PIN FAMILY OF AUXIN TRANSPORTERS: FUNCTIONAL AND EVOLUTIONARY ASPECTS

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Petr Skůpa

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SUPERVISOR
Assoc. Prof. RNDr. Eva Zažímalová, CSc.

Institute of Experimental Botany of the Academy of Sciences, Prague, Czech Republic

Department of Experimental Plant Biology, Faculty of Science, Charles University, Prague, Czech Republic

CONSULTANT
RNDr. Jan Petrášek, Ph.D.

Institute of Experimental Botany of the Academy of Sciences, Prague, Czech Republic

Department of Experimental Plant Biology, Faculty of Science, Charles University, Prague, Czech Republic

This is to certify that this thesis is not subject of any other defending procedure. It contains set of original results that have been published in the international peer reviewed scientific journals.

Prague 31st July, 2011

Petr Skůpa

On behalf of co-authors of the papers published, I hereby confirm the agreement with inclusion of the papers below into the dissertation thesis of Petr Skůpa. The papers were produced as a team work and the particular contribution of Petr Skůpa is specified at the beginning of relevant chapters below.

Prague 31st July, 2011

Eva Zažímalová

Acknowledgement

With kind memory to my dear parents …

… I would like to express my sincere gratitude to such a great number of people thank I have a hard time to figure out how to do it. I am humbled… Thank you all! First and foremost, there would be two people I would like to thank the most. I would like to thank to my supervisor Eva Zažímalová, the thanks are just even hard to comprehend and pronounce .... I am never ceasing to wonder for the twist of fate allowing me to come to your lab! Thank you so much for never-ceasing support and guidance. And I would like to thank dearly to Jan Petrášek, who had been nearby as and guidance and inspiration from the beginning, his role was actually far more than those words may gather..

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SUMMARY

Growth and development of plant body is dependent on correct and effective integration of information about current deployment of its body parts, as well as on perception and transduction of inputs from environment. Multiple developmental processes within plant body are determined by specific and tightly controlled distribution of molecule with unique signaling mission within plant development – auxin. Spatial distribution of auxin is co-determined by plethora of tightly controlled processes, and the polar auxin transport plays unique role among them. PIN proteins are the plant-specific family of secondary transporters driving movement of auxin across membranes. With their frequent asymmetrical localization within cells, specific expression patterns in developing tissues and their reactiveness to external cues they secure unique, dynamic and asymmetric distribution of auxin within the plant body.

This dissertation thesis is focused on characterization of the role different PIN proteins play in determining cellular auxin homeostasis and consequent formation of auxin gradients. Controlled overexpression of PIN proteins in tobacco cells showed, that PIN4 and to some extent also PIN6, function as the direct auxin efflux carriers. In the cellular auxin transport they play the role analogous to other canonical 'long' PINs, i.e. they are rate-limiting factors in cellular auxin efflux. On the other hand, PIN5 was proved to contribute to the cellular auxin management by entirely novel mechanism: It was shown to reside in the endomembrane-system and to mediate intracellular redistribution of auxin with consequent dramatic impact on the auxin metabolic profile. The data provided first evidence for the new mechanism underlying the establishment and maintenance of auxin homeostasis on the level of a single cell. Moreover, it was shown, that PIN family can be classified, according to proteins structure, function and evolutionary history, in two major subfamilies, PIN5 was shown to belong to a newly characterized PIN subfamily of 'short' PINs. Altogether, the results summarized in this thesis contributed to the understanding of the molecular components mediating auxin homeostasis and provide classification of the family members based on structural and evolutionary relationships. Moreover, in the perspective of the reported results, together with relationships found among the proteins, we strived to make a synthesis about the role of PIN proteins in plant

development in a more general fashion and in respect to its impact on the knowledge about plant evolution.

SOUHRN

Pro růst rostlin a vývoj rostlinného těla má zásadní význam kvalitní a efektivní zpracování signálů, resp. informací pocházejících jednak přímo z rostlinného těla, ohledně rozložení jednotlivých rostlinných částí a jednak integrace podnětů z prostředí, umožňující rostlině reagovat na vnitřní vývojové podmínky i vnější hrozby vhodnou korekcí svého růstového programu. Velké množství vývojových procesů rostlinného těla je tak determinováno charakteristickým prostorovým rozmístěním auxinu - molekuly s informační hodnotou. Prostorového rozmístění auxinu v prostoru je dosaženo kombinací a bilancí velkého množství striktně regulovaných procesů, mezi nimiž významnou a jedinečnou roli hraje polární transport auxinu. Rodina auxinových přenašečů PIN pak představuje přenašeče auxinu z buňky, které díky své asymetrické lokalizaci, specifickým expresním vzorcům a modulovatelné transportní funkci zajišťují tvorbu specifických auxinových gradientů.

Předmětem této disertační práce je charakterizace rolí, kterou jednotlivé PINy hrají při tvorbě a udržování vnitřních buněčných hladin auxinu a následně auxinových gradientů v rostlině. Kontrolovatelná nad-exprese proteinů PIN v tabákových buněčných liniích ukázala, že PIN4 a do určité míry také PIN6, fungují přímo jako přenašeče auxinu ven z buňky. V buněčném auxinovém transportu hrají roli analogickou dalším klasickým „dlouhým“ PINům umístěným na plasmatické membráně, které určují směr a limitují rychlost mezibuněčného toku auxinu. Oproti tomu pak bylo zjištěno, že PIN5 přispívá k regulaci homeostáze auxinu v buňce zcela novým mechanismem: bylo doloženo, že je lokalizován v endomembránovém systému, kde zprostředkovává vnitrobuněčnou redistribuci auxinu, v jejímž důsledku pak dochází k dramatické změně metabolického profilu auxinu. Byl tak předložen první důkaz o novém mechanismu formování a udržování vnitřní hladiny auxinu na buněčné úrovni. Dále bylo ukázáno, že rodina proteinů PIN může být klasifikována v závislosti na primární struktuře, funkční roli i evolučních vztazích členů proteinové rodiny a rozdělena na dvě základní podrodiny, kdy PIN5 náleží do nově popsané podrodiny „krátkých“ PINů. Výsledky shrnuté v této disertační práci přispěly k poznání a popsání faktorů definujících hospodaření s auxinem na buněčné úrovni a přednést možný klasifikační systém rodiny PINů, založený na evolučních vztazích i strukturní

podobnosti jednotlivých členů. Na základě dosud publikovaných poznatků a strukturně/evolučních vztahů zjištěných mezi členy PINové rodiny jsme se pak pokusili o formulaci hypotézy o roli PINů v rostlinném vývoji obecněji, a to především s ohledem na roli, kterou PINy mohly sehrát v evoluci cévnatých rostlin.

1 INTRODUCTION

Plant growth and development consists of complex and tightly regulated actions, based on both genetically given and environmentally induced processes. Polar transport of plant hormone auxin underlies these developmental processes and secures their temporal and spatial co-ordination. Transport of auxin runs on both long and short distances, and comprises flow of auxin molecules via vascular pathways as well as cell-to-cell mechanism. Polarity of auxin transport is facilitated by polar localization of some of auxin transporters. Because of physical-chemical properties of auxin molecule, the crucial importance for polarity of auxin transport belongs to the auxin efflux carriers. There are several types of auxin efflux carriers known, and the key role for polar auxin transport and for its developmental significance is ascribed to plant-specific transporters from the PIN-FORMED (PIN) family.

The main aim of this thesis is to contribute to characterization of molecular function of distinct members of PIN family, and to the understanding of their role in evolution of plants.

2 OUTLINES OF THE THESIS

PIN proteins were long known to be essential components of the polar auxin transport and proved to be defining factors for the directionality of the auxin flow, within plant tissues (in detail see Chapter 4.5). Nevertheless, with the discovery of the role of the ABC-type transporters in auxin transport (Noh et al. 2001; review by Geisler and Murphy 2006) their direct role as auxin transport transporters was challenged. It became unclear what role in the directional auxin transport ABCB transporters play and what role really belongs to PIN proteins. One possible scenario was that the PIN proteins represent just sub-component of the auxin transport complex, governing the directionality or/and substrate specificity of the auxin transport machinery, through regulation of activity of the ABCB exporters, proposed to be the real transport unit of the complex.

Approximately at the time those possibilities were considered, I started my doctoral study in the laboratory under the supervision of Dr. Eva Zažímalová and guidance of Dr. Jan Petrášek. My starting point in my experimental work was defined by availability of established model system, the tobacco BY-2 cell line and and convenient experimental framework. In the years preceding to my arrival at the laboratory, Dr. Eva Zažímalová with Dr. Jan Petrášek had established tobacco suspension cell lines as a convenient and practical model for auxin transport studies. (Zažímalová et al. 1996; Petrášek et al. 2002; Petrášek et al. 2003; Petrášek and Zažímalová 2006).

