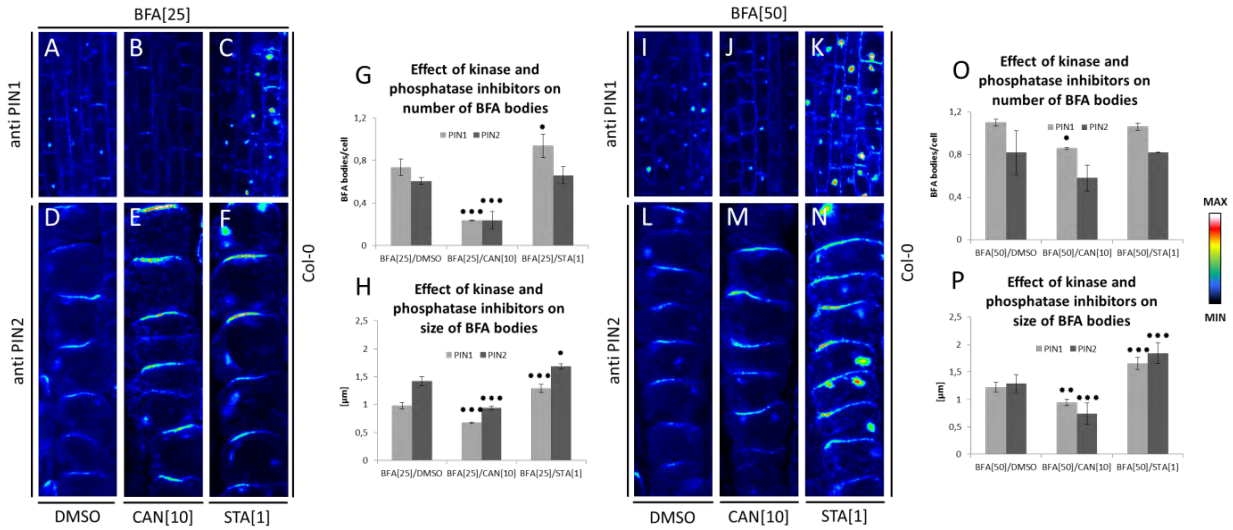

Figure 2. Early endocytosis and late trafficking of PINs in *Arabidopsis* roots are influenced by the inhibition of kinase and phosphatase activity. (A-F) Early endocytosis of PIN1 (A-C) and PIN2 (D-F) proteins, visualized by 25 µM BFA, is inhibited and promoted by treatment with 10 µM CAN (B and E) and 1 µM STA (C and F), respectively, when compared to DMSO treated control (A and D). (G) Quantification of the relative number of BFA bodies in the *Arabidopsis* roots, co-treated with 25 µM BFA and CAN or STA. n=2 independent experiments with at least six roots analyzed for each assay. (H) Quantification of an average BFA body size *Arabidopsis* roots, co-treated with 25 µM BFA and CAN or STA. n=2 independent experiments with at least six roots analyzed for each assay. (I-N) Late endocytic trafficking of PIN1 (I-K) and PIN2 (L-N) proteins, visualized by 50 µM BFA, is inhibited and promoted by treatment with 10 µM CAN (J and M) and 1 µM STA (K and N), respectively, when compared to DMSO treated control (I and L). (O) Quantification of the relative number of BFA bodies in the *Arabidopsis* roots, co-treated with 50 µM BFA and CAN or STA. n=2 independent experiments with at least six roots analyzed for each assay. (H) Quantification of an average BFA body size in *Arabidopsis* roots, co-treated with 50 µM BFA and CAN or STA. n=2 independent experiments with at least six roots analyzed for each assay. Error bars represent standard error of the mean (SEM), * significant, ** highly significant and *** extremely significant at P<0.05, P<0.01 and P<0.001 according to Student's t-test evaluation, respectively. Signal intensities are coded according to the range indicator scale corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm

Thus, different concentrations of BFA allow discriminating between effects on endocytosis for recycling and targeting for degradation (Robert et al., 2010). In our first experimental setup (Figure 2 A-H), in order to address specifically the aspect of PIN membrane stability, we have immunolocalized PIN1 (Figure 2 A-C) and PIN2 (Figure 2 D-F) proteins following co-treatments of CAN or STA with 25 µM BFA. We have observed that, compared with DMSO treated control (Figure 2A and 2D) 10 µM CAN inhibited (Figure 2B and 2E), while 1 µM STA promoted the BFA-induced formation of intracellular PIN1 and PIN2 agglomerations (Figure 2C and 2F). Above effects, were reflected by the quantitative changes in the number (Figure 2G; with exception of PIN2 after STA co-treatment), and size of BFA bodies (Figure 2H). Next, in order to dissect the vacuolar targeting of PINs, we have used similar experimental setup although with 50 µM BFA co-treatment (Figure 2 I-P). We have observed decreased, when compared to DMSO treated control (Figure 2I and 2L) size of BFA bodies after co-treatment with 10 µM CAN (Figure 2J, 2M and 2P). On the other hand, upon co-treatment with 1 µM STA, enlarged BFA bodies could be observed (Figure 2K, 2N and 2P). The above differences, with exception of PIN1 after CAN co-treatment, were not reflected in the amount of BFA bodies (Figure 2O). These results suggest that the inhibition of kinase and phosphatase activity influences the rates of PIN endocytosis and vacuolar targeting.

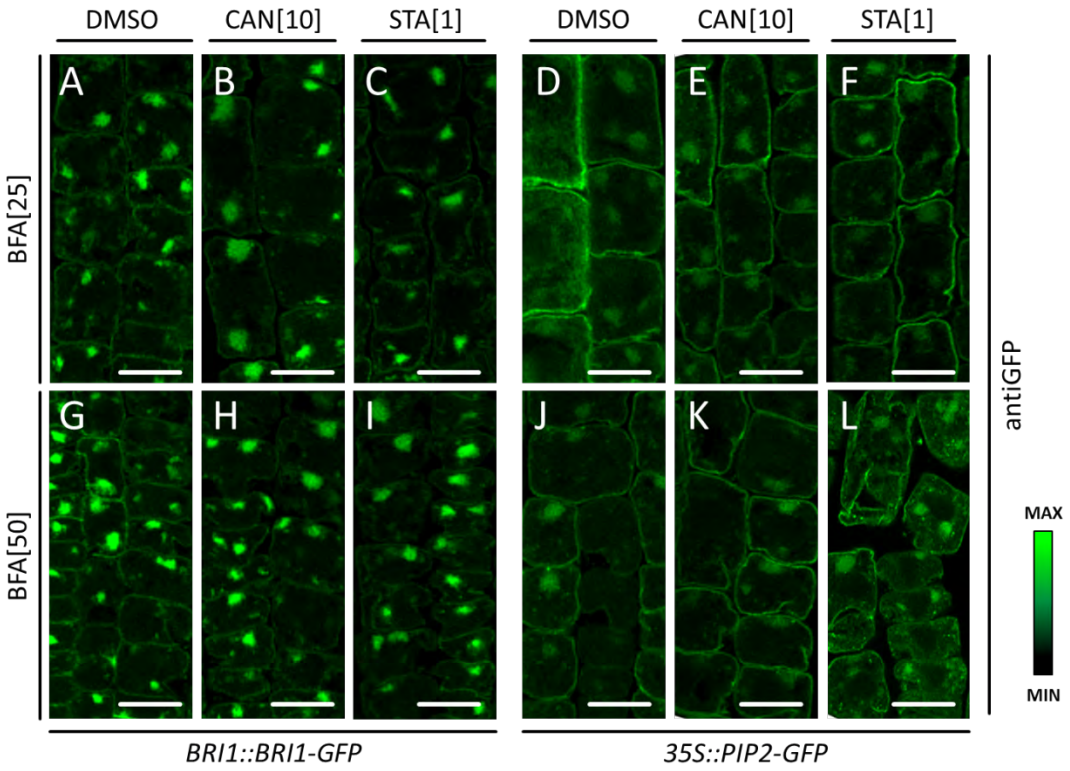

Figure 3. Inhibition of kinase and phosphatase activity does not have an effect on trafficking of apolarly localized PM proteins. (A-F) Early endocytosis of BRI1 protein (A-C) and PIP2 (D-F), visualized by 25 µM BFA treatment, is neither affected by the inhibition of phosphatase activity at 10 µM CAN (B) and (E) nor by the inhibition of kinase activity at 1 µM STA (C) and (F), when compared to DMSO treated control (A) and (D). (G-L) Late endocytic trafficking of BRI1 (G-I) and PIP2 (J-L), visualized by 50 µM BFA treatment, is neither affected by 10 µM CAN (H and K) nor by 1 µM STA (I and L) treatments, when compared to DMSO treated control (G and J). Signal intensities are coded in green corresponding to increasing intensity levels (see color scale). BFA – BrefeldinA CAN – cantharidin, STA - staurosporine. Scale bar = 10 µm.

We have continued the pharmacological part of this study by assessing whether the described above effects of CAN and STA on the protein localization are specific for PINs, or are functional also for other PM-resident proteins. We have therefore designed the experiment, based on the immunolocalization of BRASSINOSTEROID INSENSITIVE1 (BRI1)-GFP and PLASMA MEMBRANE INTRINSIC PROTEIN2 (PIP2)-GFP proteins following CAN or STA co-treatments with 25 or 50 µM BFA (Figure 3 A-L). During the first repetition of this experiment, neither for BRI1 (Figure 3B and 3C), nor for PIP2 (Figure 3E and 3F), we were able to detect any differences in the dynamics of early endocytosis, when compared to DMSO treated controls (Figure 3A and 3D). What is more, when compared to DMSO treated control (Figure 3G and 3J), both in case of BRI1 (Figure 3H and 3I) and PIP2 (Figure 3K and 3L), the late endocytic trafficking seemed also not to be affected by CAN- and STA-induced changes in the cellular phosphorylation status. We have repeated this experiment using the live cell imaging of BRI1-GFP and PIP2-GFP proteins following similar treatments with newly ordered batch of CAN and STA. We could observe an effect comparable to the effect of CAN and STA on PIN endocytosis and vacuolar targeting but of extremely low magnitude (data not shown). In order to precisely describe the consequences of pharmacological inhibition of kinase and phosphatase activity on the localization BRI1 and PIP2 proteins, the additional repetitions of the above experiment should be performed. What is more, to draw comprehensive conclusions on specificity of influence of changes in cellular phosphorylation status on protein cell surface abundance and vacuolar targeting, the above analysis should be expanded on other PM-resident proteins, both localized apolarly like ABCB transporters (Noh et al., 2001) as well as polarly like BOR4 (Miwa et al., 2007) or PIS1 (Ruzicka et al., 2010) for outer lateral, BOR1 (Takano et al., 2005) for inner lateral or AUX1 (Swarup et al., 2001) for apical polar domain.

Overall, our data suggests that CAN-induced abolishment of enzymatic phosphatase activity has an inhibitory effect on endocytosis of PINs, stabilizing them at the PM. What is more, it seems that the inactivation of protein de-phosphorylation not only influences PIN cycling between PM and early endosomal compartments, but also inhibits late endocytic trafficking of PINs. Interestingly, as revealed by STA treatments, suppression of protein phosphorylation seems to have an opposite effect, decreasing PM abundance of PINs, and recruiting them for late endocytic pathway.

The formation of ectopic auxin accumulations, profoundly affecting plant development, was previously associated with the function of PID (Christensen et al., 2000; Benjamins et al., 2001) and WAG (Dhonukshe et al., 2010) Ser/Thr protein kinases, as well as protein phosphatases from PP2A family (Garbers et al., 1996; Rashottte et al., 2001). Recent findings proposed that abnormal auxin distribution pattern, observed in *pid*, *wag* and *ppta* mutants, is a consequence of ectopic PIN polarity. It was shown, that basal-to-apical switch in PIN localization is a result of preferential, phosphorylation-dependent recruitment of PINs into apical targeting pathway (Friml et al., 2004; Michniewicz et al., 2007; Kleine-Vehn et al., 2009). To corroborate on these findings, we have decided to apply a genetics-based strategy in our research. The goal in developing this approach was to determine if phosphorylation of auxin efflux carriers, besides of acting as a signal for polar targeting, would also provide a marker for cellular recognition machinery, to maintain protein within its polar domain or designate it for vacuolar delivery. We have divided this part of our research for two sections. First we have addressed the consequences of hyper-phosphorylation on the subcellular localization of PIN proteins. We have performed live-cell-imgaging analysis of the crosses between *35S::PID* genetic background and pPIN1::PIN1-GFP, pPIN2::PIN2-GFP and pPIN2::PIN3-GFP expressing lines (Figure 4 A-H). We were addressing the PM

stability and vacuolar targeting as markers for rates of protein turn-over (see Chapter 2). We have observed that all three proteins, when expressed in wild-type genetic background, in addition to expected PM localization, exhibited diffused GFP fluorescence in the lytic vacuoles (Figure 4A, 4D and 4G). In contrast, for both PIN2 (Figure 4B and 4C) and PIN3 (Figure 4E and 4F) expressed in *35S::PID* genetic background, higher rates of PM-derived signal with concomitant decreased intensity of vacuolar GFP signal, could be observed. To lesser extent, similar effect could be observed also in case of PIN1 protein (Figure 4H).

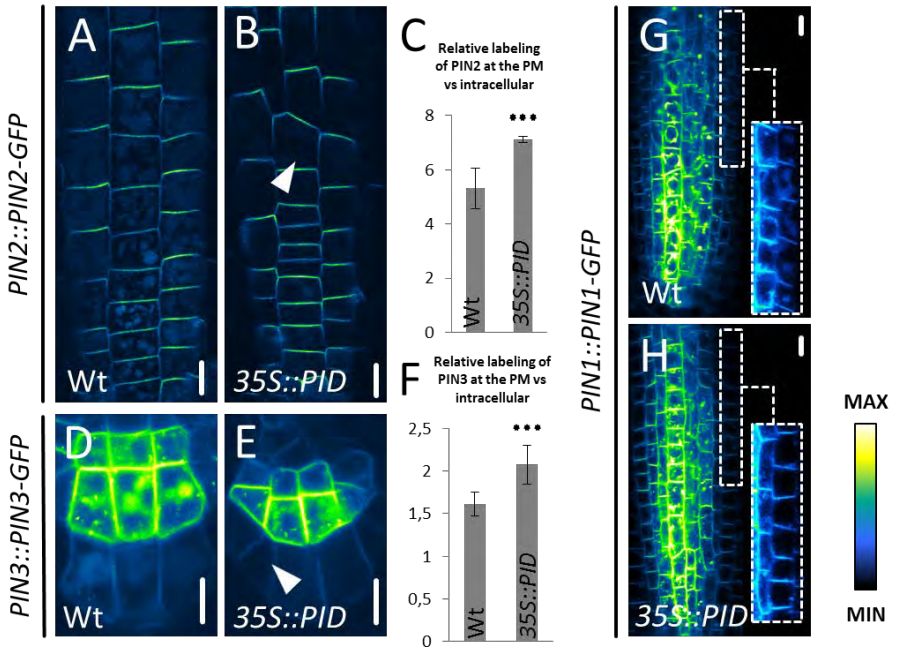

Figure 4. Increased rates of protein phosphorylation influence subcellular PIN localization in *Arabidopsis* roots. (A-B) Live cell imaging of PIN2-GFP protein in epidermal cells of wild-type (A) and *35S::PID* (B) genetic background seedlings, after 8h incubation in the dark. Note a decreased vacuolar accumulation (marked by white arrowhead) of diffused PIN2-GFP signal in *35S::PID* (B), when compared to wild-type genetic background (A). (C) Quantification of relative PIN2-GFP abundance at the PM versus the intracellular signal in the seedlings of wild-type and *35S::PID* genetic background. n=3 independent experiments with at least six roots analysed for each assay and at least six cells evaluated per root. (D-E) Live cell imaging of PIN3-GFP protein in columella cells of wild-type (D) and *35S::PID* (E) genetic background seedlings, after 8h incubation in the dark on medium supplemented with 5 µM ConA. Note a decreased vacuolar accumulation (marked by white arrowhead) of diffused PIN3-GFP signal in *35S::PID* (E), when compared to wild-type genetic background (D). (F) Quantification of relative PIN3-GFP abundance at the PM versus the intracellular signal in the seedlings of wild-type and *35S::PID* genetic background. n=3 independent experiments with at least six roots analysed for each assay and at least five cells evaluated per root. (G-H) Live cell imaging of PIN1-GFP protein in the central cylinder of wild-type (G) and *35S::PID* (H) genetic background seedlings, after 24h incubation in the dark. Note a decreased vacuolar accumulation (marked by discontinuous white line on magnified internal panel) of diffused PIN3-GFP signal in *35S::PID* background (H) when compared to wild-type background (G). Error bars represent standard error of the mean (SEM), *** extremely significant at P<0.001 according to Student's t-test evaluation, respectively. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

Next, we have explored if hypo-phosphorylation, in case of PINs, would be the signal for PM dissociation and/or late endocytic pathway targeting. Dark-incubated PIN3-GFP protein, typically exhibits GFP-positive internalizations in the corners of RAM columella cells (Kleine-Vehn et al., 2010). These intracellular structures, most likely, reflect PIN3 protein being dynamically re-localized in response to changing environmental conditions (gravity, light). We have used this phenomenon to address the PM stability of PINs. We have expressed *pPIN3::PIN3-GFP* construct in *35S::PID* and *pidwag1wag2* genetic backgrounds (Figure 5 A-D). We have observed that, compared to wild-type genetic background (Figure 5A), the amount of internalized PINs, reflected by number and size (not shown) of intracellular GFP-positive structures (Figure 5D), is decreased and increased in the kinase gain-of-function (Figure 5B) and loss-of-function (Figure 5C) background, respectively.