I have started the work by the successful transformation of inducible constructs carrying PIN4 and PIN6 genes, kindly provided by Dr. Jiří Friml, into BY-2 cells. I used these lines for characterization of PIN4 and PIN6 transport capacity, as described in Chapter 6.1 of this thesis and published in Petrášek et al. (2006) and Mravec et al. (2008). I followed the same protocol with transformation of the cell line with PIN5 gene. With increasing evidence about different character of this PIN protein, I entirely refocused my effort for this member of PIN family. With respect to plant material

available, I had combined several methodological elements into new methodological framework, describing shift in metabolic profile of auxin, allowing us to infer on auxin transport changes in subcellular resolution, Substantial part of the findings is published in Mravec et al. (2009) and described in Chapter 6.2 of this thesis. Moreover, starting initially by long, inspirational discussions about unusual character of PIN5 sequence with Jozef Mravec I started to ponder about evolutionary attributes of the PIN sequences in general. Thought originally limited to sequence/function relationship of PIN sequences were later extended to potential coevolution of PIN sequences, polar auxin transport and increased plasticity and complexity of the plant body architecture in general.

All the presented findings point at the significance of PIN proteins, for both proper genetically-based and environmentally-conditioned plant development. Unpredicted functional division within the PIN protein family, together with the developmental diversity within 'long' PINs point out the emerging need to understand not only molecular function of PINs but also, generally, the evolutionary context of auxin transport evolution, and phylogenetic relationships between PIN protein sequences in particular. (Zažímalová et al. 2007; Křeček and Skůpa et al. 2009; enclosed in the Chapter 6.3 of this thesis)

3 OBJECTIVES OF THE THESIS

Based on the outlines above, the objectives of this thesis are:

1. To contribute to characterization of PIN family, namely:
2. To characterize molecular function of representatives of the 'long' PINs (namely PIN4) and PIN6;
3. To characterize molecular function of a representative of the 'short' PINs (namely PIN5);
4. To contribute to the understanding of evolutionary aspects and relationships between evolution of PIN family and evolution of land plants.

These objectives were addressed by making use of inducible or constitutive overexpression of particular *PIN* genes in suspension-cultured tobacco BY-2 cells, followed by observation of cell phenotype, cell growth parameters, analysis of auxin transport and auxin metabolic profiling. The main aim of this thesis is to summarize the data about molecular function of distinct members of PIN family and their evolutionary history, and to contribute to understanding their role in evolution of plants.

4 LITERATURE OVERVIEW

4.1 Development of plant body plan

Higher plants, along with animals are multicellular organisms with advanced architecture of their bodies, with various sets of differentiated cells arrayed within their bodies to cope in a co-ordinative way with predicaments of the ever-changing conditions of their environment. Plants survival strategy is based on collection of light as the energy source for their bodies, and the evolution process tuned the development of the plant body-plan to formation of such organs that are the most appropriate for collecting of light as well as of inorganic nutrients from the environment. As the result, higher plants are modular and sessile organisms unable to move from their habitat.

Animals, in comparison with plants, have the advantage of mobility; therefore, body plan of any particular species of higher animals is relatively invariant and thus, we can conclude with Ottoline Leyser that "*the most responsive part of an animal's anatomy to the environment is the wiring of their brain*" (Leyser 2010). In animals, pattern formation is predominantly taking place during embryogenesis, which is the only stage within ontogenesis when organ formation occurs. In later stages of life cycle, animal body enlarges proportions of the already existing body parts (ibid).

Embryo of the new plant in a seed emerges during embryogenesis comparatively in much more rudimentary state than embryo of animals (ibid). The proper embryogenesis ends basically with the newly established apical-basal axis, with functional meristem on each end, with cotyledon(s) and with variable amount of energy reserves for the start. Nevertheless, the continuous, flexible, post-embryonic development of the plant body, which ensues onward, represents evolutionary response to the needs of the sessile organism's to react to it's environment (reviewed by Niklas and Kutschera 2009). Body plan is continuously formed from that point onward, processing all the environmental and genetic inputs into indefinite ever-growing live form.

Growth and development of plant body is dependent on correct and effective integration of information about current deployment of its body parts, as well as on

internalization of inputs from environment. Plants do not have central nervous system or any directly analogical system to convey long range information to arrange proper coordination of response of different body parts. Instead they rely on range of long-distance signals spreading through all cells of the body in much slower manner (Leyser 2011).

4.2 Auxin

Auxins represent class of such signaling molecules, with distinct roles on the cellular, tissue and whole plant level. Every cell in a plant body is capable to contribute in a way to the flow of information, represented by available auxin molecules, and to read from it back again in the context-dependent manner.

One auxin molecule might be seen in this context as a unit of information, distributed unevenly in both space and time within the growing body of the plant, creating patterns of alternating gradients, maxima and minima of auxin concentrations within the body (Benková et al. 2003; Benjamins and Scheres 2008; Petrášek and Friml 2009). Specific distribution of auxins together with the overlaid pattern of the signal perception components give rise to a wide range of possible local responses, having a coordinative value for growth responses and plant development as a whole (Kieffer et al. 2010).

As strong endogenous growth regulator, auxin is essential for control of almost any growth or developmental process in higher plants. Auxin biology actually belongs to the oldest fields of experimental plant research. F. W. Went in 1926 had shown for the first time the role of auxin in mechanism of phototropic response, i.e. the bending and differential growth of plants in relation to the source of light. Auxin was later isolated and identified by Thimann and Koepfli (1935) as indole-3-acetic acid (IAA). However, besides the principal natural auxin IAA the auxin class is much richer and there are several natural and many synthetic compounds with auxin activity (recent review by Simon and Petrášek 2011). As the research continued, it became evident, that auxin is of fundamental importance for much wider spectrum of vital processes, and that growth responses to environmental stimuli such as tropisms (Went 1926; Friml et al. 2002b) represent only small, albeit important, subset of those processes.

Survival strategy of plants is actually based on never-ending, continuous body development, their flexible form being reshaped by continuous response to the

environment through both directional growth and the *de novo* organ formation. Each event of organ formation is dependent on well-driven spatio-temporal co-ordination of division and expansion of cells within the frame of developing patterns (Friml et al. 2003). Such positional information (Wolpert 2011) is provided by the complex pattern of auxin gradients, auxin maxima and minima within the tissue, followed by short distance cell-to-cell communication, providing feedback and new refinement of the auxin gradients. In this respect, auxin seems to act analogically to the animal morphogens (Teleman et al. 2001). Growth and development is structured process, following formation of patterns of cells with differentiated cell fate. Pattern formation is genetically controlled, and in both plants and animals it requires each cell to sense its position within the pattern and to respond to it accordingly (Wolpert 2011). To provide positional information in developing tissues, different morphogens create gradients within developing tissues of animals.

Nevertheless, in contrast to morphogens, auxin presence and its information value is not restricted to short event(s) within the life cycle of the organism, as auxin provides very robust and complicated network throughout the whole body and the whole lifecycle of the plant. Each cell in plant body can respond to the auxin differently depending on its position, ontogenic and/or positional context, with range of different possible cellular responses. As the auxin gradients stimulate auxin signaling pathways differentially, various growth responses and cell differentiation events are triggered (Benjamins and Scheres 2008; Petrášek and Friml 2009). These processes may result in change of cell specifications and subsequently in formation of new cell patterns, new tissues and organs, or in responses of the existing ones to environmental stimuli such as light and gravity (Tanaka et al. 2006; Friml et al. 2002b).

Such role for auxin was shown for many developmental processes, may they involve formation of new meristem such as branching of stem (Leyser 2009), formation and positioning of lateral bud or root (De Smet et al. 2007) or may they create organs with definitive form, such as leaf or flower primordia within the process of phyllotaxis (Kuhlemeier 2007; Kuhlemeier and Reinhardt 2001; Reinhardt et al. Pesce 2003), leaf shape formation (Bilsborough et al. 2011), maturation of vessel bundles (Sachs 1975; 1991; Ye et al. 2002), patterning of root meristem (Blilou et al. 2005; Grieneisen et al. 2007) or leaf vasculature (Scarpella et al. 2006), generation and development of reproduction organs (Aloni et al. 2005; Pagnussat et al. 2009) and

maturation of the fruit (Østergaard 2009; Nishio et al. 2010), seeds and embryos within them (Friml et al. 2003).

4.3 Auxin perception

Each cell is capable to work with auxin signal and reacts to it on its own, in its own specific manner depending on positional and ontogenic context. The strength of signal in any part of the plant body can be conceived as a result of overlay of two "maps" of patterns (Kieffer 2010). First would be the auxin responsiveness map, which is determined by identity and activity of present components of auxin reception components, and the second being a map of IAA distribution within the plant (Petersson et al. 2009), which defines availability of auxin signal at given point. Both maps of patterns are subject of never-ending changes and of complex but yet flexible regulation with various degrees of feedbacks and mutual intersection (Vanneste and Friml 2009).

The intensity and mode of response on cellular level is determined by transcriptional regulation of the components of various signaling pathways, which can be present. There are several independent systems of auxin reception so far reported.

The gene expression-related auxin perception is utilizing the components of the nucleus-residing signaling pathways mediated through the TIR1 auxin receptor (Dharmasiri et al. 2005a and 2005b, Kepinski and Leyser 2005). TIR1 (TRANSPORT INHIBITOR RESISTANT 1) is the subunit of the $SCF^{TIR1/AFB\ (Auxin\ F\text{-}box)}$ complex. Auxin acts as molecular glue between TIR1 receptor and Aux/IAAs repressors (Tan et al. 2007); afterwards Aux/IAAs are targeted to degradation in a proteasome. The degradation of the repressors leads in turn to potentiation of the Auxin Response Factors (AR)-mediated transcription of the auxin-responsive genes.

However, auxin signaling utilizes also pathways that are independent of transcriptional regulation. Besides a yet unknown pathway involved in the regulation of PIN protein cycling (Paciorek et al. 2005), fast auxin response can be conveyed through reception by the Auxin Binding Protein 1 (ABP1). Albeit there is a progress in our understanding the role of ABP1 in regulation of vesicle trafficking (Robert et al. 2010), its precise function is not known yet. Interestingly, recently one more auxin perception system was suggested: According to Yin et al. (2009) auxin might be

perceived also by the Golgi-localized GLP4. Nonetheless, the role of GLP4 is not yet clear.

The strength of auxin signal conveyed to the receptor systems of the cell is determined also by availability of the active form of the signal molecule. Pool of active auxin molecules is prone to tight regulation through many sub-processes, so that the final physiological concentration of signaling molecule at given point is achieved. However, even if physiologically very important, the knowledge about this regulation is only scarce.

4.4 Auxin homeostasis

As unique, dynamic and asymmetric distribution of auxin within the body plan is required, it is controlled by very tightly regulated processes. There are two possible basic processes which both contribute to the establishment and modulation of homeostasis of any compound: metabolism and transport (Fig. 1). Auxin metabolism involves biosynthetic, conjugation/deconjugation and degradation reactions (reviewed in Woodward and Bartel 2005) which altogether result in specific auxin metabolic profile in particular cell (and its compartment) at particular time point. The complete pool of auxin molecules in each cell is modified and eventually controlled through combined contribution from auxin metabolism, it's transport and spatiotemporal feedback system from the auxin signaling in response to the pre-existing cell patterns and environmental cues (Vanneste and Friml 2009).

All the sub-processes involved in auxin homeostasis are amazingly complex, with high degree of redundancies. To the auxin production several biosynthetic pathways contribute. Auxin transport (from, to and within cell) is operated by plethora, more or less independently regulated systems and is driven by several transport protein families. Much less is known about auxin-(de)conjugation and degradation reactions as well as about particular enzymes involved; however, it is obvious that these reactions are very much species-dependent (Cooke et al. 2002, Bajguz and Piotrowska 2009).

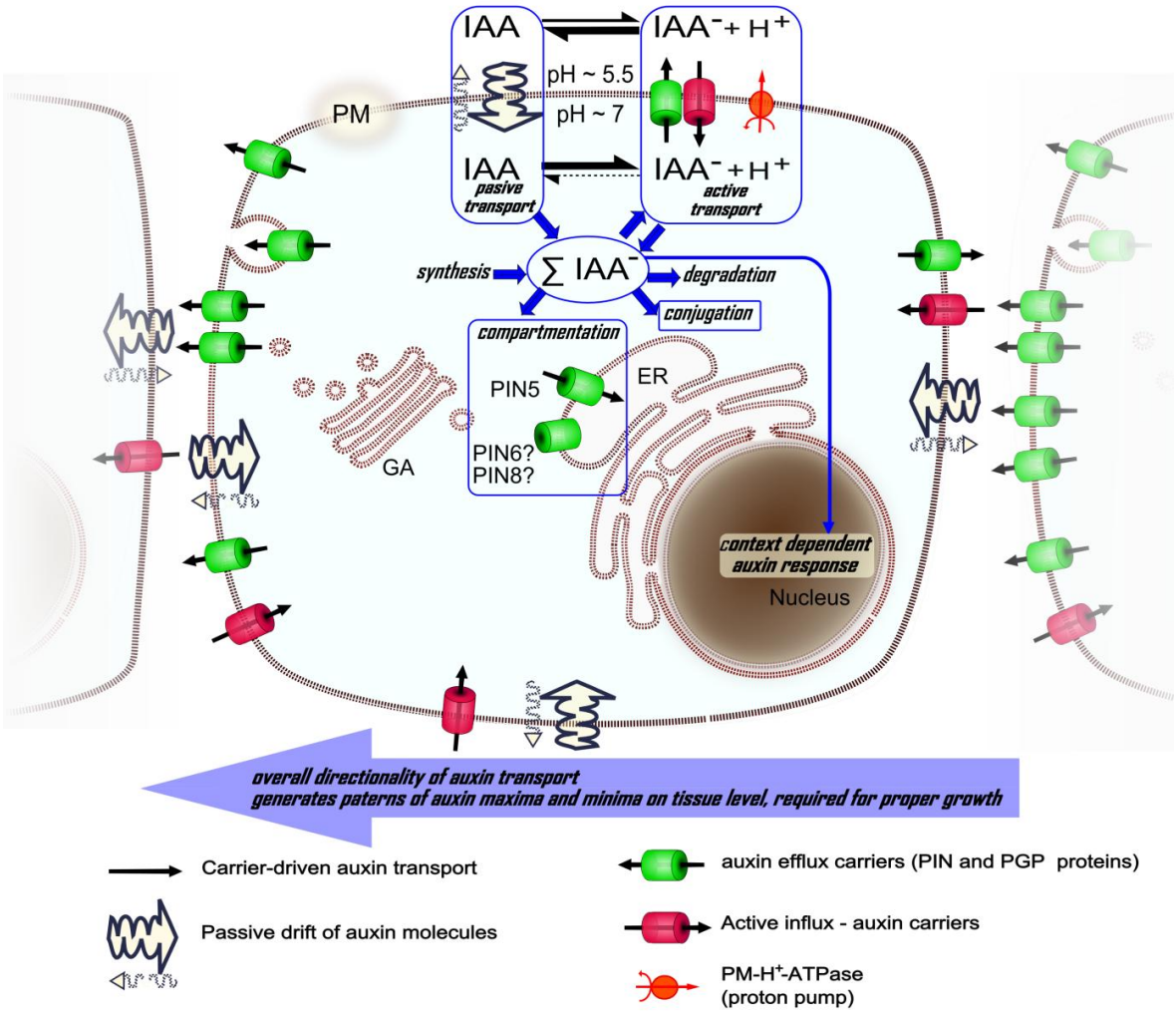

Figure 1. Schematic illustration of processes involved in management of auxin pool at cellular level. PM, plasma membrane; ER, endoplasmic reticulum; GA, Golgi apparatus; Context dependent auxin response is dependent on tissue- and cell-specific modulation of auxin responsiveness (drawing modified from Křeček and Skůpa et al. 2009, and Friml and Jones, 2010).

4.4.1 Auxin biosynthesis

For a long time, it was believed that the shoot tip is the preeminent source of auxin, which is subsequently transported basipetally (rootwards – Baskin et al. 2010) into the rest of the plant. The highest rate of auxin synthesis was later found to be in the youngest leaves, just under the new developing leaf primordia (in *Arabidopsis thaliana* just 0.5 mm long) (Ljung et al. 2001). High amount of auxin helps to establish new vasculature in the new leaves, supports the attachment of the newly founded vasculature of the leaves to the preexisting vasculature in the stem, and continuously,

auxin molecules are released into the passive flow of the phloem sap streaming rootwards. The predominant source of auxin in the tip of growing shoot helps to determine the overall polarity of the plant body and ultimately influences processes even at the other pole of the plant body. Ljung at al. (2001; 2005) had shown that along with the shoot tip, the root and young embryo are also sites with higher rates of auxin biosynthesis.

Ultimately it seems that every cell within plant body upholds the competence to synthesize IAA molecules on its own through some of the pathways known (Peterson et al. 2009), but the actual rate of synthesis is vastly differentiated and strictly dependent on the developmental context; it is also temporally and spatially regulated (review by Zhao 2010). Local auxin biosynthesis actually contributes '*de novo*' to the generation and maintenance of local auxin gradients (reviewed in Zhao 2008; Chandler 2009) and represents key regulatory factor for developmental processes such as female gametophyte development in *Arabidopsis* (Pagnussat 2009).

The native production of IAA in plants is either tryptophan-dependent (with indole-3-pyruvic acid, indole-3-acetamide, indole-3-acetaldoxime, and tryptamine as main intermediates; reviewed recently in Zhao 2010 and Normanly 2010) or tryptophan-independent (with either indole-3-glycerol and/or indole as auxin precursors; reviews by Woodward and Bartel 2005). Of those several pathways, none was fully uncovered so far; however, it seems that especially the YUC pathway appears to be the key auxin production pathway as the YUC genes are highly conserved throughout the whole plant kingdom (reviewed in Zhao 2010).

While the stream of auxin within the plant body is permanently supplemented by active biosynthesis, most of the IAA in the cell is actually not present in its active form.

4.4.2 Auxin conjugation/deconjugation and degradation

According to Normanly et al. (1993) and Cooke et al. (2002), only a small fraction of IAA is in its free form (estimated from 1% up to 25% (Ludwig-Müller 2011) depending on tissue and plant species studied). Most of the IAA molecules (up to 90%) are in the form of amide conjugates and the rest (9% by Cooke et al. 2002) as ester conjugates. As for amide conjugates, broad spectrum of them had been identified in variety of different plant species (Ludwig-Müller 2011).

The role of auxin conjugation/deconjugation in auxin homeostasis had been subject of various evolutionary studies (Sztein et al. 1999; Cooke et al. 2002; Ludwig-Müller 2011; De Smet et al. 2011) showing, that while there is a significant shift in the amount of conjugated molecules with evolution, the auxin conjugation as such is an ancient way to regulate auxin physiological activity and to control pool of active (free) auxin in the cell. The capacity to deactivate IAA through conjugation increases in time and with increased complexity of the body; in evolutionary more advanced species it becomes more dominant and its regulatory role changes and evolves in time (Sztein et al. 1999; Cooke et al. 2002 and 2003).