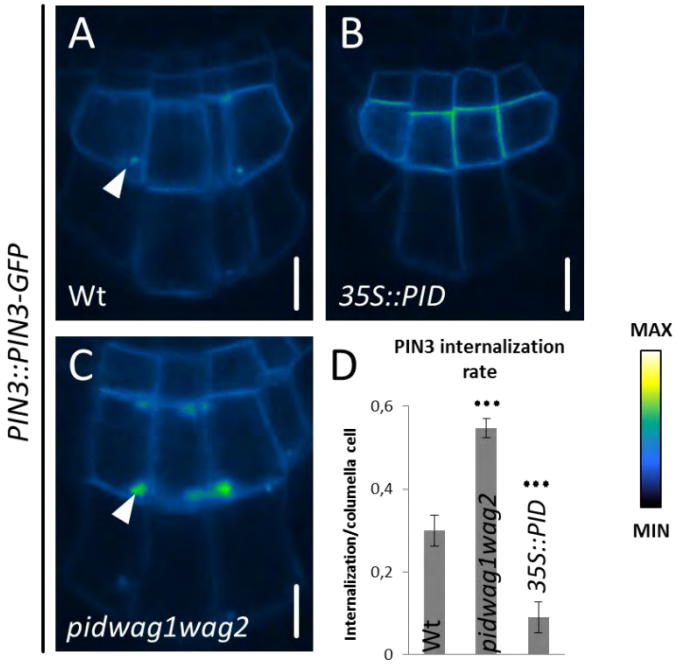

Figure 5. Decreased rates of protein phosphorylation influence subcellular PIN localization in *Arabidopsis* roots. (A-C) Live cell imaging of PIN3-GFP protein in the columella cells of wild-type (A), *35S::PID* (B) and *pidwag1wag2* (C) genetic background seedlings, after 9h dark treatment. Note increased size and amount of GFP-positive internalizations (marked by white arrowheads) in *pidwag1wag2* genetic background (C), when compared to wild-type background (A). (D) Quantification of PIN3-GFP internalization rate in the columella cells of Wt, *35S::PID* and *pidwag1wag2* genetic background. n=3 independent experiments with at least ten roots analysed for each assay. Error bars represent standard error of the mean (SEM), *** extremely significant at P<0.001 according to Student's t-test evaluation, respectively. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

We have previously shown that prolonged treatments with auxin target PINs for vacuolar degradation (see Chapter 2). Based on this observation we have designed an experiment to further analyze the PM stability of PINs in conditions of altered cellular phosphorylation status. We have immunolocalized PIN1 and PIN2 proteins following 12h treatment with 10 µM NAA in *35S::PID*,

pidwag1wag2, *rcn1*, *rcnl2* and *rcn1rcnl2* genetic backgrounds (Figure 6 A-G). *rcn1 (roots curling on NPA1),* a regulatory subunit A (PP2AA1) mutant of PP2A phosphatase complex was shown to have reduced phosphatase enzymatic acvitivy (Deruere et al., 1999). In addition, mutants in closely related, regulatory A subunits of PP2A (PP2AA2 and PP2AA3), *rcnl1* and *rcnl2* (*rcn-like1* and *rcn-like2*, respectively), when combined, caused increasingly stronger developmental defects related with increased PIN phosphorylation and thus altered auxin transport (Michniewicz et al., 2007). We have therefore hypothesized, that genetically-induced changes in the activity of kinase and phosphatases would influence the rate of PIN phosphorylation and thus the stability at the PM and vacuolar targeting of auxin transporters.

In deed, when compared with wild-type control (Figure 6A), PID over-expressing (Figure 6B) as well as *rcn1* (Figure 6D) and *rcnl2* (Figure 6E) mutant seedlings, exhibited resistance to the auxin-induced PIN turn-over. What is more, we could observe an additive effect in *rcn1rcnl2* double mutant (Figure 6F). Similar analysis in *pidwag1wag2* mutant background revealed that the PIN stability, even though decreased when compared with PID overexpressing background or phosphatase mutants, is increased when compared with wild-type (Figure 6C and 6G). Besides of the fact that high variability of signal intensity was observed following PIN immunolocalization in *pidwag1wag2*, in light of our previous findings, we did not find a rational explanation for increased resistance of this mutant line to auxin effect on PIN vacuolar targeting. It should be noted in this place that auxin is rapidly metabolized, therefore the effects observed in this experiment (treatments extended in time), could be due to certain auxin metabolites. Such a possibility should be further studied.

Earlier studies have identified an amino-acid motif TPRXS (S/N), evolutionary conserved in the hydrophylic loop (HL) of PM localized PINs, as being site of phosphorylation. Subsequently, direct PID-dependent phosphorylation was reported for central serine residues: Ser231, Ser252 and Ser290 within PIN1 HL. Substitution of these residues with Alanine (Ala) (to mimick lack of phosphorylation) or Glutamic acid (Glu) (to mimic constitutive phosphorylation), lead to preferential basal or apical, respectively, targeting of PIN1 protein (Kleine-Vehn et al., 2009; Huang et al., 2010). Similar strategy helped to identify residues: Ser337 and Thr340 as additional sites, although non-phosphorylated by PID directly, important for PIN polarity regulation (Zhang et al., 2010). Recent analysis of PIN3 HL sequence yielded characterization of overlapping, with the described above for PIN1, but also novel phosphorylation sites, determining polar localization of PIN3. Interestingly, the substitution of PIN3 HL residues: Ser209, Ser212, Ser215, Thr222 and Ser226 with Ala caused targeting of PIN3 protein to the tonoplast instead of PM (Ganguly et al., 2012; Ganguly and Cho, 2012). Considering all these facts, we have initiated the analysis of PIN protein localization using *Arabidopsis* transgenic lines expressing PIN1 version containing amino-acid substitutions mimicking (xAsp) or preventing phosphorylation (xAla), previously established in our group (Figure 7 A-J; Michniewicz, unpublished; Grones, unpublished; Zhang et al., 2010). We were concentrating specifically on the aspect of PM occurrence versus vacuolar targeting. For the convenience, we referred to the residues: Ser231, Ser252/Ser253, Ser290 and Ser337/Thr340 as phosphorylation sites 1, 2, 3 and 4 (p1, p2, p3 and p4), respectively. We have applied 12h treatment with 10 µM NAA to destabilize PIN1-GFP, PIN1p234xAla-GFP and PIN1p234xAsp-GFP proteins from the PM (Figure 7 A-D; see Chapter 2). Following quantification of the residual GFP signal, we could identify increased, when compared to un-mutated control (Figure 7A and 7D), sensitivity to the auxin effect on PIN membrane stability of *pPIN1::PIN1p234xAla-GFP* construct (Figure 7B and 7D).

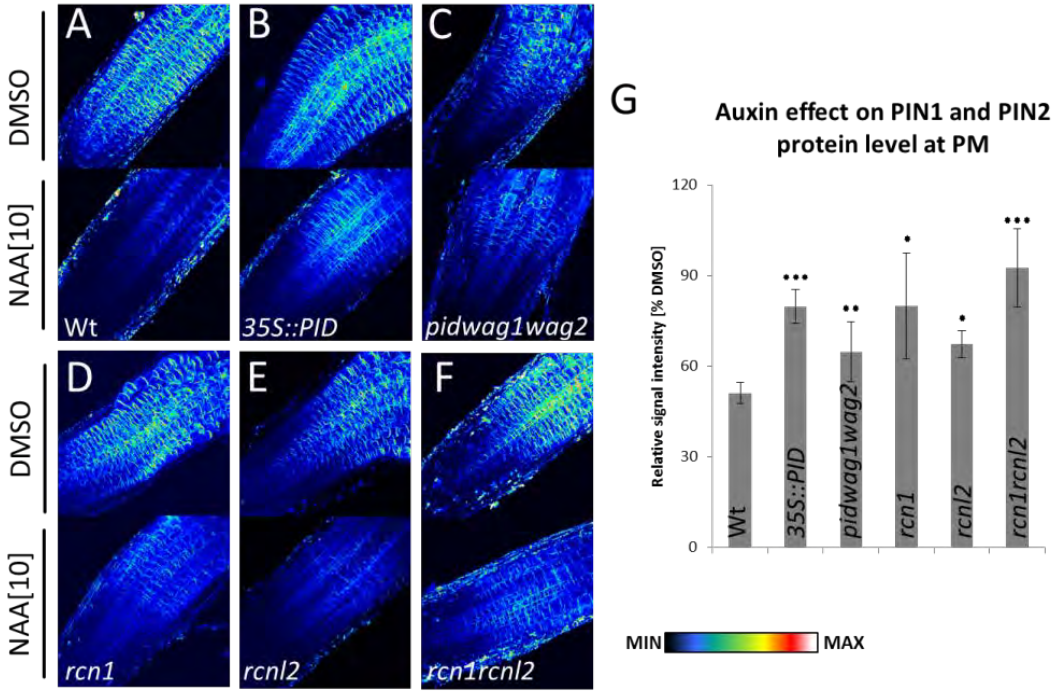

Figure 6. Auxin-induced destabilization from the cell surface and degradation of PINs in *Arabidopsis* roots is influenced by the phosphorylation status of the protein. (A-F) PIN1 and PIN2 immunolocalization in *Arabidopsis* seedlings of wild type (A), *35S::PID* (B), *pidwag1wag2* (C), *rcn1* (D), *rcnl2* (E) and *rcn1rcnl2* (F) genetic background after DMSO or 10 µM NAA treatment. (G) Quantification of the relative PIN1 and PIN2 signal intensity at the PM (% of the DMSO control). n≥2 independent experiments with at least five roots analysed for each assay. Immunolocalization pictures represent maximum intensity projection of 10 Z-sections spaced approximately 6 µm apart through the whole root. Error bars represent standard error of the mean (SEM), * significant, ** highly significant and *** extremely significant at P<0.05, P<0.01 and P<0.001 according to ANOVA two-factor with replication evaluation, respectively. Signal intensities are coded according to the range indicator scale, corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

On the other hand, the *pPIN1::PIN1p234xAsp-GFP* construct, although more stable on the PM than *PIN1::PIN1p234xAla-GFP* construct, was still oversensitive to the long-term auxin effect on PIN PM abundance, when compared to un-mutated control (Figure 7C and 7D). We have then analyzed the transgenic *Arabidopsis* lines expressing PIN1 or PIN3 proteins with phospho-mimetic and phospho-abolishing substitutions at p4 (Figure 7 E-J). Neither for PIN1 (Figure 7 E-G), nor for PIN3 (Figure 7 H-J), when compared to un-mutated controls (Figure 7E and 7H), we were able to detect any differences in protein abundance at the PM or rates of vacuolar targeting, resulting from substitutions within p4, changing phosphorylation status of the protein (Figure 7F, 7G, 7I and 7J). It should be noted that this was preliminary analysis in order to identify potential specificity of certain phosphorylation sites towards the regulation of PIN PM abundance and vacuolar targeting. This experimental design was performed only once and using only one independent transgenic line per phosphorylation variant (transgene integration site could influence the results). The promising results visualized on Figure 7

prompted us to design a detailed analysis of PIN1 phosphorylation sites in context of vacuolar targeting (see Chapter 6).

DISCUSSION

Previous studies revealed that the polar localization of PIN proteins is tightly associated with their phosphorylation status (Friml et al., 2004; Wisniewska et al., 2006; Michniewicz et al., 2007). The readjustments of this status depend on the antagonistic activity of AGC3 protein kinase PINOID (PID), its homologs WAVY ROOT GROWTH1/2 (WAG1/WAG2) (Benjamins et al., 2001; Friml et al., 2004; Santner

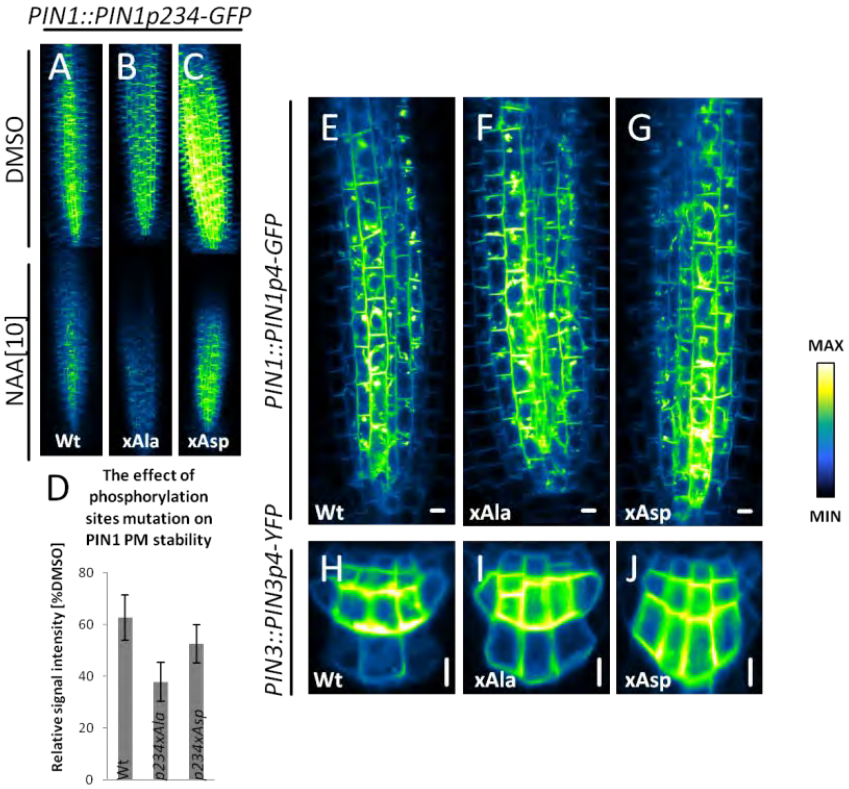

Figure 7. The consequence of genetic manipulation in the conserved phosphorylation sites within PIN HL on the stability of PIN proteins at the PM. (A-C) Live cell imaging of *Arabidopsis* transgenic lines expressing *PIN1::PIN1-GFP, PIN1::PIN1p234xAla-GFP* and *PIN1::PIN1p234xAsp-GFP* constructs, after 12h treatment with 10 µM NAA. Note a decreased stability of PIN1 at the PM when p2, p3 and p4 within PIN1HL are non-phosphorylated (B), compared to wild-type control (A). (D) Quantification of relative GFP signal intensity in seedlings expressing *PIN1::PIN1-GFP, PIN1::PIN1p234xAla-GFP* and *PIN1::PIN1p234xAsp-GFP* constructs. n = 1 with at least 8 seedlings analyzed. (E-J) Live cell imaging of *Arabidopsis* transgenic lines expressing *PIN1::PIN1-GFP* (E), *PIN1::PIN1p4xAla-GFP* (F), *PIN1::PIN1p4xAsp-GFP* (G), *PIN3::PIN3-YFP* (H), *PIN3::PIN3p4xAla-YFP* (I) and *PIN3::PIN3p4xAsp-YFP* (J), after long-term incubations in the dark. Note a lack of observable differences in the subcellular PIN localization, when compared to non-mutated controls (E and H). Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). p1 – phosphorylation site 1 (see text of Chapter 3), x - substitution. Scale bar = 10 µm

and Watson, 2006; Dhonukshe et al., 2010) and PROTEIN PHOSPHATASE2A (PP2A) (Muday and DeLong, 2001; Michniewicz et al., 2007; Ballesteros et al., 2013). These enzymes phosphorylate and dephosphorylate PINs, respectively. Recent experiments led to the formulation of an orthodox model based on which, the enzymatic activity of aforementioned protein kinases and phosphatases, contributes to the preferential recruitment of the hyper-phosphorylated PINs into the apical, BFA-insensitive pathway, independently of the GNOM function. In contrary, hypo-phosphorylated PINs, according to this model, are targeted to the basal cell side, in GNOM-dependent manner (Kleine-Vehn et al., 2009).

Interestingly, evidences in the literature suggest that the described above, trafficking-related processes for the establishment of polar PIN localization might exist in parallel with other phosphorylation-based mechanisms for the maintenance of PIN localization within specific domains of PM. For example, inhibitors of phosphatase enzymatic activity, such as CAN, were shown to elevate shootward auxin transport (Rashotte et al., 2001; Shin et al., 2005). Since this mode of auxin relocation within plant tissue is facilitated most prominently by apically, in epidermal cell files, localized PIN2 protein, these findings, at least partially, imply an increased occurrence of PIN2 at the PM upon abolishment of phosphatase activity. An opposite effect, namely a reduction in shootward auxin transport was reported when kinase function was impaired genetically (in *pid* mutant alleles), or pharmacologically (by STA treatment) (Sukumar et al., 2009). Disturbing both phosphorylation and de-phosphorylation lead to profound defects in environmental responses like root gravitropism but also in other plant developmental processes (Benjamins et al., 2001; Rashotte et al., 2001; Shin et al., 2005). On the subcellular scale CAN, even at high doses, does not influence PIN2 localization. However, it should be mentioned that the authors of the referred study addressed polarity changes rather than quantitative differences in PIN2 abundance at the PM (Shin et al., 2005). What is more, although analysis of staurosporin-treated *Arabidopsis* seedlings, expressing GFP-tagged PIN2 protein, revealed a lack of polarity defects, an increased number of PIN2-positive internal structures could be detected, suggesting decreased stability of PIN2 at the membrane in these conditions (Sukumar 2009).

The referred reports prompted us to investigate a possible dual role of PIN phosphorylation. We hypothesized, that in addition to its orthodox function during trafficking-based polar PIN delivery, the modifications of phosphorylation status could be involved in the regulation of cell surface abundance and rates of vacuolar trafficking of PIN proteins. We have used two independent, pharmacology-based, experimental approaches to dissect such a dual function of protein phosphorylation; live-cell-imaging of *Arabidopsis* seedlings expressing GFP-fused PIN proteins and immuno-localizations of PIN proteins. In both experimental setups, in order to impair the status of phosphorylation we have used staurosporin and cantharidin treatments. We were able to observe a cantharidin-induced stabilization of PIN2 protein at the PM with concomitant reduction of PIN2 vacuolar abundance. This data suggests decreased rates of endocytosis and vacuolar trafficking of auxin efflux carriers in the above conditions. The same experimental design with use of staurosporine, allowed us to observe a lower PM abundance simultaneously with increased vacuolar occurrence of PIN2 protein. In order to precisely define into which extent the phosphorylation status of PIN proteins is associated with their PM abundance, western blotting analysis of PIN1 and PIN2 microsomal fractions should be performed upon inhibition of kinase and phosphatase activity. Large scale analysis of transcriptome revealed that cantharidin negatively regulates the transcription of PIN3, 4 and 7 (Bajsa et al., 2011), it would be, however, necessary to

analyze a possible relation between the effects of kinase and phosphatase inhibitors, presented in this study and the regulation of PIN transcription.

We further investigated the role of HL phosphorylation in the regulation of PIN cell surface abundance as well as possible mechanism, by which phosphorylation of certain residues within PIN amino acid sequence could provide a signal for conditional endocytosis eventually resulting with PIN turn-over. We have expressed fluorescently tagged members of the PIN family in hypo- and hyper-phosphorylating genetic backgrounds of *pidwag1wag2* and *35S::PID*, respectively. We were able to demonstrate increased rates of PIN3 internalization in the cellular environment lacking kinase function. Although the internalized PIN3 structures did not have identity of the lytic compartment (data not shown) this experiment certainly demonstrates that, when under-phosphorylated, PIN3 protein is less stable at the PM. On the other hand, a decreased occurrence of PIN-derived GFP signal in the vacuoles concomitantly with stabilization of the protein at the PM, observed in kinase gain-of-function seedlings, led us to believe that PIN hyper-phosphorylation stabilizes protein at the PM and reduces rates of its vacuolar lysis. Again like in case of pharmacological treatments, the application of biochemistry-based approaches, like western blotting analysis of PIN content in microsomal fractions of hypo- and hyper-phosphorylating genetic backgrounds, could bring additional insights into the model presented in this study. What is more, the possibility that the PIN3-GFP internalizations observed with increased frequency in kinase loss-of-function mutant, are the result of elevated *de-novo* synthesis and secretion of the PIN3 protein, should be excluded. Therefore, the frequency of PIN3-positive internalizations in the columella cells of *pidwag1wag2* mutant should be addressed experimentally, for example in presence of protein synthesis inhibitor, cycloheximide.

In our experiments, we were able to detect significant changes in protein subcellular localization, induced by perturbations of its phosphorylation status. Generally, these changes were most prominent for PIN2 and PIN3 proteins, less pronounced but to some extent present also for PIN1 protein but absent (or of extremely low magnitude) in case of BRI1 and PIP2 proteins. We have therefore concluded that the effect of constitutive or abolished phosphorylation, on the PM abundance of the protein and rates of its degradation is specific for PINs, when compared to other PM cargos (in this case apolarly localized). To draw the comprehensive conclusions in this context, however, the analysis should be expanded on various other PM-resident proteins (mentioned in the Results section of this Chapter). Within PIN family the regulatory mechanism presented here appears to apply for apically and apolarly, rather than basally localized cargoes.