From the regulatory point of view, the role of auxin conjugation can be divided into two different outcomes. While the amide conjugation to the aspartate and glutamate serves as the first step of the degradation route for auxin molecules, both the ester conjugation and amide conjugation to at least alanine and leucine is reversible and the IAA-glc, IAA-Leu and IAA-Ala are generally thought/considered to be the auxin-storage forms. The major downstream or directly oxidative catabolic products of IAA are oxindole-3-acetic acid (oxIAA) and oxIAA-glucose (Kai et al. 2007; Petersson et al. 2009)

Interestingly orthologs of genes for the IAA-amino acid amidohydrolases ILR1, IAR3 and ILL1 had been shown to be all present even in the all investigated algae of the evolutionary very basal clade of green algae *Chlorophyta* (De Smet et al. 2011). Although the corresponding proteins had not been functionally characterized yet, conservation of the sequences across such an old division of plant-related organisms suggests that auxin conjugation/deconjugation is probably one of the primordial mechanisms to control auxin homeostasis.

With increasing complexity of the body plan of arising new species in the plant evolution, auxin presumably had recruited wider and wider spectrum of regulatory roles, to underpin the complex growth plasticity known from present flowering species; in parallel, the processes modulating auxin activity in the cell were getting more and more complex. Until recently, the subcellular localizations of components of auxin metabolism machinery did not draw any significant attention in the scientific community and subcellular localizations of most of the components has yet to be specified. Still, according to the present knowledge, some of the enzymes involved in auxin metabolism are actually known to have distinct subcellular localization (Woodward and Bartel 2005; Bartel and Fink 1995), suggesting that also auxin

compartmentalization may have significant role in both regulation of the cellular auxin pool and the developmental outcome for the organization of the plant body plan.

4.4.3 Auxin compartmentalization

When auxin molecules cross the plasma membrane and enter the cell interior, they immerse into the environment of cytoplasm. There, due to the higher pH compared to the cell wall, auxin molecules tend to dissociate rapidly into auxin anions and protons. Because charged anions cannot cross any cellular membranes freely, auxin cannot leave cytoplasm. This so-called "acid trap" phenomenon creates a prerequisite for the chemiosmotic theory (discussed below); however, it also raises the question, why should the components of auxin metabolism and signaling be compartmentalized, if the molecules of auxin cannot reach them in cell compartments. Recently, it was shown that IAA compartmentalization can be mediated through the newly found auxin transporters localized on membranes of the endoplasmic reticulum (ER) – the paper by Mravec et al. (2009; enclosed in the Chapter 6.2 of this thesis) characterized *At*PIN5, one of the non-canonical members of the PIN-FORMED (PIN) family, and proved it to mediate the intracellular auxin redistribution between cytoplasm and the lumen of the ER.

4.5 Auxin transport

The most unique of all the processes modifying auxin levels on both cellular and all higher levels is (polar) auxin transport. Although every cell in the plant body is theoretically capable to synthesize auxin on its own, it is often auxin transport, which has the decisive role in governance of the developmental pattern in plant body. In large picture, auxin is moving from growing tips of the shoots down to hypocotyl and to the growing tips of the roots.

In its movement, auxin exhibits properties with some analogy to both the hormones and morphogens (Teleman et al. 2001, and above). Generally, transport of auxin follows innate polarity of the plant body. On the long distance in the rootward direction (from the predominant source in the shoot tips to the tips of roots) significant portion of auxin molecules moves passively downwards, being carried by sap flow in the phloem vessels (Baker 2000). Auxin transported this way, is

representing long distance signal, ultimately influencing the response and overall architecture of the roots on the other than shoot pole of the growth axis.

In parallel to the above mentioned passive transport, in plants one more transport phenomenon exists: It is the active, cell-to-cell and carrier driven transport (the polar auxin transport, PAT). This is a directional and directed flow of auxin molecules through cells, to which probably every living cell in plant body contribute. It is this mostly directional movement of auxin through neighboring cells, which has serious consequences on creation and maintenance of local auxin foci and gradients with morphogenic-like properties (Friml and Palme 2002; Friml et al. 2002a; Friml et al. 2002b; Benková 2003). Polar auxin transport is an intricate process that runs via combination of passive diffusion of auxin into cell and carrier driven auxin uptake and efflux, to and from each of the cells.

Auxins are weak organic acids, so, in extra-cellular pH (ca. 5.5) auxins are partly non-dissociated (protonated). As long as the IAA molecules remain in their protonated form, they lack dipolar moment, which would prevent them from crossing the cellular membranes. As a result, there exists significant diffusion from apoplast through PM passively along concentration gradient (Fig. 1 – passive drift of auxin molecules) into the cell interior. However, at conditions of the higher pH inside cells (ca. 7.0), molecules of auxins almost completely dissociate and these auxin anions are polar, trapped in the cell and they are unable to leave cell without protein-mediated transport (Fig. 1).

On this basis, Rubery and Sheldrake (1974) together with Raven (1975) formulated convincing model - the chemiosmotic theory (summarized by Goldsmith and Goldsmith 1977), explaining possible molecular basis for PAT. So, according to the chemiosmotic theory, specific auxin efflux carriers were postulated to export trapped auxin ions out of the cell. These unevenly localized auxin efflux carriers have been believed to be the driving force behind polar auxin transport throughout plant tissues, because preferential transport activity on one side of a cell would give directionality to the flow of auxin from cell to the other one and thus subsequently through file of adjacent cells.

At present, several protein families of carriers exhibiting auxin influx or efflux activities are studied (recent reviews by Petrášek and Friml 2009; Zažímalová et al. 2010).

4.5.1 Auxin influx carriers

As noted above, protonated auxin molecules cross PM passively; however, in their anionic form auxins may also enter the cell by the action of auxin influx carriers. For the auxin influx carriers there seems to be one main candidate family – auxin permease-like AUX1/LAX proteins (Bennett et al. 1996; Kerr and Bennett 2007). These carriers are electrogenic, proton-symporters, thus they are capable of importing auxin anions into cytosol against a concentration gradient. To date, four influx carriers of the AUX1/LAX family have been described in *A. thaliana* and several others in other plant species. AUX1 and LAX3 transport function was demonstrated in the studies by Yang et al. (2006) and Swarup at al. (2008), respectively, showing import of radiolabeled IAA into heterologous *Xenopus* cells.

The AUX1 facilitates loading of auxin into the phloem of leaves (Marchant et al. 2002), in shoot AUX1 localizes in L1 layer cells to the PM (Reinhardt et al. 2003). In roots AUX1 is localized in cells of stele, columella and lateral root cap. Interestingly, Swarup et al. (2001) showed asymmetrical localization of AUX1 on the PM of protophloem cell files, albeit non-polar localization in epidermal cells (Swarup et al. 2001) and anywhere else was reported. AUX1, LAX1 and LAX3 had been all demonstrated to be essential for proper formation of lateral roots, in different stages (Marchant et al. 2002; Swarup et al. 2008). The data described above shows AUX1/LAX protein family as functional influx carriers, with substantial developmental role.

Besides AUX1/LAX proteins, ABCB4 of the ABCB transporter family (see below) has been characterized as auxin importer (Terasaka and Blakeslee et al. 2005), and recently it has been suggested to have the ability to switch directionality of its auxin transport function in concentration-dependent manner (Yang and Murphy 2009; Kubeš and Yang et al. submitted). Moreover, in the context of lateral roots development, the nitrate transceptor (transporter/sensor) NRT1.1 has been shown recently to also import auxin molecules into the cells (Krouk et al. 2010).

4.5.2 Auxin efflux carriers

In the search for putative auxin efflux carrier, two families of proteins had attracted most of the attention recently. The two main candidate protein families for auxin efflux carriers are plant specific PIN-FORMED proteins (PINs) (Petrášek et al.

2006; reviewed in Zažímalová et al. 2007, Křeček and Skůpa et al. 2009 for included publications see chapter (6.3); Zažímalová and Murphy et al. 2010) and ATP-BINDING CASSETTE subfamily B (ABCB)/P-GLYCOPROTEIN (PGP)/MULTIDRUG RESISTANCE (MDR) transporters (Noh et al. 2001; Titapiwatanakun 2009; Mravec 2008).

4.5.3 Characterization of PIN proteins' transport function

Mutant later named *pin1* was isolated in the mutant screen run by Okada et al. (1991), based on their unusual inflorescence morphology. *pin1* lacked the ability to produce floral organs properly. The impaired inflorescences finished their development by formation of knitting-needle-like structures, which gave the name PIN-FORMED (PIN) to the whole protein family later. Interestingly, application of auxin transport inhibitors on wild type plants mimicked phenotype of *pin1* (Okada et al. 1991). The work of Gälweiler et al. (1998) characterized PIN1 protein and the results confirmed that it might fulfill the role of the transporter proposed in the chemiosmotic theory model (see above). Significantly, topology prediction for PIN1 proteins revealed two transmembrane domains with one large hydrophilic loop between them and PIN1 proteins were shown to be localized polarly on the basal (rootward) ends of the cells. These findings are well in correspondence with properties predicted by chemiosmotic theory and with the observed directionality of auxin flow. In the same year, *agr1*, *eir2* and *waw6* were characterized by several independent author teams, and they were shown to be allelic mutant genes to another member of the PIN protein family – PIN2 (Luschnig et al. 1998; Utsuno et al. 1998; Müller et al. 1998; Chen et al. 1998). Luschnig et al. (1998) noted the 35-40% similarity to bacterial transport proteins. With continuing research it became more evident, that uneven distribution of PIN proteins on PM corresponds to the predicted auxin flows and as such, the PIN proteins were for a long time the only candidates for the polarly localized auxin transporters (Friml et al. 2002a; 2002b; Friml et al. 2003; Benková et al. 2003; Reinhardt et al. 2003) and studies showed that the same PIN protein in different cells can acquire different polarities suggesting existence of cell type-specific signals (Friml and Palme, 2002). Later, the direct experimental evidence confirmed that the polarity of auxin flow is determined by the asymmetrical distribution of auxin efflux carriers of PIN-type on the PM (Wisniewska et al. 2006). Gradually, it also became evident, that polar localization as well as the activity of PINs is tightly regulated on multiple levels – from regulation of

gene expression, through control of various modifications of the protein itself, vesicle trafficking, recycling between PM and cell interior, polar localization, direct biochemical regulation of their activity *in situ*, up to their degradation (reviews by Zažímalová et al. 2007 – enclosed in the Chapter 6.3 of this thesis; Benková and Hejátko 2009; Friml 2010; Peer et al. 2011).

Paper by Petrášek et al. (2006 - enclosed in the Chapter 6.1 of this thesis) clarified the role of the PIN proteins in auxin efflux machinery; it showed that PIN proteins are rate-limiting factors of the cellular auxin export. Expression of PIN proteins alone in non-plant systems, in yeast and human HeLa cells, was sufficient to create increased auxin efflux in those systems, and the degree of auxin efflux from tobacco BY-2 and *A. thaliana* cells corresponded to the degree of *PIN4*, *PIN6, PIN7,* as well as *PIN1* expression, respectively, in these model systems. These results provided the evidence for the direct auxin-transporting activity of the PM-located PINs.

4.5.4 PIN protein family

The PIN-FORMED (PIN) protein family consists of plant specific transmembrane proteins. Transport specificity towards different compounds in the auxin class varies. Predominantly, and not surprisingly, they are good transporters for the native auxin IAA, and also for closely related, synthetic auxin naphthalene-1-acetic acid (NAA) (Delbarre et al. 1996). However, some synthetic auxin-like compounds structurally related to 2,4-dichlorophenoxy acetic acid (2,4-D) are only poor substrates for PIN proteins (Simon et al., submitted).

In *Arabidopsis* this family has eight members (reviewed and discussed by Paponov et al. 2005; Zažímalová et al. 2007; Křeček and Skůpa et al. 2009 – the latter two papers are enclosed in the Chapter 6.3 of this thesis). According to phylogenetic analyses incorporated in the above mentioned papers - with increasing degree of detail and certainty - the family should be divided into two subclades (Fig. 2).

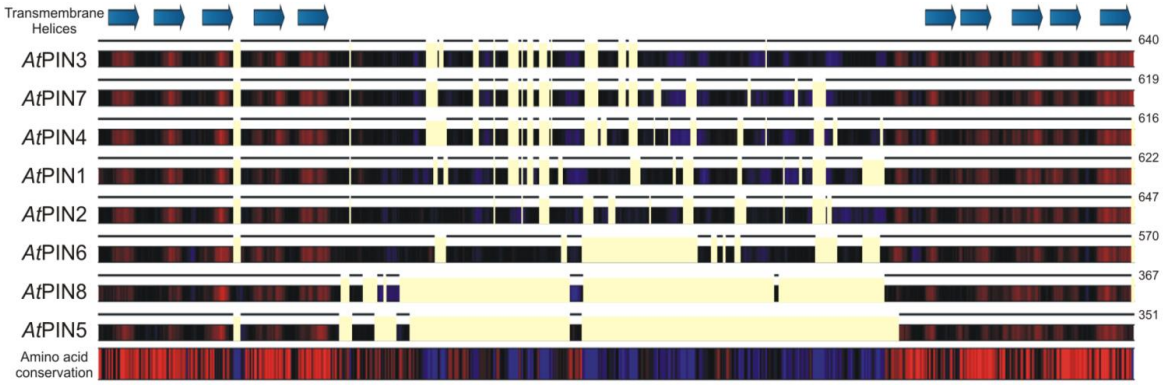

Figure 2. Alignment of *Arabidopsis* PIN protein sequences.

Arabidopsis PIN family comprises of eight members with variable length of their sequences. Sequences are represented with interrupted lines aligned according to their sequence similarities. Number of amino acid residues for each particular PIN is indicated by a small number to the right of the sequence line. Transmembrane segment position is indicated by blue arrow above the alignment. These segments of proteins present the most conserved part of all PINs. PIN protein sequences vary predominantly in the central hydrophilic region, with significant deletion/insertion as evidenced for PIN5 and PIN6.

Overall amino-acid conservation along the protein sequence is displayed in status bar bellow the alignment with red to blue scale to represent degree of conservation. In this bar, red colour represents the most conserved residues of the sequence, blue one the least.

Kyte-Doolittle Hydropathy plots (Kyte and Doolittle 1982) were used as indicators for hydrophobicity over the length of each protein sequence. Blue to red scale represents degree of hydropathy used in plot, with maximal hydrophobicity being indicated with red, minimal with blue.

According to Křeček and Skůpa et al. (2009), the first subclade in Arabidopsis lists PIN1–PIN4 and PIN7. This sub-clade of PIN proteins represents the “classical (canonical)” PINs, characteristic with their mostly asymmetrical localization on PM. Functionally, they are auxin efflux carriers driving cellular auxin efflux, and thus determining the process of intercellular polar auxin transport. Structurally, they all share long hydrophilic loop placed between the two hydrophobic regions, each composed of five transmembrane segments. Hence, they are referred to as ’long’ PINs. PIN6 is somewhat exceptional as it has its central hydrophilic loop markedly reduced and is most probably localized in the endoplasmic reticulum (Mravec et al. 2009 – enclosed in the Chapter 6.2 of this thesis); however, due its high sequence similarity in transmembrane regions, phylogenetic analysis conducted in Křeček a Skůpa (2009 – enclosed in the Chapter 6.3 of this thesis) placed PIN6 into ‘long’ PINs – i.e. along the “canonical” ones.

The second sub-clade is evolutionary distinct within PIN-FORMED family. In Arabidopsis, this subclade has either two or three members, depending on whether PIN6 is additionally included along with PIN5 and PIN8 to one group as in Mravec et al. (2009 – enclosed in the Chapter 6.3 of this thesis). In so far published phylogenetic studies of the PIN family (Paponov et al. 2005; Zažímalová et al. 2007; Mravec et al. 2009; Křeček and Skůpa et al. 2009; Miyashita et al. 2010; Nishio et al. 2010; Palovaara et al. 2010; De Smet et al. 2011), there is no clear consensus regarding the placement of PIN6. Proteins of this sub-clade are almost completely (PIN5 and 8) or partially (if PIN6 is included) lacking the typical central hydrophilic loop, and so they are dubbed 'short' PINs. As mentioned above, portion of internal loop in *At*PIN6 has been preserved and also sequence similarity in transmembrane regions is placing it evolutionary and probably functionally closer to the 'long' PINs.

Structurally there is higher variability in the sequences of PIN5 and PIN8 then in those of 'long' PINs (including PIN6). This indicates that the positions that are invariant only in long PINs must be crucial for some important function of long PINs that has not been retained in 'short' PINs (Křeček and Skůpa et al. 2009).

PIN5 was shown to have unparalleled role in mediating homeostasis and availability of the active auxin inside cell (Mravec et al. 2009 – enclosed in the Chapter 6.3 of this thesis). Based on overexpression in yeast cells, PIN5 was confirmed to be functional auxin-transport protein, with presumed direction of transport of auxin into the lumen of ER in plant cells. Here, in the ER the metabolic fate of free IAA molecules is changed due to their deposition to the compartment presumably with different auxin-metabolizing enzymatic system. All these features suggest that 'short' PINs differ significantly from those possessing typical large hydrophilic loop in their molecules, in both the subcellular localization and developmental role. Nevertheless, it also shows that PINs' transmembrane domains are still sufficient to execute auxin transport without support of the hydrophilic loop. PIN proteins had been found in all fully sequenced species of terrestrial plants but not in any of the so far sequenced unicellular species of green algae (Křeček and Skůpa et al. 2009) – see Fig. 3. Nevertheless, recently De Smet et al. (2011) analyzed EST library of much evolutionary closer, filamentous, Streptophyta algae *Spirogyra pratensis* and found a PIN ortholog with short hydrophilic loop, reminiscent of the PIN5-like subclass of the Arabidopsis PIN proteins. This might be seen as illustration of the initial transition step in the

evolution of PIN proteins, as predicted in Zažímalová et al. (2007) for Streptophyta algae.