Collectively the data discussed above, in our opinion, is convincing enough to propose an integration of another phosphorylation-based regulatory component into mechanism controling auxin fluxes during plant development. Such a mechanism would execute a dual function where not only the regulation of trafficking-based delivery of PINs to the specific polar domain of PM would be achieved (Friml et al., 2004; Michniewicz et al., 2007; Kleine-Vehn et al., 2009), but similarly to ubiquitination (Leitner et al., 2012), a signal to regulate the cell surface abundance and vacuolar delivery of PINs, would be provided by phosphorylation.

We further elaborated on the topic of PIN phosphorylation status variations and possible implications of it for both the cell surface abundance of PIN efflux carriers and rates of their vacuolar turn-over. We have analyzed the *Arabidopsis* transgenic lines expressing PIN proteins, mutated within previously described phosphorylation sites, to mimic constant or loss of phosphorylation (Grones,

unpublished; Michniewicz, unpublished; Zhang et al., 2010; Huang et al., 2010). We were able to observe quantitative decrease in the stability of PIN1 at the PM, when phospho-sites p2, p3, and p4 were modified to mimic lack of phosphorylation. On the other hand, we could not detect any differences when single p4 site was modified to mimic loss or constant phosphorylation. This data suggests possible specificity of certain phosphorylation sites towards the regulation of PIN PM abundance and vacuolar targeting.

MATERIAL AND METHODS

Plant material and growth conditions

All *Arabidopsis thaliana* mutants and transgenic lines used in this study are in the Columbia (Col-0) background and have been described previously: *PIN1::PIN1-GFP* (Benkova et al., 2003); *PIN2::PIN2-GFP* (Xu and Scheres, 2005); *BRI1::BRI1-GFP* (Russinova et al., 2004); *35S::PIP2-GFP* (Cutler et al., 2000); *35S::PID* (Benjamins et al., 2001); *pidwag1wag2* (Dhonukshe et al., 2010); *rnc1 rcnl2 rcn1rcnl2* (Zhou et al., 2004); *PIN1::PIN1p234xAla-GFP* and *PIN1::PIN1p234xAsp-GFP* (Zhang et al., 2010); *PIN3::PIN3-GFP* (Zadnikova et al., 2010). Surface-sterilized seeds were sown on half-strength Murashige and Skoog (0.5 MS) agar plates and stratified for 2 days at 4°C. Plants were grown on vertically oriented plates under continuous light conditions at 22°C for 4-5 days.

Reagents

1-Naphthalene-acetic acid (1-NAA), Cantharidin (CAN), Staurosporine (STA), ConcanamycinA (ConA) (Sigma, http://www.sigmaaldrich.com), BrefeldinA (BFA) (Invitrogen, http://www.invitrogen.com).

Immunodetection and microscopy

Whole-mount immunolocalization in *Arabidopsis* roots was performed as described previously (Sauer et al., 2006). The rabbit anti-PIN1 (Paciorek et al., 2005; 1:1000), rabbit anti-PIN2 (kindly provided by C. Luschnig; 1:1000) and mouse anti-GFP (Roche; 1:600) were used as primary antibodies. The fluorochrome-conjugated rabbit Cy3 and mouse Alexa Fluor 488 (Invitrogen; 1:600) were used as secondary antibodies.

Drug Application and Experimental Conditions

Pharmacological phospho-mimetic experiments (Figure 1) were performed as follows. Subcellular localization of PIN2 was performed after 5h incubation in the dark, on medium supplemented with 5 µM ConA and 10 µM CAN or 1 µM STA. PIN1 was analysed after 8h incubation in the dark, on medium supplemented with 10 µM CAN or 1 µM STA. For BFA immunolocalizations (Figure 2 and 3), pre-incubation with 10 µM CAN, 1 µM STA or DMSO for 30 min followed by co-incubation of respective reagents with 25 or 50 µM BFA for 90 min was applied. For the analysis of protein stability in *35S::PID*

background (Figure 4), 8h incubations in the dark for PIN2 and PIN3 (additional supplementation of medium with 5 µM ConA) and 24h for PIN1, were used. PIN3 protein stability in *pidwag1wag2* and *35S::PID* background (Figure 5) was analysed after 9h dark incubation. For immunolocalization of PIN1 and PIN2 in phosphorylation mutants (Figure 6), 12h treatment with 10 µM NAA was used. For the analysis of PIN abundance in the phospho-mimetic and phospho-preventing transgenic lines (Figure 7), 12h treatment with 10 µM NAA (*PIN1::PIN1p234xAla/Asp-GFP*), 24 h dark incubation (*PIN1::PIN1p4xAla/Asp-GFP*) and 11h dark incubation (*PIN3::PIN3p4xAla/Asp-GFP*) was used.

Image processing, and statistical analysis

Signal intensities are coded according to blue-yellow, green or range indicator scale, corresponding to the increasing intensity levels (see color scale under figure). Quantitative results are visualized as means with error bars representing standard error of the mean (SEM), * significant, ** highly significant and *** extremely significant at $P<0.05$, $P<0.01$ and $P<0.001$ according to Student's t-test or ANOVA two-factor with replication evaluation, respectively. All fluorescence signals were evaluated on the Zeiss LSM 710 or Olympus IX-81 confocal laser scanning microscopes. For imaging GFP, the 488- and 514-nm lines of the argon laser were used for excitation, and emission was detected at 510 and 530 nm, respectively. For semi-quantitative measurement of fluorescence intensities, laser, pinhole, and gain settings of the confocal microscope were kept identical among respective treatments. The mean fluorescence intensity was measured with ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij). Images were processed with Adobe Photoshop 12.0. Statistics were evaluated with Excel2010 (Microsoft).

Quantification index

The relative labelling of PIN1 and PIN2 at the membrane vs intracellular (Figure 1) was performed using 'Surface Plot' option of ImageJ software. The signal sample was collected from the region marked by discontinuous white line on Figure 1. The statistical analysis of early endocytosis (Figure 2; BFA 25 µM) and late endocytic trafficking (Figure 2; BFA 50 µM) was done by relating the number of BFA bodies to number of cells visible on the image and by measuring an average diameter of BFA bodies. In both cases the cells in central cylinder including endodermis for PIN1 and epidermis, cortex for PIN2 were analyzed. The relative labelling of PIN2 and PIN3 at the membrane vs intracellular was performed by marking the area of the apical plasma membrane of the cell and relating it to whole intracellular or vacuolar area of the same cell. The analysis of PIN3 membrane stability in *pidwag1wag2* and *35S::PID* backgrounds (Figure 5) was performed by counting the number of GFP-positive internalizations and relating it to the number of the columella cells. The analysis of PIN1 and PIN2 membrane stability was performed by the quantification of the signal intensity collected from whole root (Figure 6) or whole GFP expressing region (Figure 7).

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Chapter 4

Putative regulator of PIN polarity identified by means of forward genetics screen using strigolactone analog - GR24

Pawel Baster, Petr Valosek, Jing Zhang and Jiří Friml

Putative regulator of PIN polarity identified by means of forward genetics screen using strigolactone analog - GR24

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Abstract

Establishment of the cell polarity, considered as the generation of any asymmetry in the cell structure or distribution of the cellular components, is one of the fundamental tasks for all eukaryotic organisms. It is particularly important for plants as, due to their immobility, they have to adjust their development to the varying environmental conditions. Shape and direction of an organ growth within different developmental contexts and in response to various environmental signals is orchestrated by the phytohormone auxin. The formation of auxin gradients across the tissue, which are subsequently triggering specific developmental responses, is executed by the directional cell to cell transport of auxin, performed by efflux carriers of the PIN family. Most of PIN family members, dependent on tissue or developmental context, display subcellular localization restricted to specific polar domain witin PM. This polar localization is regulated by various processes, including PIN destabilization from the PM and vacuolar targeting for proteolysis. Here we show that the native PIN localization is disturbed, likely by the preferential depletion of PINs from the basal PM, in presence of the synthetic strigolactone-analogue GR24. As a result, *Arabidopsis* roots in presence of GR24 exhibit inhibition of the root growth and agravitropism. We have designed forward genetics screen using GR24 as a tool in order to identify novel regulators of polar PIN localization. The EMS mutant *pig1* (*PIN localization resistant to GR24 1*) displays proper root growth, gravitropism and normal PIN polarization in presence of GR24. We present data supporting the notion that *pig1* mutant is resistant to GR24-induced destabilization of PIN1 from the cell surface and vacuolar targeting.

Author Contributions: JF designed the research; PB performed most of the experiments, analyzed the data, prepared the figures and wrote the manuscript, PV mapped the *pig1* mutation, JZ performed the experiments visualized on Figure 1.

INTRODUCTION

The phytohormone auxin controls an ave-inspiring magnitude of crucial events during plant life, including the regulation of developmental processes at both embryonic (Friml et al., 2003; Schlereth et al., 2010) and post-embrionic level (Sabatini et al., 1999; Friml et al., 2002a). What is more, many environmental responses, contributing for an amazing plasticity of plant ontogenesis, like photo- or gravitropism, are instructed by auxin (Ding et al., 2011; Rakusova et al., 2011). Such a broad range of regulation, by only one molecule, can be achieved due to the implementation of relatively simple system, at the core of which lies the generation of spatio-temporarily-defined fluctuations of auxin concentration within plant tissues (Tanaka et al., 2006). The emergence of these, so called, 'auxin gradients' acts as a trigger for downstream signaling cascades (Benkova et al., 2003; Sorefan et al., 2009). Auxin, in contrary to cellular influx, has to be actively exported from the cell interior (Rubery and Sheldrake, 1974; Raven, 1975). This task is facilitated, most prominently, by carriers of the PIN family, most of which (PIN1, 2, 3, 4 and 7), dependent on the tissue and developmental context, localize to highly defined, polar domains within PM (Zazimalova et al., 2007). It is thus the abundance and localization of PIN's which regulate the rate and direction of an intercellular auxin flux, essentially contributing for the generation of auxin gradients (Petrasek et al., 2006; Wisniewska et al., 2006). Research performed over past years identified a multitude of molecular components and cellular processes involved in the establishment and maintenance of PIN polarity. These include, among others, regulated PIN transcription (Vieten et al., 2005), subcellular trafficking (reviewed in Nodzynski et al., 2012), post-translational PIN modifications (reviewed in Lofke et al., 2012) and finally, regulated protein turn-over. What is more, several cell-related factors, like composition of the PM or cell wall connections were also identified to contribute, especially for maintenance of polar PIN localization (see Chapter 1). Interestingly, by multiple PIN-related feedback mechanisms, auxin was shown to organize its own flux (see Chapter 1; Paciorek et al., 2005; Sauer et al., 2006; Robert et al., 2010). As recent research shows, not only auxin can have profound influence on PIN localization, but other hormones or signaling molecules can have as well (see Chapter 1).

The field of experimental plant biology continuously faces the discovery of new signaling molecules. These novel substances bring a potential for further expanding of our understanding of processes underlying plant development at the subcellular scale. Recently a hormonal activity was designated to a group of carotenoid-derived compounds termed Strigolactones (SL) (Matusova et al., 2005; Zwanenburg et al., 2009). These molecules are known for few decades now, and were initially described as rhizospheral plant exudates, stimulating seed germination of the *Striga* and *Orobanche* parasitic weed genera (Cook et al., 1966; Lopez-Raez et al., 2009). SLs were also reported to attract the symbiosis between plants and arbuscular fungi (Akiyama et al., 2005; Besserer et al., 2006). In flowering plants nutrient limitation appears to activate SL biosynthesis and thus plant-fungi, mycorrhizal interaction, which consecutively improves the uptake of compounds essential for plant development (Kohlen et al., 2011; Ruyter-Spira et al., 2011). Recent discovery that SL act also as signaling molecules was described in the context of the shoot branching regulation (Gomez-Roldan et al., 2008; Umehara et al., 2008). Indeed, mutations in the *Arabidopsis thaliana* MORE AXILLARY GROWTH (MAX) and orthologous genes in *Pisum sativum*, *Oryza sativa* and *Petunia hybrida* all lead to increased number of axiliary branches (Beveridge, 2000; Ishikawa et al., 2005; Snowden et al., 2005; Zou et al., 2005 and

2006; Johnson et al., 2006; Arite et al., 2007; Simons et al., 2007). Similarly, in lower plant species, like *Physcomitrella patens*, SLs through the control of protonemal branching were implicated in the regulation of single colony growth and inter-colony competition (Proust et al., 2011; Delaux et al., 2012). *De novo* synthesis of SLs is regulated by DWARF27 (D27), an isomerase-activity possessing protein, which provides substrate for MAX3-mediated reaction (Lin et al., 2009; Waters et al., 2012a). MAX3 and MAX4, also known as CAROTENOID CLEAVAGE DIOXYGENASES 7 and 8 (CCD7 and CCD8), are responsible for the biosynthesis of carlactone, an intermediate form of biologically active strigolactone (Sorefan et al., 2003; Booker et al., 2004; Alder et al., 2012). MAX1, Cytochrome P450 Monooxygenase, acts downstream of MAX3 and MAX4, most likely, to further modify carlactone (Booker et al., 2005). Mutant phenotypes of all the above players could be rescued by exogenous SL application. In contrary, DWARF14 (D14), α/β hydrolase proposed with role during SL activation or signal transduction (Arite 2009; Hamiaux et al., 2012; Waters et al., 2012b) and MAX2, an F-Box protein and constituent of SCF complex, being putative SL receptor, were shown to be SL insensitive (Stirnberg et al., 2002 and 2007).

In this study, we were exploring the effects of exogenous application of strigolactone on polar localization and abundance of PIN auxin transporters. Using GR24, a synthetic strigolactone analogue, we have performed a forward genetic screen for the regulators of PIN polarity, which yielded identification of PIG1 protein. PIG1, possibly by modulation of intracellular auxin or auxin-conjugate levels, contributes to the regulation of PIN cell surface abundance, by the modulation of protein vacuolar targeting.

RESULTS

In order to identify potentially novel regulators of polar PIN localization a forward genetics screen was designed (see Material and Methods), based on the observation that synthetic strigolactone analogue - GR24 - interferes with PIN polarity. In native conditions, PIN1 and PIN2 proteins are localized to the basal (rootward) side of the endodermal and young cortical cells of the RAM, respectively. Upon treatment with GR24, both proteins were observed to exhibit preferential, when compared to untreated control, apical (shootward) PM localization (Figure 1 A-C). What is more, substantial differences in morphology of plants treated with GR24 were found, when compared with control plants. These included: inhibition of the root growth and impaired root gravitropism (Figure 1 D-G). Such morphological phenotypes were previously associated with defective localization or abundance of PIN proteins (Gälweiler et al., 1998; Luschnig et al., 1998; Okada et al., 1991).

Identification of PIN insensitive to GR24 1 (PIG1) protein

As a result of the forward genetic screen, a *pig1-1* (*PIN insensitive to GR24 1-1*) mutant was identified. First, we have confirmed the resistance of *pig1-1* mutant to the GR24 effect on root growth and subcellular localization of PIN proteins (Figure 2 A-G). Root growth of control, *pPIN1::PIN1-GFP* expressing plants, upon transfer to medium supplemented with 50 µM of GR24, was inhibited and gravitropism disturbed (Figure 2A and 2C). In contrast, roots of *pig1-1* mutant, upon transfer to GR24-supplemented medium, maintained growth and responded properly to gravistimulation (Figure 2B and 2C). Moreover, the GR24-induced change in polar localization of endodermal PIN1 and cortical PIN2

proteins, observed in wild-type seedlings (Figure 2D and 2E), was absent in *pig1-1* mutant (Figure 2F and 2G). This experiment was performed twice with similar results obtained.

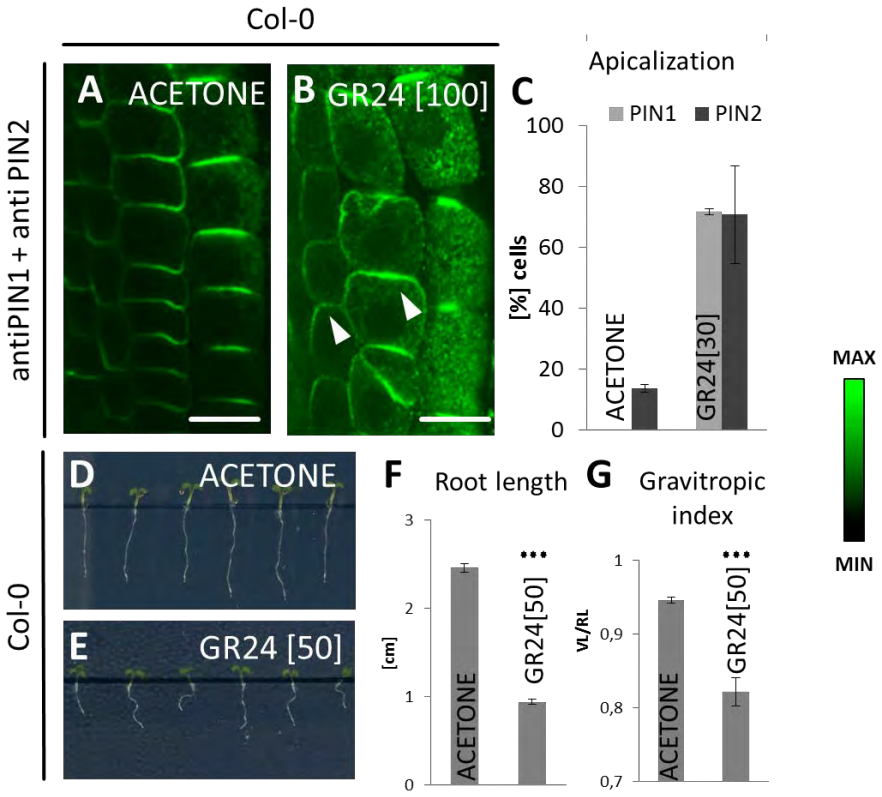

Figure 1. The effect of GR24 on the root morphology and subcellular localization of PIN1 and PIN2 proteins in *Arabidopsis* seedlings. (A-B) PIN1 and PIN2 immunolocalization in roots of *Arabidopsis* seedlings germinated on the control medium (A) or medium supplemented with 30 µM GR24 (B). Note a preferential apical localization of PIN1 in endodermis and PIN2 in the cortex, marked by white arrowheads. (C) Quantification of percentage of cells with apical localization of PIN1 in the endodermis and PIN2 in the cortex. n=1 with at least 5 roots analysed. (D-E) Morphology of *Arabidopsis* seedlings germinated on the control medium (D) and medium supplemented with 50 µM GR24 (E). (F) Quantification of the root length of *Arabidopsis* seedlings germinated on the control medium vs. medium supplemented with 50 µM GR24. n=1 with at least 18 roots analysed. (G) Quantification of the root gravitropic index of *Arabidopsis* seedlings germinated on the control medium vs. medium supplemented with 50 µM GR24. n=1 with at least 18 roots analysed. Error bars represent standard error of the mean (SEM), *** extremely significant at P<0.001 according to Student's t-test evaluation. Signal intensities are coded in red corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm