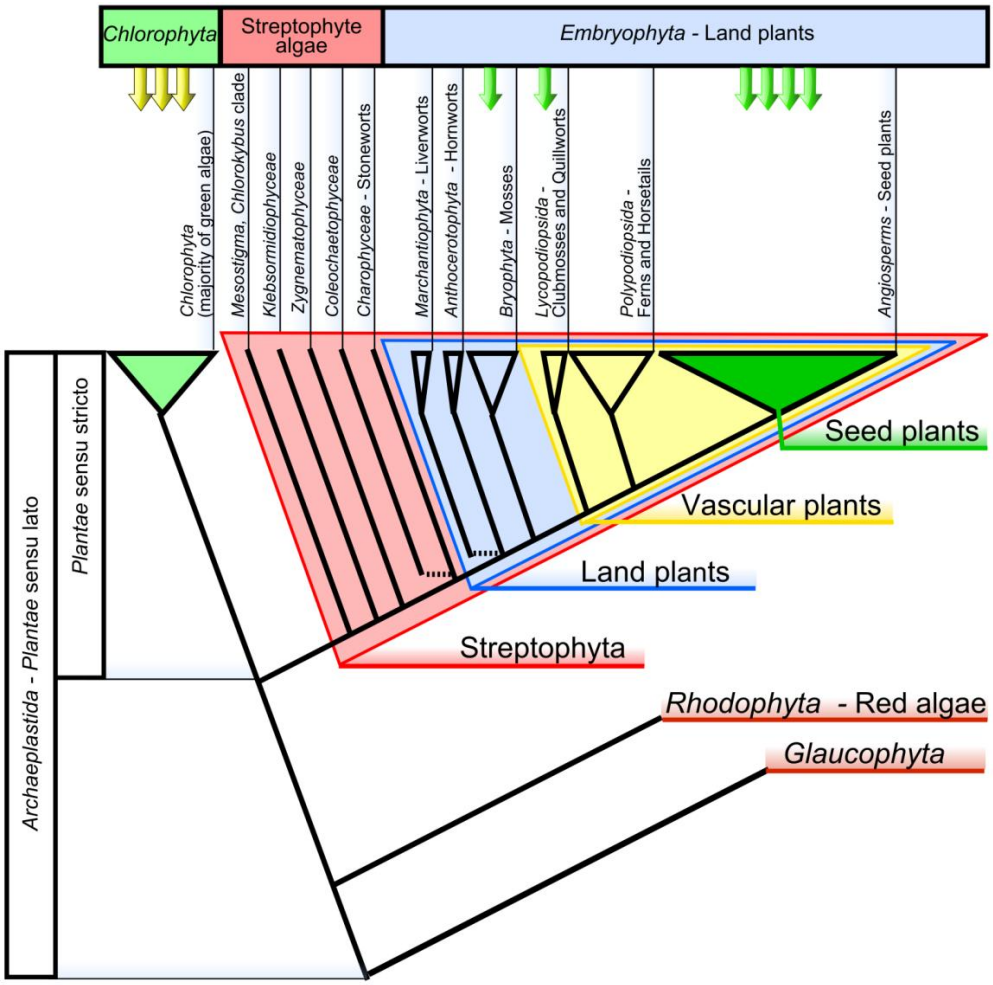

Figure 3. Simplified evolutionary cladogram of the Plantae illustrates the known distribution of PIN sequences in the plants and its direct ancestors. Yellow arrows represent species within the evolutionary branch with fully sequenced genome lacking PIN-like sequence; green arrows represent fully sequenced genomes of species with at least one copy of PIN-like sequence. (Figure adopted from Křeček and Skůpa et al. 2009).

All the above mentioned data point at the significance of PIN proteins for both proper genetically-based and environmentally-conditioned plant development. Unpredicted functional division within the PIN protein family, together with the developmental diversity within 'long' PINs point out the emerging need to understand not only molecular function of PINs but also, generally, the evolutionary context of auxin transport evolution, and phylogenetic relationships between PIN protein sequences in particular.

5 MATERIAL AND METHODS

5.1 Chemicals

All chemicals were obtained from Sigma (St. Louis, MO, USA) unless stated otherwise. 1-naphthylphthalamic acid (NPA) was obtained from OlChemIm (Olomouc, Czech Republic). NPA was dissolved in ethanol to give 10 mM stock solution. Dexamethasone (DEX) was dissolved in DMSO to give 30mM stock solution. For accumulation and metabolic assays following radiolabeled compounds were used: [^{3}H]IAA, [^{3}H]2,4-D and [^{3}H]benzoic acid (specific radioactivity 20 Ci/mmol each, American Radiolabeled Chemicals, ARC, Inc., St. Louis, MO, USA), and [^{3}H]NAA which was either supplied from ARC with the same properties as compounds described above or it was synthesized at the Isotope Laboratory, Institute of Experimental Botany, Prague, Czech Republic (specific radioactivity 25 Ci/mmol).

5.2 Plant material and cultivation conditions

BY-2 tobacco cells (*Nicotiana tabacum* L., cv. Bright Yellow-2; Nagata et al. 1992) were used as the original material for all experiments. Tobacco BY-2 cells were stably transformed by Arabidopsis cDNA of PIN4, PIN5 and PIN6. BY-2 cells were transformed according to An et al. (1985) as described in Petrášek et al. (2003). 4 or 6 ml of tobacco cells were co-cultivated with *Agrobacterium tumefaciens* strains C58C1 or GV2260 containing appropriate binary vector in the presence of 20 μM acetosyringon. Constructs pTA-AtPIN4, 5, 6, carrying *At*PIN4, 5 and 6 coding sequences, respectively, cloned into the binary transformation plasmid pTA7002 (with complete two-component glucocorticoid-inducible system; Aoyama and Chua, 1997) were kindly provided by Jiří Friml and Jozef Mravec (PSB, VIB, Ghent University, Belgium).

The BY-2 cell lines were cultivated in darkness at 25°C on an orbital incubator (IKA KS501, IKA Labortechnik, Staufen, Germany; 120 rpm, orbital diameter 30 mm) in liquid medium (3% [w/v] sucrose, 4.3 g.l^{-1} Murashige and Skoog salts, 100 mg.l^{-1} inositol, 1 mg.l^{-1} thiamin, 0.2 mg.l^{-1} 2,4-dichlorophenoxyacetic acid, and 200 mg.l^{-1} KH_2PO_4, pH 5.8) and subcultured weekly. Stock BY-2 calli were maintained on media solidified with 0.6% (w/v) agar and subcultured monthly. Transgenic cells and calli

were maintained on the same media supplemented with 100 $mg.ml^{-1}$ cefotaxim and 20 $mg.ml^{-1}$ hygromycine.

Cell density was determined by counting cells using Fuchs-Rosenthal haemocytometer slide during the whole cultivation cycle. Expression of PIN genes in tobacco cells was induced in cell suspensions from regenerated cell line by the addition of DEX (1 µM, except for stated otherwise) at the beginning of the subcultivation period.

5.3 Verification of transgene presence and expression

Genomic DNA of transformed tobacco cell lines was isolated using DNeasy Plant Mini Kit (Qiagen®). The genomic fragment of particular gene was amplified with proper pair of primers. For amplification of *PIN5* combination of forward primer (5'-TGTCCCTCGTTTGGTTGAAG-3') and reverse primer (5'-CGTTCCTGTTCCTGCTTTTG-3') was used, giving product 201bp long. The combination of forward primer (5'-ACTCTATTCTCAATCGCTAC -3') and reverse primer (5'- CAAAACATGATCTCGTTACT -3') for was used for amplification of *PIN6* gene fragment giving a product of 500bp.

The expression of transgenes was examined typically in one-day old cells after induction of gene expression with DEX. If needed, cells of other age were used. Total RNA was isolated using the Plant RNA Qiagen Mini-Prep and RT-PCR performed using Qiagen® OneStep RT-PCR or Invitrogen SuperSriptII kits according to the manufacturer's protocols. *AtPIN4* gene fragment was amplified using forward primer (5'- TCGTTGTTCTTCAGTGTAT -3') and reverse primer (5'- CTCCACACCATTATCAAT -3') giving a product of 976bp. *AtPIN5* gene fragment was amplified using forward primer (5'- CCGCTCGAGGAAGCTTTGTTTTCTTTCCTTTCGT-3') and reverse primer (5'-AAGAAGATCCTCCTCAGAAT-3') giving a product of 1200bp. *AtPIN6* gene fragment was amplified using forward primer (5'- ACTCTATTCTCAATCGCTAC -3') and reverse primer (5'- CAAAACATGATCTCGTTACT -3') as above.

5.4 Immunocytochemistry

Modified indirect immunofluorescence protocol for tubulin visualization, described in Petrášek et al. (2003) was used for visualization of PIN4, 5 and 6 in tobacco cells. BY-2 cells in exponential growth phase were used if not stated otherwise. Cells were filtered through nylon mesh attached to small container (pore size 20 µm), one container per sample, and all following manipulations took place in the containers.

Scheme of a container is shown in Nick et al. (2000). Cells were prefixed 20min with 120μM MBS (m-maleinimido-benzoyl-N-hydroxy-succinimide ester) and 40min with 3.7% (w/v) PFA (paraformaldehyde) in a buffer consisting of 50 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$, PMSF (200 μM), 1% Triton X-100 (w/v) pH 6.9, at 25°C. After three-times repeated wash-out of the cells with stabilizing buffer (SB) (50 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$), cells were treated for 7 min with the enzyme cocktail (1% (w/v) macerozyme, 0,05% (w/v) pectolyaze and inhibitors of proteases) followed by another three-times 10 min wash-out in SB. After treatment with 0.5% (w/v) bovine serum albumin in PBS (PBSA) for 30 min, cells were transferred into Eppendorf tubes and incubated with polyclonal rabbit antibodies in PBSA against PIN4 and 6 at 1:2000 or monoclonal mouse anti-c-Myc antibody (Sigma) at 1:500 for PIN5 lines. After three-times repeated 10 min washing in PBS, the secondary antibody was applied for 1 h. Optionally 0.2 μl of DNA-staining solution was added and cells were observed immediately.

5.5 Microscopy and image analysis

For the determination of cellular dimensions and phenotypes, microscope Nikon Eclipse E600 equipped with Nomarski DIC was used. DIC images were grabbed with digital camera (DVC 1310C, USA) and digitally stored. Lucia image analysis software (Laboratory Imaging, Prague, Czech Republic) was used for measurement of cell length and diameters, 100 cells per sample were interactively measured and displayed in dot plot as values representing distribution of cellular dimensions in the sample. Nikon Eclipse E600 epifluorescence microscope and Zeiss LSM 5 Duo confocal microscope (Carl Zeiss, Jena, Germany) both equipped with filter sets and/or excitation/emission settings appropriate for particular secondary antibodies, were used for observation of immunofluorescently labeled cells.

5.6 Auxin accumulation assays

Auxin accumulation in suspension-cultured tobacco BY-2 cells was measured as described in Petrášek et al. (2003), originally modified from the protocol by Delbarre et al. (1996). From each cell suspension, cultivation medium was removed by filtration through nylon cloth (20 μm mesh), cells were re-suspended in the uptake buffer (20 mM MES, 40 mM sucrose, and 0.5 mM $CaSO_4$, pH adjusted to 5.7 with KOH), and equilibrated for 45 min under continuous orbital shaking. After 45 min the equilibrated

cells were re-suspended in fresh uptake buffer, and incubated on the orbital shaker for another 1.5 h in darkness at 25°C. The density was precisely adjusted to $6x10^5$ cells/ml. At the beginning of the assay, radiolabeled auxins were added to the cell suspension to give a final concentration of 2 nM. Labeled [^{3}H]IAA or [^{3}H]2,4-D or [^{3}H]NAA were used. The accumulation was then measured in 0.5-mL aliquots of cell suspension at certain measured time points. Aliquots of suspension were taken from the batch and accumulation of radiolabel in the aliquot was terminated by rapid filtration under reduced pressure on 22-mm-diameter cellulose filters. Filters with the cells were transferred to scintillation vials, extracted in ethanol for 30 min, and radioactivity was determined by liquid scintillation counting (Packard Tri-Carb 2900TR scintillation counter, Packard Instrument Co., Meriden, CT) and corrected automatically for quenching error. Inhibitors (namely NPA, at final concentration typically 10 μM) were added from stock solution at the time zero (just after addition of radiolabeled auxin) in volumes required for final concentration. Relative radiolabel retention was measured as a radioactivity retained inside cells at particular time points after addition of the labeled auxin and expressed as pmols of the accumulated auxin per million cells. Values of accumulation were also corrected for surface radioactivity by subtracting counts obtained for aliquots of cells collected immediately after the addition of labeled auxin. All measurements were done at least in triplicates and calculated standard errors of the mean have been shown.

5.7 IAA metabolic profiles

Twenty four hours after inoculation, cells were prepared for the experiment by equilibration protocol identical to the treatment of the cells for the accumulation essay. Afterwards, cells were incubated in 20 nM [^{3}H]IAA under standard cultivation conditions for the period of 2.5 h. Then, cells were collected and frozen (200 mg of fresh weight per sample) in liquid nitrogen and stored until extraction. Extraction and purification of auxin metabolites was performed as described in Dobrev and Kamínek (2002). Briefly, samples of BY-2 cells, 200 mg each, were transferred to Eppendorf tubes, then 1.5 ml of cold Bieleski's solution was added; cells were homogenized using Retsch homogenizator at 30 Hz for 4 min. After overnight incubation of the homogenized samples at -20°C the supernatant was collected by twice repeated centrifugation at 18000 rpm (at 4°C; Jouan AM2.18, France). Supernatant collection process was repeated with pellet newly re-suspended in 1ml of cold Bieleski's solution,

then supernatants were blended and force-evaporated. Dry fraction after the evaporation was diluted in 5 ml of 1 M formic acid and purified on an Oasis MCX mixed mode, cation-exchange, reverse phase column (Waters, 6 cc/150 mg). IAA metabolites were eluted from the columns by 5 ml of methanol and evaporated to dryness.

For the following HPLC analysis, samples were dissolved in 20 µl of 50% (v/v) acetonitrile and diluted by 80 µl of distilled water. The radioactive metabolites of [^{3}H]IAA were separated on HPLC, using two methods. First one was pilot method with lower resolution. For this method column Luna C8(2), 50x4.6mm, 3µm (Phenomenex, Torrance, CA) was used with mobile phase A: 40 mM HCOOH adjusted with NH_4OH to pH 3.0. The second method was with higher resolution with column Luna C18(2), 150x4.6, 3µm and mobile phase A: 40 mM CH_3COOH adjusted with NH_4OH to pH 4.0. The HPLC instrumentation and parameters common for both methods were: Series 200 autosampler and quaternary pump (both PerkinElmer, Wellesley, MA), column heating at 35°C and two detectors coupled in series; 235C diode array detector (PerkinElmer) and Ramona 2000 flow-through radioactivity detector (Raytest, Straubenhardt, Germany). Solvent B was CH_3CN/CH_3OH, 1/1, v/v. Flow rate was 0.6 ml/min with linear gradient 30%–50% B for 10 min, 50%–100% B for 1 min, 100%B for 2 min, 100%–30% B for 1 min. The column eluate was monitored at 275 nm by the diode array detector, and after online mixing with 3 volumes (1.8 ml/min) of liquid scintillation cocktail (Flo-Scint III, Packard BioScience Co., Meriden, CT) was monitored by a Ramona 2000 radioactivity detector. The identity of IAA peak was verified and metabolites of [^{3}H]IAA were identified on the basis of comparison of their retention times with authentic standards.

5.8 Alignment of protein sequences:

Multiple sequence alignment of PIN proteins for figure 2 was constructed with program CLC Main Workbench 4,0 using cost value 10 for gap opening and 1 for gap extension. Kyte-Doolittle model (Kyte and Doolittle 1982) for hydrophobicity over the length of the protein sequence was built with window length value was set to 9. Blue to red scale represents degree of hydropathy used in plot.

6 RESULTS

Papers included in this section have in common the focus on the PIN protein family, with respect to transport function of its members, to its evolutionary history and to functional and structural diversification within the protein family.

Paper by Petrášek et al. (2006) brought direct evidence that PIN proteins are not only providing directionality to the auxin flow, but that they are real transporters with the rate-limiting role in auxin export from cells. Paper by Mravec et al. (2008) characterized complementary transport function of two groups of auxin efflux transporters - PIN carriers and ABCB transporters. Papers by Zažímalová et al. (2007) and by Křeček and Skůpa et al. (2009) represent reviews focused on PIN proteins as well as on regulation of their activity, and they contain original contribution to the knowledge on the PIN proteins structure and evolution. Paper by Mravec et al. (2009) is focused on members of the PIN protein family with reduced internal loop, predominantly on *At*PIN5, and on their until then unexpected role in establishment and/or maintenance of intracellular auxin homeostasis. Results in this section are divided topically into three chapters.

6.1 Plasma membrane-targeted PINs: a key role in polar auxin transport

This chapter encompasses two published papers:

Petrášek J, Mravec J, Bouchard R, Blakeslee JJ, Abas M, Seifertová D, Wiśniewska J, Tadele Z, Kubeš M, Čovanová M, Dhonukshe P, **Skůpa P**, Benková E, Perry L, Křeček P, Lee OR, Fink GR, Geisler M, Murphy AS, Luschnig C, Zažímalová E, Friml J. PIN proteins perform a rate-limiting function in cellular auxin efflux. SCIENCE 2006;312(5775):914-8**.** (IF 2010: 31.364)

Mravec J, Kubeš M, Bielach A, Gaykova V, Petrášek J, **Skůpa P**, Chand S, Benková E, Zažímalová E, Friml J. Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. DEVELOPMENT 2008;135(20):3345-54 (IF 2010: 6.898)

As described in the introduction (Chapter 4.5), since the chemiosmotic theory was created in 1974, predicted auxin efflux carriers became an important part of the understanding of how could auxin be unevenly and yet coordinately distributed throughout the plant. However, the knowledge on the molecular players was missing completely till the characterization of two proteins from the PIN family in 1998. Even later, their role as putative auxin efflux carriers/facilitators was not fully clear. In the papers presented, it was shown, that PIN proteins are independent auxin transporters and that ABCB- and PIN-dependent transport systems interact functionally on the system level, rather than through direct protein-protein interaction.