In order to molecularly characterize the *pig1* mutation, we have applied a map based cloning analysis. We have evaluated the recombination events in approximately 900 chromosomes of F_2 progeny derived from the cross between EMS mutagenized Col-0 (selected in the screen to poses *pig1-1* mutant phenotype) line and wild-type *Landsberg erecta* ecotype. We have found a substitution of a single nucleotide in the coding region of At5g50850 (Figure 3A). This gene is coding for PYRUVATE

DEHYDROGENASE E1-β subunit (PD E1-β) (Luethy et al, 1994). PYRUVATE DEHYDROGENASE COMPLEX (PDC) is composed, in plants, of three main components: E1 - PYRUVATE DEHYDROGENASE (PD), E2 - DIHYDROLIPOYL ACETYLTRANSFERASE (DA) and E3 - DIHYDROLIPOYL DEHYDROGENASE (DD), which are catalyzing three sequential reactions. The E1 part of the PDC forms heterotetramer of two α and two β subunits. Plant PDCs are unique, in that they exist in two spatially and functionally separated forms (Mooney et al., 2002). The mitochondrial PDC (mtPDC) catalyzes the oxidative decarboxylation of pyruvate, derived from glycolysis in the cytoplasm, to acetyl-CoA. Acetyl-CoA is subsequently used as a carbon source for the production of energy and reducing agent NADH, during Krebs cycle (Lernmark and Gardestrom 1994; Randall et al., 1996). On the contrary, the plastid form (plPDC) provides the same substrate (Acetyl-CoA) for *de novo* fatty acid biosynthesis (Camp and Randall, 1985). In *Arabidopsis*, the

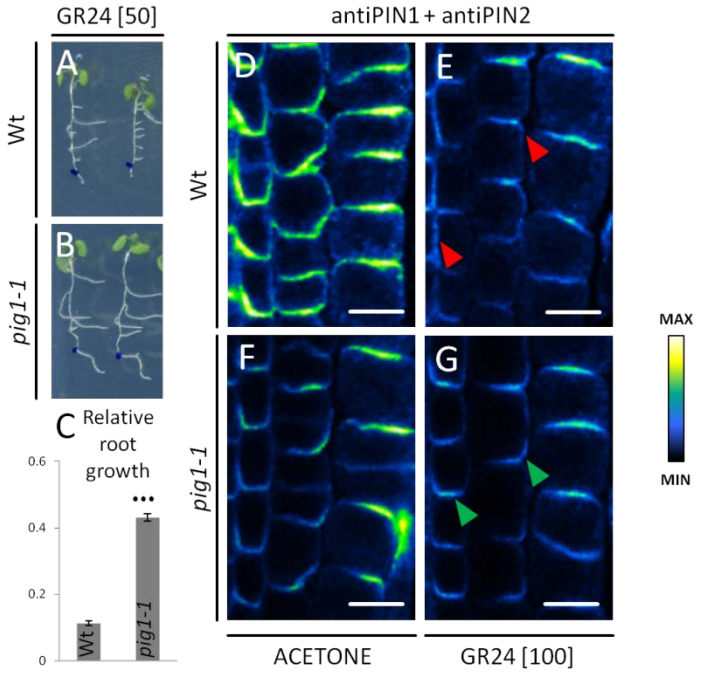

Figure 2. *pig1-1* is resistant to the GR24 effect on root morphology and subcellular PIN1 and PIN2 localization. (A-B) The morphology of *Arabidopsis* seedlings of wild-type (A) and *pig1-1* mutant (B), 7DAG transferred to medium supplemented with 50 µM GR24 and gravistimulated twice. (C) Quantification of the relative root growth of *Arabidopsis* seedlings on medium containing 50 µM of GR24 vs un-supplemented medium. n=1 with at least 60 seedlings evaluated. (D-G) PIN1 and PIN2 immunolocalization in *Arabidopsis* wild-type (D) and (E) and *pig1-1* mutant (F) and (G) seedlings, incubated for 8h on the control medium (D) and (F) or medium supplemented with 100 µM GR24 (E) and (G). Note a preferential apical/lateral and basal localization of PIN1 in endodermis and PIN2 in the cortex cells, marked by red and green arrowheads, respectively. This experiment was performed twice with similar results obtained. Error bars represent standard error of the mean (SEM), *** extremely significant at P<0.001 according to Student's t-test evaluation. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

mitochondrial PDC is encoded by two genes for E1-α (At1g59900 and At1g24180; Luethy et al. 1995; Quint et al. 2009), one gene for E1-β (At5g50850, Luethy et al. 1994), three genes for E2 (At3g52200,

At3g13930 and At1g54220; Guan et al. 1995; Thelen et al. 1999; Taylor et al. 2004) and two genes for E3 (At1g48030 and At3g17240; Lutziger and Oliver, 2001).

To confirm that the mutation we have identified within At5g50850 sequence, is responsible for the *pig1-1* mutant phenotype, we have transformed the *pig1-1* mutant with genomic construct expressing the wild-type version of the At5g50850 gene from its own 5' regulatory sequence, fused with a fluorescent tag at the N- or C-terminal part of the protein (see Materials and Methods). The expression of *pPIG1::PIG1-RFP* construct complemented, while *pPIG1::RFP-PIG1* did not complement the phenotype of *pig1-1* mutant (Figure 4A; see Material and Methods). The *in vitro* interaction between E1-α and E1-β subunits of PDC was previously presented in heterologous system (Szurmak et al., 2003). In plant genome, this interaction was identified as one of the most conserved (Geisler-Lee et al., 2007). Thus, to further address the involvement of PDC in plant response to GR24, we have analyzed the root growth and gravitropic response of *iar4-2* mutant (mtPD E1-α; LeClere et al., 2004) as well as *m132* and *RNAi>>At3g52200* (PD E2-1; Yu et al., 2012) lines. We have observed significantly increased, when compared to wild-type, root length of *iar4-2, m132* and *RNAi>>At3g52200* mutant seedlings in presence of GR24 (Figure 4B). This result suggests that PDC E1 and E2 components activity is required to mediate the GR24 effect on *Arabidopsis* primary root elongation and gravitropism. To draw a comprehensive conclusions the E3 subunit should be also analyzed. To further analyze the function of the *Arabidopsis* mtPD E1-β subunit we have examined its subcellular localization. We have transformed wild-type Col-0 seedlings with *pPIG1::PIG1-GFP* and *pPIG1::GFP-PIG1* constructs. The analysis of T_2 generation revealed a dot-like and diffused, cytosol-like expression pattern for *pPIG1::PIG1-GFP* and *pPIG1::GFP-PIG1* constructs, respectively (Figure 4C and 4D). Due to the predicted, for E1-β (http://bbc.botany.utoronto.ca) as well as presented, for E1-α (Quint et al., 2009), mitochondrial localization, we have co-localized the expression of PIG1-GFP protein with MitoTracker dye for mitochondria staining. We could observe a full co-localization, suggesting that, similarly to E1-α, the E1-β subunit of PDC localizes to mitochondria (Figure 4 E-G).

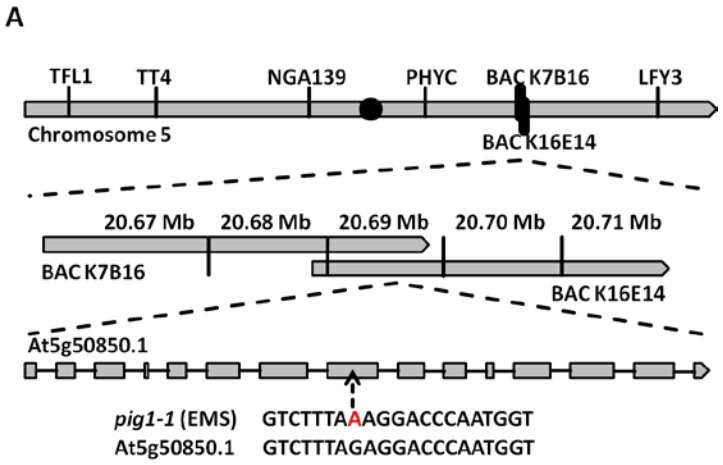

Figure 3. PIG1 encodes a putative PD-1 β, a protein with conserved function among eukaryotes. (A) Scheme of putative PD E1-β coding locus and organization. The position of *pig1-1* allele (discontinuous arrow) and the point mutation (red letter) are depicted.

On the tissue scale, the promoters of the mtPDC subunits, characterized to date, were shown to have largely ubiquitous activity throughout the plant. Interestingly, the expression of E1-α and E2-1 is complementary in RAM. E1-α exhibits a strong expression, while promoter activity of E2-1 is absent from root tip region (Quint et al., 2009; Yu et al., 2012). To analyze the tissue expression of E1-β subunit, we have generated *pPIG1::GUS* construct (see Material and Methods), and transformed it into wild-type Col-0 background seedlings. We are currently selecting homozygous plants from T_3 generation of this line. To analyze the morphological and subcellular consequences of PIG1 protein overproduction we have generated *35S::PIG1-GFP* and *35S::GFP-PIG1* constructs (see Material and Methods), homozygous lines of which are currently selected from T_3 generation.

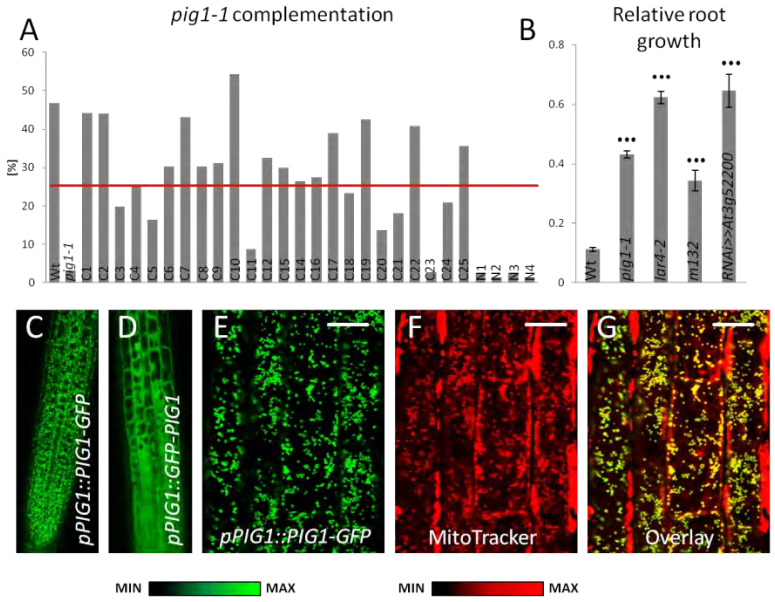

Figure 4. The complementation of *pig1-1* mutant and localization of PD E1-β protein in *Arabidopsis* roots. (A) Complementation of *pig1-1* mutant phenotype with *pPIG1::PIG1-GFP* or *pPIG1::GFP-PIG1*. Consecutive numbers indicate independent transformation lines. C – C-terminal phusion, N – N-terminal phusion. Red line indicates an expected threshold of 25% seedlings exhibiting wild-type phenotype in segregating T_2 population. See Material and Methods for the description of the complementation experiment. (B) Quantification of the relative root growth of *Arabidopsis* seedlings on medium containing 50 µM of GR24 vs un-supplemented medium. n=1 with at least 15 seedlings evaluated. (C-D) Live cell imaging of translational phusions *pPIG1::PIG1-GFP* (C) and *pPIG1:: GFP-PIG1* (D). (E-G) Co-localizing of *pPIG1::PIG1-GFP* phusion with mitochondrial dye (MitoTracker). Error bars represent standard error of the mean (SEM), *** extremely significant at P<0.001 according to Student's t-test evaluation. Signal intensities are coded in green or red corresponding to increasing intensity levels (see color scales). Scale bar = 10 µm.

We have followed our research with the analysis of *pig1-1* mutant morphology, without additional hormonal supplementation (Figure 5 A-D). We were able to identify significant differences in *pig1-1* mutant phenotype, when compared to wild-type control. These included increased root length (Figure 5A and 5B) and decreased hypocotyl elongation (Figure 5A and 5C). Interestingly we have additionally noticed that roots of *pig1-1* mutant exhibit skewing to the right direction (Figure 5A and 5D). These kinds of phenotypes are often associated with auxin regulated processes (Zhao et al., 2001;

Stepanova et al., 2008). We have, therefore, decided to test the response of *pig1-1* mutant roots to the exposure to various auxinic compounds (Figure 6 A-D). Intriguingly, we have noticed interesting trend. Roots of *pig1-1* mutant are slightly oversensitive, when compared to wild-type seedlings roots, to auxinic compounds activating DR5-visualized auxin signaling, like IAA, NAA (Figure 6A and 6B) while they are slightly resistant to auxinic compounds inactive in terms of SCF^{TIR1}-mediated auxin signaling like IBA or IAA-L-Ala (Figure 6C and 6D; Simon et al., 2013). These changes are not statistically significant, possibly due to the fact that this analysis was performed on non-backcrossed *pig1-1* mutant population, thus other mutations in the *pig1-1* genome could influence the results. What is more, the root elongation of wild-type and *pig1-1* seedlings, as mentioned before, vary on unsupplemented medium and thus the comparative analysis could be difficult.

Nevertheless, we hypothesized that tissues of *pig1-1* could have abnormal levels of certain auxins or auxin conjugates. Consequently, the morphological phenotypes as well as lack of GR24-induced changes in PIN polarity, observed in *pig1-1* mutant seedlings, may be associated with these abnormalities. Another possibility is that the balance between endogenous levels of strigolactones and auxin or auxin conjugates could be important for maintenance of PIN polarity. Affecting this balance, through changes in PIN localization, would lead to developmental aberrations.

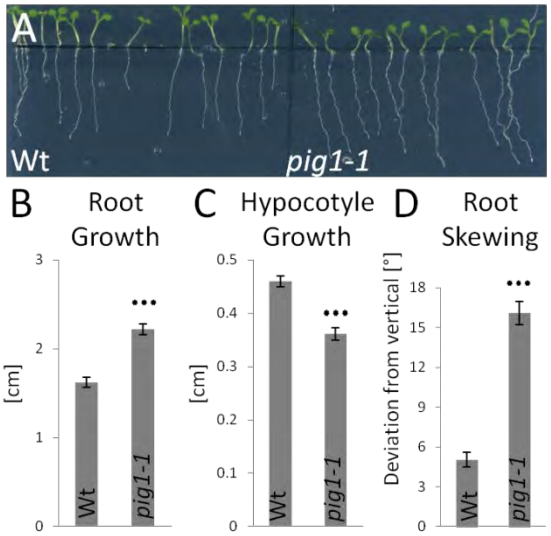

Figure 5. The morphological phenotype of *pig1-1* mutant. (A) The morphology of the wild-type Col-0 and *pig1-1* mutant seedlings, germinated on un-supplemented medium. (B) Quantification of *pig1-1* mutant root growth on un-supplemented medium. n=1 with 3 biological replicas and at least 20 seedlings evaluated for each replica. (C) Quantification of *pig1-1* mutant hypocotyl growth on un-supplemented medium. n=1 with 3 biological replicas and at least 30 seedlings evaluated for each replica. (D) Quantification of *pig1-1* mutant root skewing on un-supplemented medium. n=3 with at least 25 seedlings evaluated for each experiment. Error bars represent standard error of the mean (SEM), *** extremely significant at $P<0.001$ according to Student's t-test evaluation.

Multiple components of the subcellular trafficking machinery, contributing for the establishment and maintenance of an intrinsic polarity of PIN efflux carriers, were identified to date (see

Chapter 1). In our next experimental design, we aimed to dissect an involvement of specific parts of this machinery in context of GR24-induced changes in polar PIN localization. We have immunolocalized PIN 1 protein after co-treatments of 25 or 50 µM BFA with GR24, NAA, applied on wild-type and *pig1-1* seedlings (Figure 7 A-P). In plants, the secretory pathway is readily blocked by BFA, upon additional inhibition of endocytosis by auxin (at approx. 25 µM of BFA), PIN proteins no longer end up in BFA-compartments (Paciorek et al., 2005; Men et al., 2008; Kitakura et al., 2011). As shown recently, at higher concentrations (approx. 50 µM), BFA along with influencing exocytosis, also inhibits vacuolar targeting and degradation of PIN proteins (Kleine-Vehn et al., 2008 and 2008a; Robert et al., 2010). Thus, different concentrations of BFA allow discriminating between effects on endocytosis for recycling and targeting for degradation (Robert et al., 2010).

As expected, in both wild-type and *pig1-1* mutant background, NAA inhibited the formation of 25 µM BFA-induced intracellular PIN1 accumulations (Figure 7 A-B and I-J). GR24, in both genetic backgrounds, appeared not to have influence on the formation of 25 µM BFA-induced, PIN1 accumulations (Figure 7C and 7K). When 25 µM BFA was used together with NAA and GR24, withouth differences between wild-type and *pig1-1* seedlings, auxin effect exceeded that of strigolactone, causing inhibition of the BFA-body formation (Figure 7D and 7L). Since, as mentioned before, BFA at lower concentrations (approx. 25 µM), impacts ARF-GEFs regulating PM recycling, we have concluded that there are no differences between wild-type and *pig-1-1* seedlings in the dynamics of this part of subcellular PIN trafficking. Our following experiments revealed that BFA at concentration of 50 µM causes intracellular accumulation of PIN1 in both wild-type and *pig1-1* mutant seedlings (Figure 7E and 7M). In agreement with previous findings, NAA, by inhibiting an endocytosis step of continuous PIN cycling, prevented the formation of PIN-containing BFA bodies (Figure 7F and 7N; Paciorek et al., 2005; Robert et al., 2010). On the other hand co-treatment of BFA with GR24 did not seem to have an effect on the formation of BFA bodies (Figure 7G and 7O). Surprisingly, the addition of GR24 to 50 µM BFA/NAA co-treatments counteracted, in wild-type seedlings, the auxin-mediated inhibition of BFA body formation (Figure 7H). In contrary, the auxin inhibitiory mechanism, as revealed by similar experiment, was operational in *pig1-1* mutant seedlings (Figure 7P). Since BFA at higher concentrations (approx. 50 µM) influences additionally ARF-GEFs controlling late endocytic trafficking events, we have concluded that it is likely that the dynamics of late endocytic trafficking is disturbed in *pig1-1* mutant. This experiment was performed however only once, and it should be repeated in order to draw comprehensive conclusions.

The above results prompted us to concentrate on the description of PIN vacuolar targeting in *pig1-1* mutant. It was recently reported that exogenously applied strigolactone can trigger a rapid depletion of PIN1 from the PM (Ruyter-Spira et al., 2011; Shinohara et al., 2013), however further subcellular fate of internalized this way PINs was not addressed. To corroborate on this findings and to further investigate the role of late endocytic trafficking in the regulation of PIN polarity, we have treated the *Arabidopsis* seedlings expressing *pPIN2::PIN2-GFP* protein in *eir1-1* mutant background with GR24 (Figure 8 A-C). When compared to untreated control (Figure 8A), we could observe a reduction of PIN2-GFP levels at the PM concomitantly with an increase of a diffused vacuolar GFP signal following 24h treatment with 10 µM GR24 (Figure 8B and 8C). Next, we have designed the experiment in which, upon treatment with GR24, we have immunolocalized PIN1 protein, expressed ectopically under the control of PIN2 promoter and fused with HA tag (*pPIN2::PIN1-HA*; Figure 8 D-E). This line was previously shown

to exhibit predominant basal and non-polar localization of PIN1 in epidermal cells (Figure 8D; Feraru et al., 2011). Upon 48h treatment with 50 µM GR24, we could observe the residual PIN1 abundance prevalently on the apical polar domain (Figure 8E). The analysis of PIN stability upon GR24 challenge in pPIN2::PIN1-HA transgenic line was performed once and it is necessary to repeat it. Nevertheless, together with the results of the experiment visualized on Figure 8 A-C, it suggests that, indeed, strigolactone destabilizes PINs from the cell surface and targets them for vacuolar delivery. What is more, it appears that this destabilization occurs preferentially from the basal side of PIN-expressing endodermal cells. To determine if the lack of sensitivity to the GR24 effect on root morphology and subcellular PIN localization could be a result of increased PIN membrane stability in *pig1-1* mutant background, we have analyzed the PM abundance and vacuolar targeting of *pPIN1::PIN1-GFP* in *pig1-1* versus wild-type backgrounds (Figure 9 A-D). We could observe that, in wild-type seedlings treated for 24h with 10 µM GR24, the cell surface abundance of PINs is strongly reduced while the occurrence of GFP-positive vacuolar signal is increased (Figure 9 A-B). This effect was absent in *pig1-1* mutant genetic background (Figure 9 C-D). This experiment was repeated twice with similar results obtained.

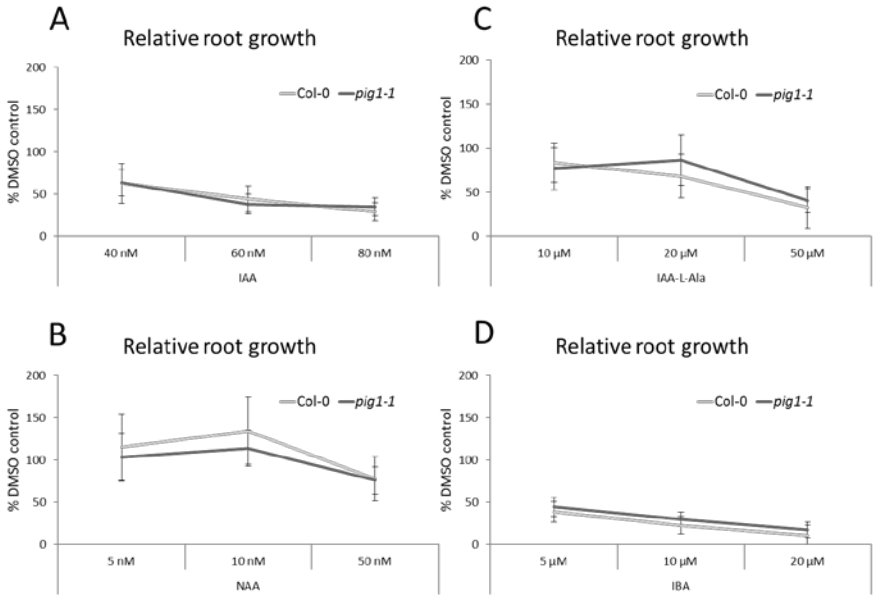

Figure 6. The effect of various auxinic compounds on root elongation of *pig1-1* mutant. (A-D) Quantification of the relative root growth of *Arabidopsis* seedlings on medium containing 40, 60 and 80 nM IAA (A); 5, 10 or 50 nM NAA (B); 10, 20 or 50 µM IAA-L-Ala (C); 5, 10 or 20 µM IBA (D) vs un-supplemented medium. Error bars represent standard deviation (sd). n=1 with at least 18 seedlings evaluated. Differences are not statistically significant, according to ANOVA two-factor with replication evaluation.

DISCUSSION

The initial results of our study indicated that GR24, a synthetic strigolactone analogue, can influence polar localization of PIN auxin carriers. Based on this observation we have performed a forward genetic screen for regulators of PIN polarity, which yielded identification of *pig1-1* mutant. Upon exposure to exogenously applied GR24, seedlings of *pig1-1* mutant, in contrast to wild-type

seedlings, maintain root growth, correct gravitropic response and typical PIN polarization. We have described the genomic context of the mutation, and confirmed that single substitution in the At5g50850 gene encoding for E1-β subunit of PDC is responsible for *pig1-1* mutant phenotype.

The characterization of the morphological phenotype revealed, that when compared to Columbia wild-type, *pig1-1* mutant exhibits increased primary root length. This is in contrast with previously published reports, stating that the primary root of mutants for both E1-α and E2-1 subunits of *Arabidopsis* mtPD display short root phenotype (LeClere et al., 2004; Quint et al., 2009; Yu et al., 2012). These previous observations together with the fact that the expression of the PD E1-β transcript, as revealed by qRT-PCR, was reduced to about 60% in *pig1-1* roots (data not shown), prompted us to investigate the effects of PIG1 complete loss-of-function. Since we were unsuccessful in identifying a homozygous knock-out insertion for At5g50850 gene from available public collections, we have designed artificial microRNA lines for silencing of *PIG1* transcript (see Material and Methods). These lines are currently in T_2 generation status.

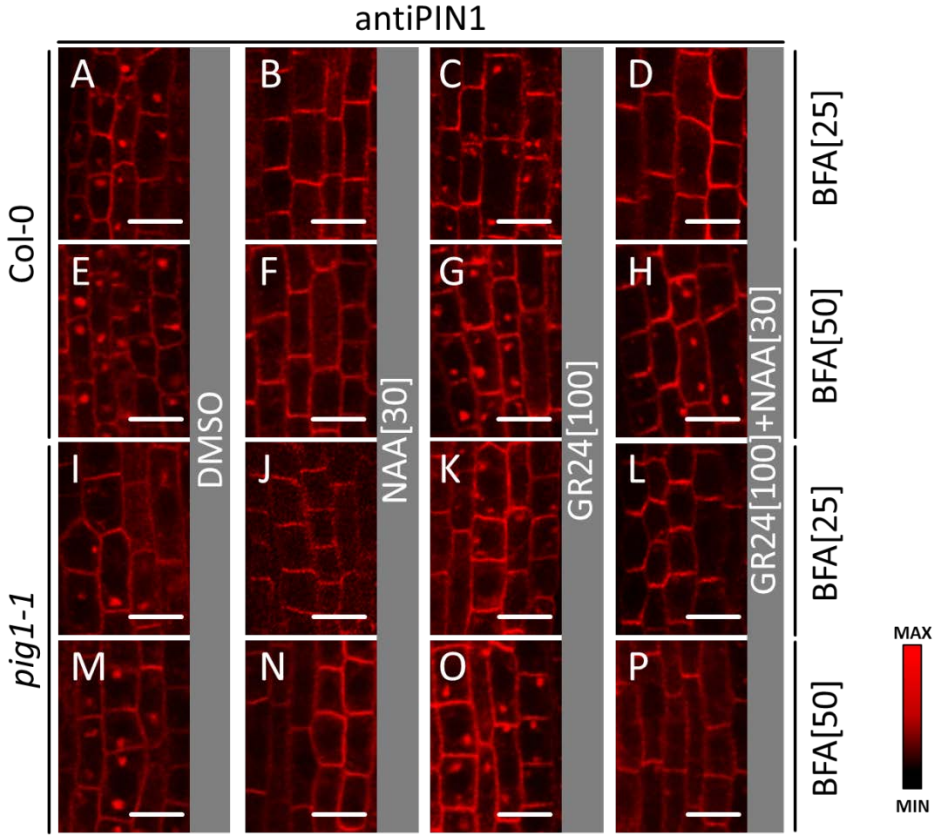

Figure 7. The effect of GR24 on the formation of BFA-induced PIN1 intracellular accumulations in the roots of *Arabidopsis pig1-1* mutant. (A-P) Immunolocalization of PIN1 protein in roots of Col-0 (A-H) and *pig1-1* (I-P) mutant seedlings, co-incubated with 25 µM BFA and DMSO (A and I); 30 µM NAA (B and J); 100 µM GR24 (C and K); 30 µM NAA and 100 µM GR24 (D and L) or 50 µM BFA and DMSO (E andM); 30 µM NAA (F and N); 100 µM GR24 (G and O); 30 µM NAA and 100 µM GR24 (H and P). Note an absence of BFA-induced PIN1 internalizations in *pig1-1* mutant background after co-treatment with 50 µM BFA, 30 µM NAA and 100 µM GR24. Signal intensities are coded in red corresponding to increasing intensity levels (see color scale). Scale bar = 10 µm.

The lack of *pig1-1* phenotype complementation by N-terminally tagged *RFP-PIG1* protein, can be explained by the fact that the *PIG1::RFP-PIG1* construct is most likely non-functional. To support this statement we have used TargetP software (Emanuelsson et al., 2000), algorithm of which predicts protein localization based on the presence of mitochondrial targeting peptides (MTPs) in the N-terminal amino acid sequence of the protein. We have also used MITOPRED software, predicting protein localization based on multiple criteria like patterns of specific Pfam domains occurrence, their amino acid composition and the pI value differences between mitochondrial and non-mitochondrial proteins (Guda et al., 2004). Both programs predicted mitochondrial localization for PD E1-β subunit, validating our results for the localization of *pPIG1::PIG1-RFP* construct. Importantly the used algorithms predicted the presence of MTPs in the N-terminal part of PD E1-β, crucial for localization of the protein. We therefore believe that the introduction of the fluorescent tag (in case of *pPIG1::RFP-PIG1* construct) in proximity of this region could interfere with native protein folding and therefore influence the localization and function of the *RFP-PIG1* protein.

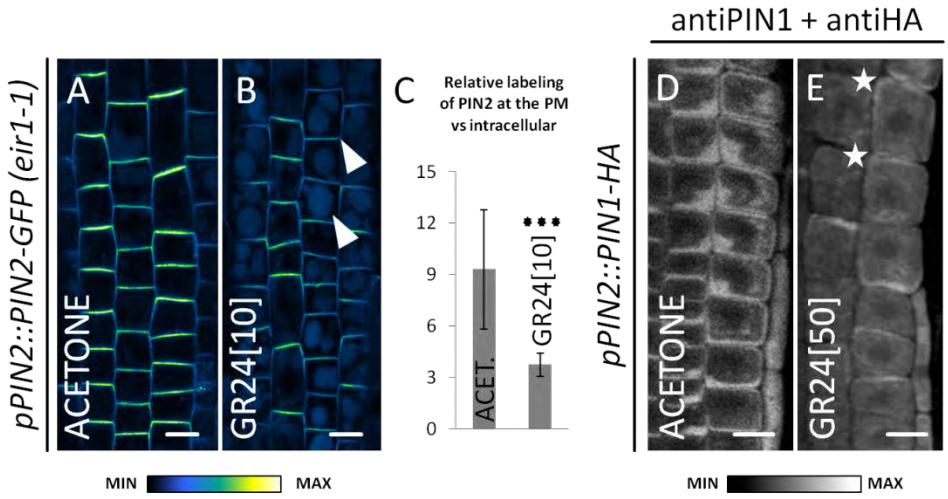

Figure 8. The effect of GR24 on membrane stability and vacuolar targeting of PIN proteins in *Arabidopsis* roots. (A-B) Intracellular localization of *pPIN2::PIN2-GFP* (*eir1-1*) protein in seedlings incubated for 24h with acetone (A) or with 10 µM GR24 (B). (C) Quantification of relative PIN2-GFP abundance at the PM versus the intracellular signal in the Arabidopsis roots treated with GR24. n=2 independent experiments with at least six roots analysed for each assay and at least six cells evaluated per root. White arrowheads highlight differences in PIN2 protein retention at the plasma membrane and accumulation in the vacuoles. (D-E) Immunolocalization of *pPIN2::PIN1-HA* protein in seedlings incubated for 48h with acetone (D) or 50 µM GR24 (E). Note a preferential apical localization of residual PIN1 in epidermal cells, marked by white asterisks. Error bars represent standard error of the mean (SEM), *** extremely significant at P<0.001 according to Student's t-test evaluation. Signal intensities are coded blue to yellow or black to white corresponding to increasing intensity levels (see color scales). Scale bar = 10 µm.

The PDC catalyzes the oxidative decarboxylation of pyruvate to Coenzyme A, which is subsequently acetylated to form acetyl-CoA (Mooney et al., 2002). It is not clear how the activity of mtPD contributes to the resistance of root growth to auxin amino-acid conjugates, observed in mutants for E1-α subunit (LeClere et al., 2004). One possibility proposed by previous research was that auxin

metabolism could be indirectly affected by reduced levels of acetyl-CoA resulting from defective or delayed pyruvate conversion. However substrates of Krebs cycle, downstream of acetyl-CoA, like citric acid, failed to rescue the phenotypes of *iar4-2* mutant (LeClere et al., 2004). Alternatively, it was proposed that mtPD could cooperate with other subunits of PDC during conversion of indole-3-pyruvate (IPA) to indole-3-acetyl-CoA (IAA-CoA) which, as shown, can be subsequently hydrolyzed to release free IAA; or act as a precursor for IAA conjugates (Koga et al., 1995; LeClere et al., 2004). In this case certain tissues of mtPD mutant could have disturbed levels of auxin or auxin-conjugates. Indeed, as shown by recent research, the abundance of IAA-amino acid conjugates in tissues of PD E1-α mutant was doubled relatively to wild-type plants (Quint et al., 2009).

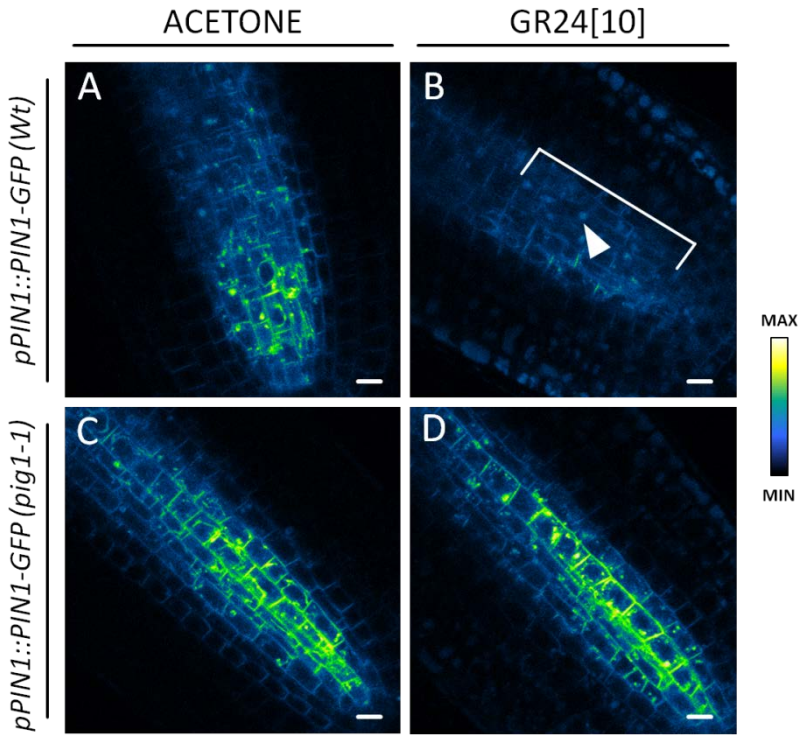

Figure 9. PIN1-GFP protein is resistant to GR24-induced destabilization from the PM in roots of Arabidopsis *pig1-1* mutant genetic background. (A-D) Live cell imaging of the PIN1-GFP protein in *Arabidopsis* seedlings of wild-type (A) and (B) or *pig1-1* (C) and (D) mutant background, incubated for 24h on un-supplemented medium (A) and (C) or medium containing 10 µM GR24 (B) and (D). Note a reduced PM expression of PIN1-GFP and increased number of GFP-positive vacuolar structures, marked on panel B, by white line and white arrowhead, respectively. These effects are absent in *pig1-1* mutant genetic background. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see color scales). Scale bar = 10 µm.

The mtPD mode of action, based on its involvement in conversion of IPA, and as a consequence, potential modulation of auxin or its conjugates content, could provide an explanation for auxin related phenotypes observed in mtPDC subunits mutants. The *iar4* (E1-α) was shown to have reduced number of lateral roots and root hairs. Additionally, the hypocotyl elongation of *iar4* was decreased, which could

be rescued by growth in higher temperature or introduction of *35S::YUC* transgene, both increasing endogenous levels of auxin (Quint et al., 2009). These facts, together with identification of auxin-related defects, like hypocotyl elongation, in *pig1-1* mutant, prompted us to explore the response of *pig1-1* to various hormonal compounds. We have noticed that roots of *pig1-1* mutant are oversensitive, to auxinic compounds activating DR5-visualized auxin signaling, like IAA, NAA or 2,4-D, while they are resistant to auxinic compounds inactive in mediating SCF^{TIR1}-based auxin signaling, like IBA or IAA-L-Ala (Simon et al., 2013). To address the possibility that imbalances in certain auxinic compounds could be responsible for defects observed in *pig1-1*, the response of the mutant should be analyzed in presence of auxin conjugates, what is more the levels of free IAA and auxin conjugates should be determined by biochemistry-based approaches.

Previous reports demonstrated that PINs can be rapidly depleted from the PM as a result of elevated strigolactone level (Ruyter-Spira et al., 2011; Shinohara et al., 2013). Yet, the subsequent fate of PINs, endocytosed by this mechanism, was not addressed. Exogenously applied or endogenously produced strigolactones were shown to have a dampening effect on auxin transport (Crawford et al., 2010). What is more, GR24-induced and auxin-related phenotypes, like defective root hair development, could be rescued by exogenous application of auxin (Koltai et al., 2010), suggesting that not only auxin transport rates, but auxin levels could be affected by strigolactone. The results of our research presented in Chapter 2 indicated, that not only prolonged increase but also decrease in cellular auxin levels induce targeting of PIN transporters to the vacuole, thereby regulating their abundance at the PM. We have therefore hypothesized that altered levels of certain auxinic compounds within tissues of *pig1-1* mutant through modulation of PIN vacuolar trafficking could influence the PIN polarity and contribute to the generation of *pig1-1* mutant phenotypes.

We were able to visualize the differences in intracellular trafficking of PINs in presence of 50 µM but not 25 µM BFA, suggesting that alterations of late endocytic trafficking rather than early endocytosis of PINs maybe related to *pig1-1* mutant phenotypes. What is more, we were able to present that upon long term exposure to exogenously applied strigolactone PINs are depleted from the membrane and targeted for vacuolar delivery. It would be beneficial to further validate these findings by western-blot analysis of PIN presence in microsomal fractions upon GR24 treatment. Interestingly, our data suggests that GR24-induced depletion of PINs occurs preferentially from the basal domain of PM. Finally, we could show that PIN1 protein, upon GR24 challenge, is stabilized at the PM of *pig1-1* mutant and targeted less for vacuolar delivery.

All together the results of our study indicate an alternative, to polarity-switch-based, interpretation of *pig1-1* phenotypes and generally PIN-dependent regulation of auxin fluxes in *Arabidopsis* roots in presence of elevated strigolactone levels. Based on our experimental data we have conceived a hypothetical model in which strigolactone induces depletion from the plasma membrane and vacuolar targeting of PINs. PIN depletion, from ambiguous reasons, occurs preferentially from the basal cell side (in endodermal and cortical cells). This results in prevalent apical localization of endodermal PIN1 and cortical PIN2 proteins upon strigolactone exposure. Strigolactone action would potentially depend on presence of specific auxinic compounds (auxin conjugates) at certain levels in the root tissues. *pig1-1* mutant due to altered metabolism of auxin exhibits altered rates of strigolactone-induced PIN vacuolar targeting. Lack of preferential PIN1 and PIN2 depletion from basal side of

endodermal and cortical cells would contribute to generation of *pig1-1* mutant GR24 resitancy. Validity of this putative mechanism of strigolacton-auxin interplay should be addressed by further research.

MATERIAL AND METHODS

Plant material and growth conditions

All *Arabidopsis thaliana* mutants and transgenic lines used in this study are in the Columbia (Col-0) background and have been described previously: *pPIN1::PIN1-GFP* (Benkova et al., 2003); *pPIN2::PIN2-GFP* (Xu and Scheres, 2005); *pPIN2::PIN1-HA* (Wisniewska et al., 2006); *iar4-2* (LeClere et al., 2004), *m132*, *RNAi>>At3g52200* (Yu et al., 2012). Surface-sterilized seeds were sown on half-strength Murashige and Skoog (0.5 MS) agar plates and stratified for 2 days at 4°C. Plants were grown on vertically oriented plates under 16h light/ 8h dark photoperiod at 18°C for 6-7 days.

EMS Mutagenesis, Mutant Forward Genetic Screen and Map Based Cloning

The forward genetic screen was performed using the 3% EMS mutagenized population of *Arabidopsis thaliana* plants carrying PIN1 protein tagged with GFP in the wild-type Columbia background. Seeds were germinated on ½ MS medium. 7 DAG seedlings were transferred to the ½ MS medium supplemented with 50 µM of GR24. Plants were gravistimulated twice, by consecutive, 90° clockwise and counterclockwise rotation of the plates, with 48h incubation after each rotation. Following this plates were scored for the presence of seedlings exhibiting root growth resistant to the GR24 and proper gravitropic response. 188 pools were screened in the primary screen (of the 294 pools in total – approximately 64 % of the EMS mutagenized population). Each pool consisted of M_2 seeds being progeny of 20 pooled M_1 plants. For each pool approximately 300 seedlings were screened (in total approximately - 3760 M_1 plants; 56400 M_2 plants). In the primary screen 401 candidates of M_2 generation were identified, in which upon treatment with 50 µM of GR24 root growth followed the gravity vector. From 401 primary candidates the gravitropic growth, resistant to the 50 µM of GR24, was confirmed in 9 candidates in the next generation. The mutations responsible for GR24 resistancy were mapped in 3 candidates. These mutations were identified in At5g50850 (PIG1), At5g16530 (PIN5, unsuccesfull complementation), At1g25540 (PFT1, GR24 resistancy confirmed in other allel of the gene). Additionally 5 candidates were identified showing resistance to 25 µM of GR24. Confirmed candidates were crossed to *Landsberg* ecotype to establish mapping population.

Reagents

Abscisic Acid (ABA); *2*,4-Dichlorophenoxyacetic acid (2,4-D); Brassinolide (BL); Naphthalene acetic acid (NAA); 6-Benzylaminopurine (BAP); Indole-3-butyric acid (IBA); 1-Aminocyclopropanecarboxylic acid (ACC); Indole-3-acetic acid (IAA); Indole-3-acetyl-L-alanine (IAA-L-Ala); MitoTracker Red (Sigma, http://www.sigmaaldrich.com); BrefeldinA (BFA) (Invitrogen, http://www.invitrogen.com); stock solutions were prepared in DMSO. GR24 was prepared by Prof. Dr. Binne Zwanenburg at Department of

Organic Chemistry, Institute of Molecules and Materials, Radboud University Nijmegen; stock solution was prepared in acetone. During experiments with GR24, acetone was used as control and itself did not interfere with PIN localization.

Cloning and *Arabidopsis Thaliana* transformation

The PIG promoter analysis, localization and overexpression lines were generated according to:
http://www.psb.ugent.be/gateway;
http://www.lifetechnologies.com/be/en/home/life-science/cloning/gateway-cloning.html;
Karimi et al., 2002; by amplification of genomic At5g50850 using primers:
p35S::PIG1-GFP
attB1 – GGGGACAAGTTTGTACAAAAAAGCAGGCTTCatgttgggaatcttgaggcaa
attB2 - ACCACTTTGTACAAGAAAGCTGGGTGtttcgatctgtaacaagctctct
and pK7FWG2 binary vector
p35S::GFP-PIG
attB1 - GGGGACAAGTTTGTACAAAAAAGCAGGCTTCatgttgggaatcttgaggcaa
attB2 - ACCACTTTGTACAAGAAAGCTGGGTGttatttcgatctgtaacaagctc
and pK7WGF2 binary vector
pPIG1::PIG1-GFP
attB1 - GGGGACAAGTTTGTACAAAAAAGCAGGCTTCttgcttgtatcagctcagtaac
attB2 - ACCACTTTGTACAAGAAAGCTGGGTGtttcgatctgtaacaagctctct
and pK7FWG,0 binary vector
pPIG1::GFP-PIG1
PROMOTER
attB4 - GGGGACAACTTTGTATAGAAAAGTTGTCttgcttgtatcagctcagtaac
attB1R - GGGGACTGCTTTTTTGTACAAACTTGGtctctctgattaacaaacaaa
and pDONR P4P1R entry vector
CDS
attB2R - GGGGACAGCTTTCTTGTACAAAGTGGTCatgttgggaatcttgaggcaa
attB3 - GGGGACAACTTTGTATAATAAAGTTGGttatttcgatctgtaacaagctc
and pDONR P2RP3 entry vector
GFP in pEN-L1-F-L2
pK7m34GW was used as a destination vector
pPIG1::GUS
attB4 - GGGGACAACTTTGTATAGAAAAGTTGTCttgcttgtatcagctcagtaac
attB1R - GGGGACTGCTTTTTTGTACAAACTTGGtctctctgattaacaaacaaa
and pDONR P4P1R entry vector
GUS in pEN-L1-S-L2
pK7m24GW,3 was used as a destination vector
pPIG1::GFP-GUS
attB1 - GGGGACAAGTTTGTACAAAAAAGCAGGCTTCttgcttgtatcagctcagtaac
attB2 - ACCACTTTGTACAAGAAAGCTGGGTGtctctctgattaacaaacaaa

pKGWFS7 was used as destination vector

The PIG amiRNA lines were generated according to:
http://wmd3.weigelworld.org/cgi-bin/webapp.cgi
using primers:
PIG1 amiRNA version A

```
miR-s gaTAGTTACTATCGTTACGTCCCctctcttttgtattcca
miR-a agGGGACGTAACGATAGTAACTAtcaaagagaatcaatga
miR*s agGGAACGTAACGATTGTAACTTtcacaggtcgtgatatg
miR*a gaAAGTTACAATCGTTACGTTCCctacatatatattccta
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PIG1 amiRNA version B

```
miR-s gaTTTTGTACGATCTAACGACGCctctcttttgtattcca
miR-a agGCGTCGTTAGATCGTACAAAAtcaaagagaatcaatga
miR*s agGCATCGTTAGATCCTACAAATtcacaggtcgtgatatg
miR*a gaATTTGTAGGATCTAACGATGCctacatatatattccta
```

Immunodetection

Whole-mount immunolocalization in *Arabidopsis* roots was performed as described previously (Sauer et al., 2006). The rabbit anti-PIN1 (Paciorek et al., 2005; 1:1000), rabbit anti-PIN2 (kindly provided by C. Luschnig; 1:1000) and mouse anti-HA (Sigma; 1:500) were used as primary antibodies. The fluorochrome-conjugated rabbit Cy3 and mouse Alexa Fluor 488 (Invitrogen; 1:600) were used as secondary antibodies.

Drug Application and Experimental Conditions

For PIN1 and PIN2 immunolocalization seedlings were: germinated for 6 days on medium supplemented with 30 µM GR24 or 24h with 100 µM GR24 (Figure 1); incubated for 8h in liquid medium supplemented with 100 µM GR24 (Figure 2); pre-incubated with 30 µM NAA, 100 µM GR24 or 30 µM NAA and 100 µM GR24 for 30 min followed by co-incubation of respective reagents with 50 µM BFA for 90 min (Figure 7); incubated for 48h on solid medium supplemented with 50 µM GR24 (Figure 8). Live cell imaging experiments were performed by incubation of seedlings for 24h in the dark on solid medium supplemented with 10 µM GR24 (Figure 8 and 9).

Image processing, and statistical analysis

Signal intensities are coded according to blue-yellow, black to white, green or red, corresponding to the increasing intensity levels (see color scale under figure). Quantitative results are visualized as means with error bars representing standard error of the mean (SEM), * significant, ** highly significant and *** extremely significant at $P<0.05$, $P<0.01$ and $P<0.001$ according to Student's t-test evaluation, respectively. All fluorescence signals were evaluated on the Zeiss LSM 710 or Olympus IX-81 confocal laser scanning microscopes. For imaging GFP, the 488- and 514-nm lines of the argon laser were used for excitation, and emission was detected at 510 and 530 nm, respectively. For semi-quantitative

measurement of fluorescence intensities, laser, pinhole, and gain settings of the confocal microscope were kept identical among respective treatments. The mean fluorescence intensity was measured with ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij). Images were processed with Adobe Photoshop 12.0. Statistics were evaluated with Excel2010 (Microsoft).

Quantification index

Gravitropic index of the roots was evaluated by measuring of vertical length (straight vertical line between the hypocotyl-root junction and root tip – VL) and relating it to the root length (RL) (Figure 1). Relative root growth was evaluated by relating portions of the root growth from before and after the transfer to hormone-supplemented medium (Figure 2 and 4). Relative root growth is presented as percentage of the root growth on unsupplemented medium (Figure 6). For the pig1-1 mutant complementation experiment (Figure 4) the T_2 generation of *pPIG1::PIG1-GFP* and *pPIG1::GFP-PIG1* in *pig1-1* mutant background was evaluated. The average root elongation of Col-0 control seedlings after transfer to medium supplemented with 50 µM GR24 was evaluated (0.2 cm). Chart represents the percentage of individual seedlings from each independent transformation line, root growth of which after transfer to GR24-supplemented medium was not exceeding 0.2 cm.

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Chapter 5

Additional scientific contributions

Pawel Baster and Jiří Friml

Additional scientific contributions

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Author Contributions:

(Robert et al., 2010)
PB analysed the effect of PEO-IAA and 5-F-IAA on BFA-visualized constitutive cycling of PIN1 and PIN2 proteins and DR5rev::GFP-visualized auxin signaling (Figure 3).

(Whitford et al., 2012)
PB analysed the plasma membrane abundance of PIN2 protein in GLV3OE and amiRglv3 genetic backgrounds (Figure 3M).

(Remy et al., 2013)
PB analysed the plasma membrane abundance of PIN2 protein in ZIFL overexpressing and *zifl* mutant background (Figure 8).

(Simon et al., 2013)
PB analysed the effect of various auxin analgues on the inhibition of endocytosis of PIN1 and PIN2 proteins (Figure 7).

(Remy et al., 2013)
PB analysed the plasma membrane abundance of PIN1 protein in ZIFL overexpressing and *zifl* mutant background (Figure 3) and contributed for manuscript writing.

Cell

ABP1 Mediates Auxin Inhibition of Clathrin-Dependent Endocytosis in *Arabidopsis*

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SUMMARY

Spatial distribution of the plant hormone auxin regulates multiple aspects of plant development. These self-regulating auxin gradients are established by the action of PIN auxin transporters, whose activity is regulated by their constitutive cycling between the plasma membrane and endosomes. Here, we show that auxin signaling by the auxin receptor AUXIN-BINDING PROTEIN 1 (ABP1) inhibits the clathrin-mediated internalization of PIN proteins. ABP1 acts as a positive factor in clathrin recruitment to the plasma membrane, thereby promoting endocytosis. Auxin binding to ABP1 interferes with this action and leads to the inhibition of clathrin-mediated endocytosis. Our study demonstrates that ABP1 mediates a nontranscriptional auxin signaling that regulates the evolutionarily conserved process of clathrin-mediated endocytosis and suggests that this signaling may be essential for the developmentally important feedback of auxin on its own transport.

INTRODUCTION

The plant signaling molecule auxin is an important regulator of plant developmental processes, including embryogenesis, organogenesis, tissue patterning, and growth responses to external stimuli (Santner and Estelle, 2009; Vanneste and Friml, 2009). Current models on auxin signaling and action focus on the paradigm that auxin regulates the expression of subsets of genes, thus eliciting different cellular and, consequently, developmental responses. Nuclear auxin signaling involves the F box protein transport inhibitor response 1 (TIR1), which acts as an auxin coreceptor (Kepinski and Leyser, 2005; Dharmasiri et al., 2005a, 2005b; Tan et al., 2007), and downstream Aux/IAA and ARF transcriptional regulators (Dharmasiri and Estelle, 2004). This pathway controls a remarkable number of auxin-mediated processes, but some rapid cellular responses to auxin are not associated with TIR1-based signaling (Badescu and Napier, 2006; Schenck et al., 2010).

Decades ago, the plant-specific protein AUXIN-BINDING PROTEIN 1 (ABP1) was proposed to be an auxin receptor (Hertel et al., 1972; Löbler and Klämbt, 1985). ABP1 in both monocot and dicot plant species shows physiological affinities toward natural and synthetic auxin ligands (Jones, 1994). ABP1, despite carrying a KDEL-endoplasmic reticulum (ER) retention motif, is secreted to some extent to the extracellular space where it is active (Jones and Herman, 1993; Tian et al., 1995; Henderson et al., 1997). ABP1 is essential for embryogenesis (Chen et al., 2001) and postembryonic shoot and root development (Braun et al., 2008; Tromas et al., 2009) and mediates auxin effect on cell elongation, but the underlying mechanism remains unclear (Jones et al., 1998; Leblanc et al., 1997).

An important regulatory level in auxin action is its differential distribution within tissues (Vanneste and Friml, 2009). Such auxin gradients result from local auxin biosynthesis and directional,

Developmental Cell
Short Article

GOLVEN Secretory Peptides Regulate Auxin Carrier Turnover during Plant Gravitropic Responses

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SUMMARY

Growth and development are coordinated by an array of intercellular communications. Known plant signaling molecules include phytohormones and hormone peptides. Although both classes can be implicated in the same developmental processes, little is known about the interplay between phytohormone action and peptide signaling within the cellular microenvironment. We show that genes coding for small secretory peptides, designated GOLVEN (GLV), modulate the distribution of the phytohormone auxin. The deregulation of the GLV function impairs the formation of auxin gradients and alters the reorientation of shoots and roots after a gravity stimulus. Specifically, the GLV signal modulates the trafficking dynamics of the auxin efflux carrier PIN-FORMED2 involved in root tropic responses and meristem organization. Our work links the local action of secretory peptides with phytohormone transport.

INTRODUCTION

Phytohormones can be synthesized and perceived in distinct tissues and, thus, relay information over a long range. Plant cells also deploy a repertoire of secretory peptides that carry short-range cell-to-cell signals (Butenko et al., 2009). These two classes of molecules are important for growth and development of multicellular plants and can be involved in the same processes, such as cell elongation (Santner and Estelle, 2009), cell proliferation control (Matsubayashi and Sakagami, 2006), organization of apical and cambial meristems (Hirakawa et al., 2008; Whitford et al., 2008; Benková and Hejátko, 2009; Jun et al., 2008; Matsuzaki et al., 2010), and organ abscission (Lewis et al., 2006). Nevertheless, little is known about the integration between hormonal actions and peptide signaling in the cellular microenvironment.

Plants respond to light and gravity by changing the organ growth direction and auxin is a key regulator during these tropic responses. Auxin gradients controlling tropic bending and root patterning (Vanneste and Friml, 2009) depend on intercellular polar auxin transport (PAT) mediated by influx carriers of the AUX1/LIKE-AUX1 family and efflux carriers of the PIN-FORMED (PIN) family (Vieten et al., 2007). Chemical inhibitors of PAT (Rashotte et al., 2000; Parry et al., 2001) and mutations in the *AUX1*, *PIN2*, and *PIN3* genes (Friml et al., 2002; Luschnig et al., 1998; Bennett et al., 1996; Chen et al., 1998; Swarup et al., 2005; Wiśniewska et al., 2006) abolish the lateral distribution of auxin upon gravistimulation and inhibit gravitropic responses.

Auxin gradients established and maintained by the PIN protein network also control the position of the stem cell niches, from embryogenesis on through the development of the adult plant. In addition, auxin influences cell division as well as cell expansion, and multiple *pin* mutants have a reduced root apical meristem (RAM) (Blilou et al., 2005). Plant peptides are also involved in stem cell homeostasis (Stahl and Simon, 2010). Recently, a family of secreted peptides, called root growth factors (RGFs), has been reported to be required for root meristem maintenance (Matsuzaki et al., 2010). The triple *rgf1 rgf2 rgf3* mutant has reduced root meristem size, and the phenotype was restored by the addition of a corresponding peptide. The structural characterization of the RGF1 mature peptide sequence revealed that it is posttranslationally modified by tyrosine sulfation (Matsuzaki et al., 2010). Posttranslational modifications have been described in several plant secretory peptide families and are important for their function. Specific posttranslational modifications considerably increase peptide bioactivity

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A Major Facilitator Superfamily Transporter Plays a Dual Role in Polar Auxin Transport and Drought Stress Tolerance in *Arabidopsis*[W]

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Many key aspects of plant development are regulated by the polarized transport of the phytohormone auxin. Cellular auxin efflux, the rate-limiting step in this process, has been shown to rely on the coordinated action of PIN-formed (PIN) and B-type ATP binding cassette (ABCB) carriers. Here, we report that polar auxin transport in the *Arabidopsis thaliana* root also requires the action of a Major Facilitator Superfamily (MFS) transporter, Zinc-Induced Facilitator-Like 1 (ZIFL1). Sequencing, promoter-reporter, and fluorescent protein fusion experiments indicate that the full-length ZIFL1.1 protein and a truncated splice isoform, ZIFL1.3, localize to the tonoplast of root cells and the plasma membrane of leaf stomatal guard cells, respectively. Using reverse genetics, we show that the ZIFL1.1 transporter regulates various root auxin-related processes, while the ZIFL1.3 isoform mediates drought tolerance by regulating stomatal closure. Auxin transport and immunolocalization assays demonstrate that ZIFL1.1 indirectly modulates cellular auxin efflux during shootward auxin transport at the root tip, likely by regulating plasma membrane PIN2 abundance. Finally, heterologous expression in yeast revealed that ZIFL1.1 and ZIFL1.3 share H^+-coupled K^+ transport activity. Thus, by determining the subcellular and tissue distribution of two isoforms, alternative splicing dictates a dual function for the ZIFL1 transporter. We propose that this MFS carrier regulates stomatal movements and polar auxin transport by modulating potassium and proton fluxes in *Arabidopsis* cells.

INTRODUCTION

The phytohormone auxin, particularly its predominant endogenous form indole-3-acetic acid (IAA), plays a critical role in the spatial and temporal coordination of plant development. Auxin regulates a variety of unrelated processes, such as embryo, root and vascular patterning, postembryonic organogenesis, and tropisms, by directing cell division and expansion (reviewed in Woodward and Bartel, 2005). The diversity of developmental responses mediated by auxin is determined by a specific cellular signal transduction mechanism involving perception by the Transport Inhibitor Response 1/Auxin Signaling F-Box receptor proteins and interpretation by a downstream nuclear signaling pathway that ultimately mediates transcriptional developmental reprogramming (reviewed in Paciorek and Friml, 2006). Many if not all aspects of auxin action rely on its differential distribution within plant tissues manifested by local auxin maxima and minima, also referred to as auxin gradients (reviewed in Tanaka et al., 2006), with the steady-state level of auxin within a particular cell triggering the developmental output of auxin signaling.

Along with metabolism, the asymmetric auxin distribution is mainly sustained by its intercellular transport, which, uniquely among plant signaling molecules, is strictly regulated in a directional fashion termed polar auxin transport. In the shoot, auxin flows in a single direction from its primary sites of synthesis, such as the apical meristem and developing leaves, down toward the root through the stem vascular tissues (Okada et al., 1991; Rashotte et al., 2003). By contrast, two distinct antiparallel streams of auxin movement occur in the root. Shoot-derived auxin travels over the whole-root distance through the central stele downwards to the root apex, where after loading into the outer cell layers it is redirected over a short distance toward the base of the root (Mitchell and Davies, 1975; Tsurumi and Ohwaki, 1978; Rashotte et al., 2000). According to the current paradigm, the mechanistic basis for the polarized auxin cell-to-cell movement is better described by the chemiosmotic hypothesis, in which the proton gradient generated primarily by plasma membrane H^+-ATPases between the neutral cytoplasm and the acidic extracellular space drives cellular auxin uptake and efflux (Rubery and Sheldrake, 1974; Raven, 1975). This model postulates the existence of plasma membrane–localized auxin influx and efflux carriers, whose coupled asymmetrical localization between adjacent cells provides the directionality of

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Research

Defining the selectivity of processes along the auxin response chain: a study using auxin analogues

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Summary

- The mode of action of auxin is based on its non-uniform distribution within tissues and organs. Despite the wide use of several auxin analogues in research and agriculture, little is known about the specificity of different auxin-related transport and signalling processes towards these compounds.
- Using seedlings of *Arabidopsis thaliana* and suspension-cultured cells of *Nicotiana tabacum* (BY-2), the physiological activity of several auxin analogues was investigated, together with their capacity to induce auxin-dependent gene expression, to inhibit endocytosis and to be transported across the plasma membrane.
- This study shows that the specificity criteria for different auxin-related processes vary widely. Notably, the special behaviour of some synthetic auxin analogues suggests that they might be useful tools in investigations of the molecular mechanism of auxin action. Thus, due to their differential stimulatory effects on DR5 expression, indole-3-propionic (IPA) and 2,4,5-trichlorophenoxy acetic (2,4,5-T) acids can serve in studies of TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALLING F-BOX (TIR1/AFB)-mediated auxin signalling, and 5-fluoroindole-3-acetic acid (5-F-IAA) can help to discriminate between transcriptional and non-transcriptional pathways of auxin signalling.
- The results demonstrate that the major determinants for the auxin-like physiological potential of a particular compound are very complex and involve its chemical and metabolic stability, its ability to distribute in tissues in a polar manner and its activity towards auxin signalling machinery.

Introduction

The auxin class of plant growth regulatory compounds controls different growth and developmental events in plants. Charles and Francis Darwin first noted the existence of an endogenous substance with biological activity in plants (Darwin & Darwin, 1980). This substance was characterized and reported as indole-3-acetic acid (IAA) (Kögl *et al.*, 1934; Went & Thimann, 1937) and it has been proved to be the major endogenous auxin present in plants. Other compounds with auxin characteristics, such as indole-3-butyric acid (IBA) (Zimmerman & Wilcoxon, 1935), phenylacetic acid (PAA) (Koepfli *et al.*, 1938) and 4-chloroindole-3-acetic acid (4-Cl-IAA) (Porter & Thimann, 1965), were later identified as endogenous auxins. Chemically more stable synthetic auxins, such as naphthalene-1-acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), are used as auxins in various biological applications, and some of these auxins and their structural analogues, such as, for example, polychloroaromatic acids or chlorinated picolinic acids, are efficient herbicides (Walsh *et al.*, 2006; Grossmann, 2010; Müller & Appleby, 2010). A number of other substances, mostly various carboxy derivatives of benzene, phenol, naphthalene or indole, show varying degrees of auxin-like activity in different bioassays (Koepfli *et al.*, 1938; Porter & Thimann, 1965; Ferro *et al.*, 2006; Christian *et al.*, 2008).

The coordinated growth and development of plants require the maintenance of optimum concentration gradients of active auxin(s) in specific organs, tissues and/or cell types, where they often act in co-operation with other plant hormones (Depuydt & Hardtke, 2011; Leyser, 2011; Rosquete *et al.*, 2012). The regulated development and maintenance of appropriate auxin concentration gradients in tissues are achieved by local metabolic processes, including biosynthesis, conjugation/deconjugation and degradation (Ljung *et al.*, 2002; Chandler, 2009; Ljung, 2013), and/or by polar transport and intracellular auxin compartmentalization (Grunewald & Friml, 2010). These processes result in the

SHORT COMMUNICATION

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ZIFL1.1 transporter modulates polar auxin transport by stabilizing membrane abundance of multiple PINs in *Arabidopsis* root tip

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Keywords: *Arabidopsis thaliana*, Major Facilitator Superfamily, PIN transporters, polar auxin transport, root development

Cell-to-cell directional flow of the phytohormone auxin is primarily established by polar localization of the PIN auxin transporters, a process tightly regulated at multiple levels by auxin itself. We recently reported that, in the context of strong auxin flows, activity of the vacuolar ZIFL1.1 transporter is required for fine-tuning of polar auxin transport rates in the *Arabidopsis* root. In particular, ZIFL1.1 function protects plasma-membrane stability of the PIN2 carrier in epidermal root tip cells under conditions normally triggering PIN2 degradation. Here, we show that ZIFL1.1 activity at the root tip also promotes PIN1 plasma-membrane abundance in central cylinder cells, thus supporting the notion that ZIFL1.1 acts as a general positive modulator of polar auxin transport in roots.

Multiple key aspects of plant development, including root patterning, growth and gravitropism, are controlled by the intercellular polarized transport of the predominant endogenous form of the phytohormone auxin, indole-3-acetic acid (IAA). While cellular auxin efflux, the rate-limiting step in this process, has been shown to rely on the coordinated action of PIN-formed (PIN) and B-type ATP binding cassette (ABCB) carriers,[1-3] the directionality and rate of auxin transport are mainly attributable to the highly regulated polar localization of PIN transporters.[4,5] Dynamic polar sorting of PINs at the plasma membrane is sustained by repeated steps of endocytic internalization and recycling back to the plasma membrane via exocytosis,[6,7] with this constitutive cycling controlling not only PIN subcellular localization, but also their plasma-membrane abundance and consequently their activity.[8] Notably, auxin appears to be the main regulator of its own asymmetric distribution (reviewed in Löfke et al.[9]), in particular through the dual effect it exerts on PIN fate. Indeed, while short-term IAA applications inhibit the internalization step of PIN cycling promoting their stability at the plasma membrane,[10,11] extended IAA treatments trigger PIN protein degradation through lytic vacuolar targeting and proteasomal activity, thus reducing their plasma-membrane incidence.[12-15] Importantly, the combination of these auxin antagonistic effects on directional vesicular trafficking and proteasome-mediated degradation allows the positional control of PIN2 activity sustaining root gravitropism.[14,15]

Membrane transporters from the Major Facilitator Superfamily (MFS) are single-polypeptide secondary carriers capable only of transporting small solutes in response to chemiosmotic ion gradients.[16] The few plant MFS carriers examined to date have been implicated in sugar, oligopeptide, nitrate and phosphate transport.[17-19] In addition, one *Arabidopsis thaliana* MFS member, ZIF1 (Zinc-Induced Facilitator 1), has been described as a tonoplast-localized transporter promoting zinc (Zn) tolerance by affecting vacuolar partitioning of nicotianamine, a low molecular mass chelator with high affinity for a range of transition metals.[20,21] Recently, we reported the functional characterization of the closest *Arabidopsis* ZIF1 homolog, the ZIFL1.1 (ZIF-Like 1) transporter.[22] In contrast to ZIF1, our results indicate that *ZIFL1.1* expression is not regulated by the Zn external status and that the activity of the encoded carrier does not contribute to plant Zn tolerance (**Fig. 1**), as already stated by Haydon and Cobbett (2007). Instead, we found that the ZIFL1.1 transporter regulates various root auxin-related processes, such as primary root elongation upon extended challenge with the phytohormone, lateral root development and gravitropic bending.[22] We further showed that the proton-coupled potassium transport activity of this root tonoplastic carrier indirectly modulates cellular auxin

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Chapter 6

Conclusions and Perspectives

Pawel Baster

Conclusions and perspectives

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In **Chapter 2** of this work we have addressed specifically a part of events following gravistimulation of *Arabidopsis thaliana* roots, namely how auxin influences the turnover of PIN2 protein thus regulating auxin flow from the place of gravity perception (root tip) to the responsive tissues in the elongation zone. We have quantitatively described a correlation between spatio-temporal regulation of the auxin distribution and differential regulation of the PIN2 abundance at the lower and upper side of gravistimulated roots. Specifically, the increase of auxin response at the lower side of the root was accompanied with initial increase of PIN2 abundance followed by its gradual decrease. On the other hand, at the upper side of the root, we have detected decrease in auxin response that is accompanied with initial decrease in PIN2 abundance followed by its gradual increase. Importantly, the differential auxin accumulation in all observed cases preceeded changes in PIN2 abundance at the plasma membrane. We have therefore concluded that an "optimal" auxin concentration is required to maintain PIN protein levels and thus auxin transport capacity at the PM during root gravitropism. We have further validated our gravitropism-based observations by the analysis of this auxin dual-action model in other developmental contexts. We have shown that exogenously applied or endogenously produced auxin mediates the PIN targeting to the vacuole and promotes PIN2 degradation. Similarly, pharmacological reduction in auxin levels or interfering with cellular auxin sensing leads to destabilization of PIN2 from the PM and higher rates of its vacuolar targeting. Subsequently we have experimentally proved that $SCF^{TIR1/AFB}$-dependent signaling is required for auxin-induced PIN2 degradation, clarifying findings of Pan et al., 2009. Moreover, we have identified molecular components (ARF2 protein) downstream of auxin $SCF^{TIR1/AFB}$ receptor, potentially more specifically related to the regulation of PIN turn-over (Figure 1).

Future research should address how the same outcome (PIN vacuolar targeting) is achieved by two seemingly opposite signals (increased or decreased auxin levels). Different sets of proteins transcriptionally regulated by different auxin levels might possibly target different subcellular trafficking processes. To analyze this hypothesis, one could screen for genes differentially regulated by decreased and increased auxin levels. For this purpose the results of microarray experiment, in which transcription profiling was analyzed in response to exogenous auxin and in conditional *axr3* auxin signaling mutant, mimicking increased and decreased auxin signaling environment, respectively, could be used. Alternatively, the response of AFB auxin receptors to different auxin levels in various cells, could be analyzed to address possible opposite effects on the downstream signaling, as recently suggested for AFB4 (Greenham et al., 2011). This could be performed by treatment with auxins (NAA, IAA), auxin antagonists (PEO-IAA, Auxinole) and inhibitors of auxin biosynthetic pathway (L-Kynurenine), followed by cell sorting and analyzis of AFB's expression. Alternatively a promoter shuffling-based approach could be used to target the expression of AFB's to various cells followed by treatment with auxin and auxin-antagonists.

It was previously reported that PIN degradation is impaired in presence of proteasome inhibitor (Abas et al., 2006). Given the fact that proteasome complex targets mainly soluble and not membrane proteins (Vierstra, 2009), but also considering our observation that other (than PINs) PM proteins (although less effectively) are rerouted to the vacuole upon fluctuations in cellular auxin levels, it is reasonable to believe that $SCF^{TIR1/AFB}$ signaling could potentially affect a more general trafficking regulator. Such a master regulator of vacuolar targeting could be subject to proteasome modifications and in turn direct post-translational modifications of PINs and other proteins. Such a hypothesis would

integrate the involvement of both proteasomal and vacuolar lytic degradation in the regulation of PIN abundance. Therefore a genetic screen for such a master regulator would definitely be valuable for further research on auxin instructed plant development, especially in context of PIN localization and abundance. Such a screen could be based on restoration of assymetric PIN2 localization during root gravitropism or gravitropic response of the roots itself in presence of proteasome inhibitors. Finally, it would be beneficial to test if auxin effect on PIN protein degradation, besides regulating root gravitropism, might contribute to other processes, such as auxin transport-mediated auxin maxima establishment during *de novo* organ formation (Benková et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Vernoux et al., 2010) where PIN degradation has been recently shown to play an important role (Marhavý et al., 2011).

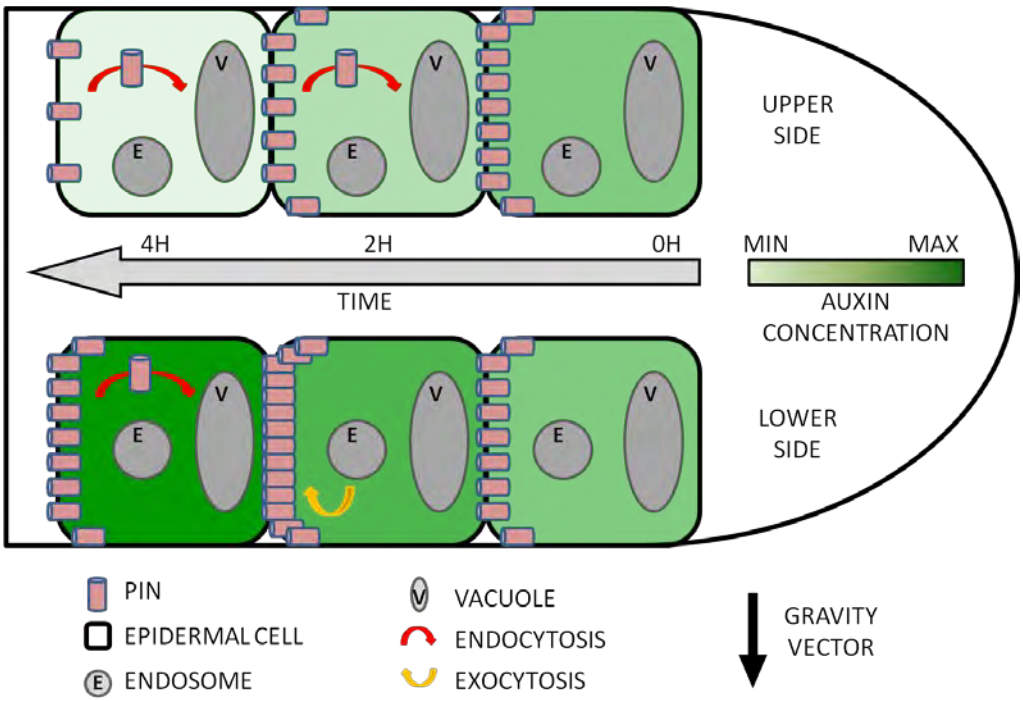

Figure 1. The model of dynamic changes in auxin distribution and localization of PIN2 protein during root gravitropic response. At the lower side of the gravistimulated root increase of auxin response is associated with initial increase of PIN2 abundance, presumably as a result of inhibited PIN2 endocytosis. Subsequent gradual decrease of PIN2 membrane abundance, coinciding with elevated auxin response, is a result of increased vacuolar targeting of PIN2 protein. At the upper side of the root, decreased auxin response is accompanied by initial decrease in PIN2 abundance followed by its gradual increase. Effects of increased and decreased auxin response on PIN vacuolar targeting are mediated by $SCF^{TIR1/AFB}$ nuclear auxin signalling.

In **Chapter 3** of this study, we were addressing the mechanism of PIN polarity maintenance, in context of post-translational modification of PIN HL loop, influencing its plasma membrane abundance. We were able to observe the stabilization of PIN2 protein at the PM with concomitant reduction of PIN2 vacuolar abundance, induced by pharmacological inhibition of phosphatase activity or kinase overexpression. In contrary, lower PM abundance simultaneously with increased vacuolar occurrence of

PIN2 protein was observed following the pharmacological inhibition of kinase activity. What is more, expression of fluorescently tagged PIN3 protein in hypo-phosphorylating genetic background of *pidwag1wag2* loss-of-function mutant allowed us to demonstrate increased rates of PIN3 internalization, reflecting most likely decreased PM abundance in the cellular environment lacking kinase function. In addition, we were able to identify quantitative differences in the PM stability of PIN proteins, amino-acid sequences of which were modified to mimic absence of, or constitutive phosphorylation.

In conclusion, in this part of our research we propose an integration of a novel mechanism involved in the regulation of auxin fluxes during plant development. Such a mechanism, based on protein phosphorylation would execute a dual function where not only the regulation of trafficking-based delivery of PINs to the specific polar domain of PM would be achieved (Friml et al., 2004; Michniewicz et al., 2007; Kleine-Vehn et al., 2009), but similarly to ubiquitination (Leitner et al., 2012), a signal to regulate the cell surface abundance and vacuolar delivery of PINs, would be provided (Figure 2).

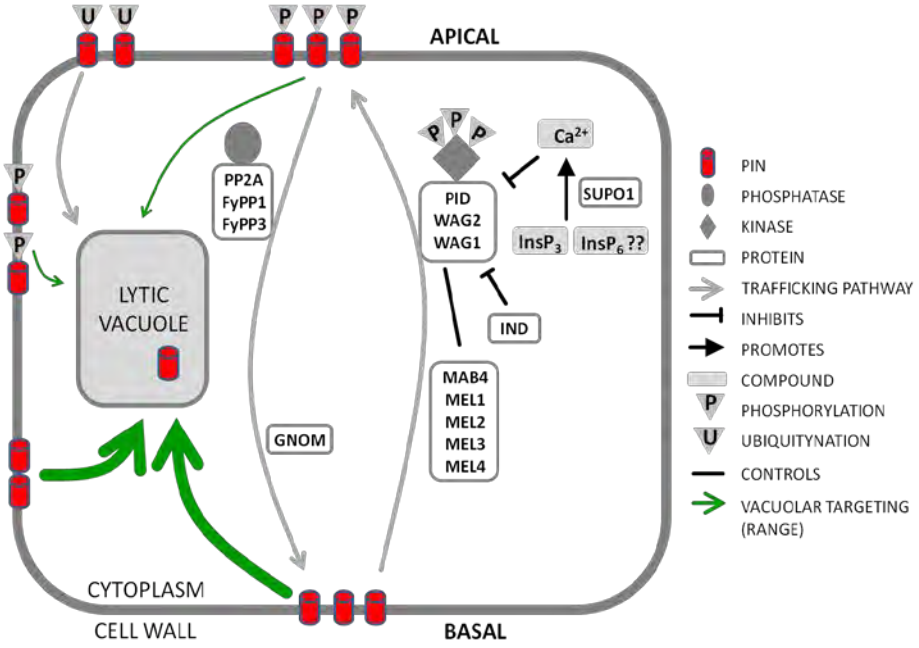

Figure 2. The model of phosphorylation-based regulation of PIN protein localization. Enzymatic activity of protein kinases and phosphatases contributes to the preferential recruitment of the hyper-phosphorylated PINs into the apical whereas hypo-phosphorylated PINs, to the basal targeting pathway. Decreased phosphorylation of PIN HL appears to additionally destabilize PINs from the cell surface and target them for the lysis in the vacuolar compartment. In contrary, when cellular phosphorylation status is increased the rate of PIN vacuolar targeting is decreased concomitantly with protein stabilization at the PM.

In order to precisely define to which extent the phosphorylation status of PIN proteins is associated with their PM abundance, western blotting analysis of PIN1 and PIN2 microsomal fractions should be performed upon pharmacological inhibition of kinase and phosphatase activity as well as in microsomal fractions of hypo- and hyper-phosphorylating genetic backgrounds of kinase loss-of-function and gain-of-function and phosphatase loss-of-function mutants, respectively. What is more, although the large

scale analysis of transcriptome revealed that cantharidin negatively regulates the transcription of PIN3, 4 and 7 (Bajsa et al., 2011), it would be necessary to analyze a possible relationship between the effects of kinase and phosphatase inhibitors presented in the Chapter 3 of this study and the regulation of PIN transcription. Another aspect which would benefit from furher investigation is the possibility that the PIN3-GFP internalizations observed with increased frequency in kinase loss-of-function genetic background are the result of elevated *de-novo* synthesis and secretion of the PIN3 protein. Therefore, the frequency of PIN3-positive internalizations in the columnella cells of *pidwag1wag2* mutant should be addressed experimentally, for example in presence of protein synthesis inhibitor, cycloheximide.

The preliminary analysis of certain phosphorylation sites, previously described as decisive for PIN polarity determination (Huang et al., 2010; Zhang et al., 2010), in context of the regulation of PIN protein abundance at the PM and turn-over, yielded promising results. We were able to observe quantitative decrease in the stability of PIN1 at the PM, when phospho-sites p2, p3, and p4 were modified to mimic lack of phosphorylation. On the other hand, we could not detect any differences when single p4 site was modified to mimic loss or constant phosphorylation. This data suggests possible specificity of certain phosphorylation sites towards the regulation of PIN PM abundance and vacuolar targeting. These promising results, prompted us to design an experiment for detail characterization of these sites in context of PIN vacuolar trafficking rates. We have cloned a PIN1 protein and introduced Ala or Glu substitutions at p1, p2, p3 and p4. This approach will hopefully allow us to dissect PIN1 phosphorylation site(s) crucial for the regulation of PIN membrane stability and degradation dynamics. PIN activity at the plasma membrane play a rate-limiting role during intercellular auxin translocation (Petrasek et al., 2006; Wisniewska et al., 2006), prominently contributing to the generation of auxin gradients crucial for various aspects of plant development (Tanaka et al., 2006). We therefore believe that this work, by addressing specifically the context of PIN cell surface abundance, brings substantial novel insights into the regulation of PIN polarity maintenance, and thus regulation of auxin-flux-dependent plant development, in general.

Chapter 4 of this dissertation is dedicated to the identification of putatively novel regulators of polar PIN localization by means of forward genetics screen using synthetic strigolactone analogue – GR24. As a result of the genetic screen we were able to identify a *pig1* (*PIN insensitive to GR24 1*) mutant, which, when exposed to GR24, maintains the growth of the root and its gravitropism. What is more, upon GR24 treatment proper PIN polar localization is preserved in roots of *pig1* mutant. Our study revealed that *pig1* mutant is defective in the E1-β subunit of Pyruvate Dehydrogenase Complex, encoded by At5g50850 gene. The *pig1* mutant displays several auxin related phenotypes. Moreover, the root elongation of *pig1* mutant is oversensitive to auxinic compounds activating DR5-visualized auxin signaling, like IAA or NAA while it is resistant to auxinic compounds inactive in mediating SCF^{TIR1}-based auxin signaling, like IBA or IAA-L-Ala (Simon et al., 2013). To further address the specificity of *pig1-1* defects, the response of the mutant should be analyzed in presence of various auxin conjugates. Additionally, the levels of free IAA and auxin conjugates should be determined biochemically in tissues of *pig1* mutant (preferentially in absence vs presence of exogenous strigolactone). In **Chapter 4** we show that strigolactone application induces depletion of PINs from the plasma membrane and its subsequent targeting for vacuolar lysis. In addition, we show that GR24-induced destabilization of PINs from PM occurs preferentially from the basal side of the cells. Finally, we provide the evidence that PIN localization in roots of *pig1* mutant is insensitive to this destabilizatory effect of GR24 (Figure 3). The

involvement of E1-β enzymatic activity in generation of *pig1* mutant phenotypes requires further studying. It should be also addressed if the hypothetical mechanism, based on disturbed differential degradation from upper vs lower side of plasma membrane upon strigolactone application, indeed is contributing for the PIN localization insensitive to GR24 in *pig1* mutant. This could be achieved, for example, by the analysis of *pPIN2::PIN1-HA* localization in *pig1* mutant background.

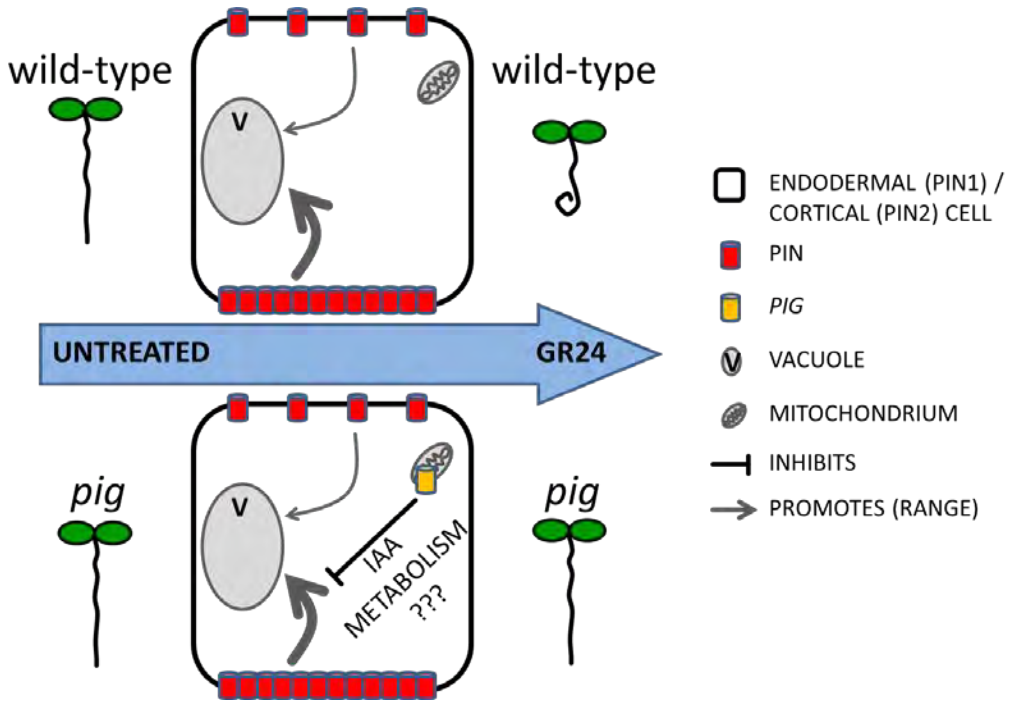

Figure 3. The model of PIG protein function. The exogenous application of synthetic strigolactone analogue (GR24) induces destabilization, preferentially from the basal PM domain, and vacuolar targeting of PINs. *PIG* mutant protein, possibly through modulation of auxin metabolism or auxin/strigolactone balance, inhibits GR24 effect on PIN degradation. As a consequence, the roots of *pig* mutant seedlings, as opposed to wild-type seedlings, maintain root growth and gravitropism in presence of GR24 in the medium.

Generally, in recent years there has been a significant advance achieved in our understanding of the basic rules governing generation, maintenance and refining of the cellular polarity in plants. It becomes clear how polar protein localization at the subcellular scale can underpin basic functionality of an organism and instruct its development. In particular, the studying of asymmetric distribution displayed by PIN auxin transporters allowed us to deepen the knowledge about polarity. At the same time it provided excellent means to comprehend auxin transport machinery and its contribution, by establishment of asymmetric auxin distribution, to the regulation of various developmental processes. An important open question remains; to which extent cellular mechanisms and molecular components of the PIN polar targeting machinery can be translated into proteins localized at different polar domains in plants. Another important aspect is the evolution of auxin transport machinery and thus mechanisms governing polarity generation in more ancient plant species. For this, the examination of evolutionary older than Angiosperms plant species like moss or algae, which are rapidly gaining popularity in the

plant field, will hopefully significantly contribute to our understanding of the polarity phenomenon in the close future.

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Summary

Natural habitat can be characterized by dynamic changes of the conditions, such as availability of light or nutrients. These changes are very often extremely unfavorable for inhabitants of certain environmental niche. Plants, in order to compensate for their immobility, developed an amazing plasticity of ontogenesis, which allows them to keep up and adjust to continuously changing environmental conditions. Polarization processes can be found at the basis of most, if not all, aspects of this, unseen in animal species, adaptability. Surprisingly, the coordination of such a broad range of processes is facilitated most prominently by one, centrally acting signaling molecule - phytohormone auxin. Due to chemi-osmotic properties of the cells, the tightly regulated generation of local auxin accumulations across plant tissues, triggering downstream signaling cascades, is to largest extent facilitated by polarly localized PIN efflux carriers. This group of proteins performs a rate and direction limiting role during intercellular auxin flux. PIN transporters serve as perfect model system to study the phenomenon of polarity.

In the introductory, review chapter entitled “Auxin on the road navigated by cellular PIN polarity”, the cellular requirements and molecular components, were described, constituting for a multi-level mechanism regulating the establishment and maintenance of PIN polarity. First, differences between animal and plant solutions for the generation of protein polarity were briefly presented. Subsequently, an emphasis was given to subcellular trafficking machinery relevant for PIN polarity. Next, cargo-based followed by cell-structural determinants for targeting and maintaining of PINs at their polar domains were presented. Finally, the feedback mechanisms for PIN polarization, including the ones specific for auxin and other signalling molecules, were discussed **(Chapter 1)**.

In the next part of this dissertation, the identification of a dual auxin-based mechanism by which PIN2 protein abundance is dynamically and differentially controlled at the upper and lower side of a gravistimulated root, was described. Prolonged fluctuations in cellular auxin levels, above or below certain native threshold, respectively, were shown to induce targeting of PIN auxin transporters to the vacuole, thereby regulating the abundance of the auxin carriers at the plasma membrane. These effects of opposite auxin concentrations on PIN trafficking to the vacuole apparently depend on the canonical auxin signaling pathway, involving auxin-dependent degradation of Aux/IAA transcriptional repressor proteins. Moreover, the data was provided to clarify inconsistencies of previous research, about the involvement of $SCF^{TIR1/AFB}$-based signaling in auxin-mediated PIN turn-over. These findings link auxin-mediated regulation of vesicle transport and asymmetric growth control during gravitropic response **(Chapter 2)**.

In another scientific project undertaken during this PhD study, we have identified a novel mechanism potentially contributing for the regulation of cell surface abundance and rates of PIN protein degradation. Our data suggests that such a mechanism, based on protein phosphorylation, executes a dual function. Not only the regulation of trafficking-based delivery of PINs to the specific polar domain of PM is achieved, but similarly to ubiquitination, a signal for conditional PIN endocytosis and trafficking to vacuole for lysis, is provided. What is more, based on the experimental results we have suggested a

possible specificity of certain phosphorylation sites towards the regulation of PIN PM abundance and vacuolar targeting **(Chapter 3)**.

The following chapter is dedicated to identification and characterization of potentially novel regulators of polar PIN targeting, by means of forward genetic screen using synthetic strigolactone analogue – GR24. This approach enabled us to identify the *pig1* (*PIN insensitive to GR24 1*) mutant, insensitive, at both morphological and subcellular level, to GR24. *pig1* mutant was shown to be defective in the E1-β subunit of Pyruvate Dehydrogenase Complex. Subsequent analysis of *pig1* phenotypes and responses revealed that the activity of PD E1-β, while not influencing PIN polarity per se, contributes most probably for the maintenance of PIN cell surface abundance by regulating rates of PIN vacuolar trafficking **(Chapter 4)**. In the final, compediary part of this thesis, illustrates an additionall, collaborative scientific input, related to the elucidation of broadly understood process for the regulation of PIN abundance and polarity in plant development, that author performed within and outside of a research group **(Chapter 5)**.

In conclusion, the data gathered during this PhD study and presented in this dissertation brings substantial novel insights into the mechanisms of PIN polarity maintenance. Successfully applied biochemical, pharmacological and genetic approaches allowed us to identify and characterize post-translational signals, of both hormonal (auxin), and non-hormonal (protein phosphorylation) nature, involved, specifically, in the regulation of cell surface abundance and rates of PIN vacuolar targeting for degradation. What is more, forward genetics approach allowed us to identify novel molecular players, potentially involved in the regulation of PIN polarity.

Curriculum Vitae

PERSONAL DETAILS

Name:	Pawel Baster
Date of Birth:	11th July 1984
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EDUCATION

2009-present PhD study in Plant Biochemistry and Biotechnology - VIB Department of Plant Systems Biology and Faculty of Sciences; Department of Plant Biotechnology and Genetics, Ghent University, Belgium; Institute of Science and Technology Austria, Klosterneuburg, Austria - Supervisor: Prof. Dr. Jiri Friml

2006-2008 Master study in Biotechnology of Plants and Microorganisms - Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland.
Thesis work entitled: "*Arabidopsis tanmei/emb2757 embryo mutant is defective for in vitro plant morphogenesis*" Supervisor: Prof. Dr. Małgorzata D. Gaj

2003-2006 Bachelor study in Biotechnology, Faculty of Biology and Environmental Protection, University of Silesia, Katowice, Poland.
Thesis work entitled: "*DNA-based computers – future or utopia?*" Supervisor: Dr. Mirosław Kwaśniewski

1999-2003 Secondary education at the 1st General/Comprehensive Secondary School, Bytom, Poland

PUBLICATIONS

Baster P, Friml J (2013) Auxin on the road navigated by cellular PIN polarity *Chapter for the Book: '*Auxin and Its Role in Plant Development' Biomedicine/Life Sciences; Wien, New York (submitted)

Simon S, Kubes M, **Baster P**, Robert S, Dobrev P, Friml J, Petrasek J, Zazimalova E (2013) Defining selectivity of processes along the auxin response chain: a study using auxin analogues. *New Phytologist* (accepted, in press)

Remy E, **Baster P**, Friml J, Duque P (2013) ZIFL1.1 transporter modulates polar auxin transport by stabilizing membrane abundance of multiple PINs in *Arabidopsis* root tip. *Plant Signaling and Behavior* (accepted, in press)

Remy E, Cabrito TR, **Baster P**, Batista RA, Teixeira MC, Friml J, Sá-Correia I, Duque P (2013) A Major Facilitator Superfamily Transporter Plays a Dual Role in Polar Auxin Transport and Drought Stress Tolerance in *Arabidopsis*. *The Plant Cell* 253:901-26

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Baster P, Ledwoń A, Gliwicka M, Trojanowska A, Gaj MD (2009) *Arabidopsis tanmei/emb2757* embryo mutant is defective for in vitro plant morphogenesis. *Plant Cell Tissue and Organ Culture* 99:305-312

PRESENTATIONS AT SCIENTIFIC MEETINGS

The 21st International Conference on Plant Growth Substances, June 2013, Shanghai, China - "*Putative regulator of PIN polarity identified by means of forward genetics screen using strigolactone analogue - GR24*" (Poster presentation)

VIB Conference, April 2012, Blankenberge, Belgium - "*Super- and sub-optimal auxin levels mediate PIN degradation during root gravitropism*" (Poster presentation)

PhD Symposium of the Ghent University, March 2012, Ghent, Belgium - "*Forward genetics approach to identify novel regulators of PIN polarity*" (Oral presentation)

4th International PhD School on Plant Development, October 2011, Zellingen-Retzbach, Germany - "*SCFTIR1/AFB signaling pathways mediates asymmetric PIN degredation during root gravitropism*" (Oral presentation)

The 20th International Conference on Plant Growth Substances, July 2010, Tarragona, Spain - "*Molecular characterization of PIN vacuolar targeting*" (Poster presentation)

TEACHING ACTIVITIES

Supervision of four Bachelor students in their Bachelor Project: *Mapping and characterization of strigolactone-resistant mutants of Arabidopsis* (3rd Bachelor Biochemistry and Biotechnology, Ghent University)

GRANTS

Fonds Wetenschappelijk Onderzoek (FWO) Vlaanderen, Belgium - Grant for participation at a congress abroad: IPGSA 2013, Shanghai, China

LANGUAGES

Polish – Mother Tonque
English – Proficient (TOEFL score: 105/120)
German – Basic
Russian – Basic

TECHNICAL SKILLS

- Molecular biology: techniques associated with DNA and RNA (extraction, purification, gel electrophoresis, restriction enzyme analysis etc.), Gateway-based cloning (handling bacterial cultures), PCR and qRT-PCR
- Experience with robotized systems: Biomek2000 (plasmid isolation), Janus (qRT-PCR), InsituPro Vsi (immuno-chemistry)
- Cell biology: epi-fuorescence, laser scanning confocal microscopy visualization of GFP and fluorescent probes (FM4-64), immuno-fuorescence, pharmacological trafficking inhibitors (BrefeldinA, Wortmanin, ConcanamycinA, Tyrfostine, Filipin etc.), biosynthesis inhibitors (L-Kynurenine, Cycloheximide etc.), phytohormones and analogs (auxin, cytokinin, PEO-IAA, Auxinole etc.)
- Good command of Olympus, Zeiss and Leica microscopes and of imaging analysis software, such as ImageJ and Olympus FluorView
- Experience with *Arabidopsis thaliana* system
- Biochemistry: western blotting
- Tissue Culture: sterile techniques for maintaining *Arabidopsis* cell cultures, somatic embryogenesis, callus cultures, biolistic transformation of *Nicotiana tabacum* cell culture and aseptic growth of seeds

- Genetics: cross pollination of *Arabidopsis*, generation of stable transgenic lines in *Arabidopsis* via floral dip, generation of the mutated plant populations (EMS mutagenesis), morphological and subcellular phenotypical characterization of the mutant *Arabidopsis* lines
- Bioinformatics: basic skills in gene and protein analyses using BLAST, ClustalW, Vector NTI
- Scientific writing and editing, graphical preparation of the manuscripts (Adobe Illustrator), handling of intellectual property
- Experience with establishing and moving of the biological laboratory

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