Both plant and heterologous overexpression systems were created by transformation with genes from the *PIN* family from *A. thaliana*. Well-established cell line of suspension-cultured tobacco BY-2 cells (*Nicotiana tabacum* L., cv. Bright Yellow-2; Nagata et al. 1992) was used as advantageous system for the study of auxin transport at the cellular level. *Arabidopsis* genes At*PIN4b,* At*PIN6a*, At*PIN7 and* At*PGP19 (*At*ABCB19)* were placed under the control of glucocorticoid-inducible system (Aoyama and Chua, 1997) and transformed into the BY-2 cells, and then transformed

cell lines were established and characterized. The overexpression of the putative auxin transporters PIN4, PIN7 and ABCB19 resulted in the increase in auxin efflux, and in changes in cell morphology characteristic for the response to auxin depletion. These changes included cessation of cell division, stimulation of cell elongation and exaggerated amyloplast formation. PIN6 showed somewhat different, less pronounced phenotype. Importantly, unlike for ABCBs, the PIN-dependent cell phenotype changes could be rescued by the well-established inhibitor of auxin efflux, NPA. *Arabidopsis* cell suspension line, derived from the *XVE-PIN1 A. thaliana* plants with estradiol-inducible overexpression system confirmed this function for the *At*PIN1 protein. Auxin-transporting capacity of PIN2 and PIN7 consistent with their auxin-transporter function were also confirmed using heterologous expression in yeast and human HeLa cells. The auxin-transport function of the PIN proteins was related to the degree of protein expression and rate-limiting. Experiments with *Arabidopsis* seedlings have shown, that PIN-dependent and ABCB-dependent auxin transport works complementary in terms of developmental effects. Taken together, these results confirmed that PIN1, 2, 4, 7, and to some extent also PIN6 are direct auxin efflux transporters, and that ABCB- and PIN-dependent transport machineries form functionally distinct auxin transport systems, which interact on the level of development, rather than through direct protein-protein interaction(s).

My particular contribution to these studies was transformation of BY-2 cells and following establishment of the suspension-grown *GVG-PIN4* and *GVG-PIN6* cell lines. Share on the characterization of these lines (measurements and description of cell growth parameters and cell morphology) as well as auxin accumulation measurements were my other tasks contributing to this work.

mobility and yield a charge of $12 \pm 2\ e^-$ per tubulin dimer under physiological conditions. This value may be important to elucidate the effect of in vivo electric forces on microtubules. Endogenous physiological electric fields, with a typical value up to 10^3 V/m, are shown to be involved in cell division, wound healing (*35*), and embryonic cell development (*36*), but their microscopic effect has so far not been understood. The application of biomotors in nanofabricated environments is an exciting development, offering novel possibilities for future developments in lab-on-chip sorting or purification applications.

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30. The use of the linear Grahame equation is strictly speaking only valid for $\zeta \ll k_BT/e = 26$ mV. However, at $\zeta = 50$ mV, the use of the linearized Grahame equation introduces an error in σ of only 14%. The use of the nonlinear version of the Grahame would invoke an unknown source of error, because we would then have to assume a value for the double-layer capacitance of the microtubule.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/312/5775/910/DC1
Materials and Methods
SOM Text
Figs. S1 and S2
References and Notes
Movie S1

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PIN Proteins Perform a Rate-Limiting Function in Cellular Auxin Efflux

Jan Petrášek,[1,2] Jozef Mravec,[3] Rodolphe Bouchard,[4] Joshua J. Blakeslee,[5] Melinda Abas,[6] Daniela Seifertová,[1,2,3] Justyna Wiśniewska,[3,7] Zerihun Tadele,[8] Martin Kubeš,[1,2] Milada Čovanová,[1,2] Pankaj Dhonukshe,[3] Petr Skůpa,[1,2] Eva Benková,[3] Lucie Perry,[1] Pavel Křeček,[1,2] Ok Ran Lee,[5] Gerald R. Fink,[9] Markus Geisler,[4] Angus S. Murphy,[5] Christian Luschnig,[6] Eva Zažímalová,[1]* Jiří Friml[3,10]

Intercellular flow of the phytohormone auxin underpins multiple developmental processes in plants. Plant-specific pin-formed (PIN) proteins and several phosphoglycoprotein (PGP) transporters are crucial factors in auxin transport–related development, yet the molecular function of PINs remains unknown. Here, we show that PINs mediate auxin efflux from mammalian and yeast cells without needing additional plant-specific factors. Conditional gain-of-function alleles and quantitative measurements of auxin accumulation in *Arabidopsis* and tobacco cultured cells revealed that the action of PINs in auxin efflux is distinct from PGP, rate-limiting, specific to auxins, and sensitive to auxin transport inhibitors. This suggests a direct involvement of PINs in catalyzing cellular auxin efflux.

Auxin, a regulatory compound, plays a major role in the spatial and temporal coordination of plant development (*1*–*3*). The directional active cell-to-cell transport controls asymmetric auxin distribution, which underlies multiple patterning and differential growth processes (*4*–*7*). Genetic approaches in *Arabidopsis thaliana* identified candidate genes coding for regulators of auxin transport, among them permease-like AUX1 (*8*), plant-specific PIN proteins (*9*) (fig. S1), and homologs of human multiple drug resistance transporters PGP1 and PGP19 (*10*, *11*). PGP1 has been shown to mediate the efflux of auxin from *Arabidopsis* protoplasts and heterologous systems such as yeast and HeLa cells (*12*). Similarly, PIN2 in yeast conferred decreased retention of structural auxin analogs (*13*, *14*). Plants defective in PIN function show altered auxin distribution and diverse developmental defects, all of which can be phenocopied by chemical inhibition of auxin efflux (*1*, *4*–*7*, *9*). All results demonstrate that PINs are essential components of the auxin transport machinery, but the exact mechanism of their action remains unclear.

Studies of the molecular function of PINs have been hampered mainly by the technical inability to quantitatively assess auxin flow across the plasma membrane (PM) in a multicellular system. We therefore established *Arabidopsis* cell suspension culture from the *XVE-PIN1* line, in which we placed the *PIN1* sequence under control of the estradiol-inducible promoter (*15*). Treatment with estradiol led to the activation of *PIN1* expression as shown by the coexpressed green fluorescent protein (GFP) reporter and reverse transcription polymerase chain reaction (RT-PCR) of *PIN1* in seedlings (Fig. 1A) and cultured cells (fig. S2). In estradiol-treated *XVE-PIN1* cells, the overexpressed PIN1 was localized at the PM (Fig. 1, B and C). The syn-

thetic auxin naphthalene-1-acetic acid (NAA) enters cells easily by diffusion and is a poor substrate for active uptake but an excellent substrate for active efflux (*16*). Therefore, change in accumulation of radioactively labeled NAA inside cells provides a measure of the rate of auxin efflux from cells. Untreated *XVE-PIN1* cells as well as nontransformed cells displayed [^{3}H]NAA accumulation kinetics indicative of saturable auxin efflux and sensitive to a well-established (*1*, *9*) noncompetitive inhibitor of auxin efflux: 1-naphthylphthalamic acid (NPA) (Fig. 1D). Estradiol did not influence control cells but led to substantial decrease of [^{3}H]NAA accumulation in *XVE-PIN1* cells (Fig. 1, D and E). This demonstrates that PIN1 overexpression leads to the stimulation of efflux of auxin from *Arabidopsis* cultured cells.

Arabidopsis cultured cells are not sufficiently friable to be useful in transport assays. Instead, we used tobacco BY-2 cells, a well-established model for quantitative studies of cellular auxin transport (*17*). PIN7, the most representative member of the subfamily including *PIN1*, *PIN2*, *PIN3*, *PIN4*, *PIN6*, and *PIN7* (fig. S1), was placed under the control of a dexamethasone (DEX)–inducible system (*18*) and stably transformed into BY-2 cells. The resulting line (*GVG-PIN7*) showed up-regulation of *PIN7* expression as early as 2 hours after DEX treatment and the up-regulated PIN7 protein was detected at the PM (Fig. 2A). Nontransformed cells displayed saturable, NPA-sensitive [^{3}H]NAA efflux, which was unaffected by DEX (Fig. 2B). Induction of expression of PIN7 or its close (PIN4) and the most distant (PIN6) homologs (fig. S1) resulted in a decrease in [^{3}H]NAA accumulation, to roughly half of the original level (Fig. 2C). The kinetics of NAA efflux after the initial loading of BY-2 cells (Fig. 2D), as well as displacement curves using competitive inhibition by nonlabeled NAA (fig. S3A), clearly confirm that PIN7 overexpression stimulates saturable efflux of auxin from cells. The efflux of other auxins—such as synthetic 2,4-dichlorophenoxyacetic acid (2,4-D) or naturally occurring indole-3-acetic acid (IAA), but not its precursor tryptophan—was also stimulated (Fig. 2, E and G). The PIN7-dependent efflux of all auxins was NPA sensitive (Fig. 2G), competitively inhibited by nonlabeled NAA, and unaffected by the structurally related but biologically inactive weak organic acid, benzoic acid (BeA) (fig. S3B). Furthermore, the increasing levels of induced PIN7, as achieved with the use of different concentrations of DEX for induction, and monitored by dot blot, clearly correlated with the gradual increase in [^{3}H]NAA efflux (Fig. 2F). These data imply that different PIN proteins are rate-limiting factors in NPA-sensitive, saturable efflux of auxins from BY-2 cells. This similarity in the molecular function of PINs, together with the diversity in their regulation, provides a basis for their complex functional redundancy observed in planta (*6*, *19*, *20*).

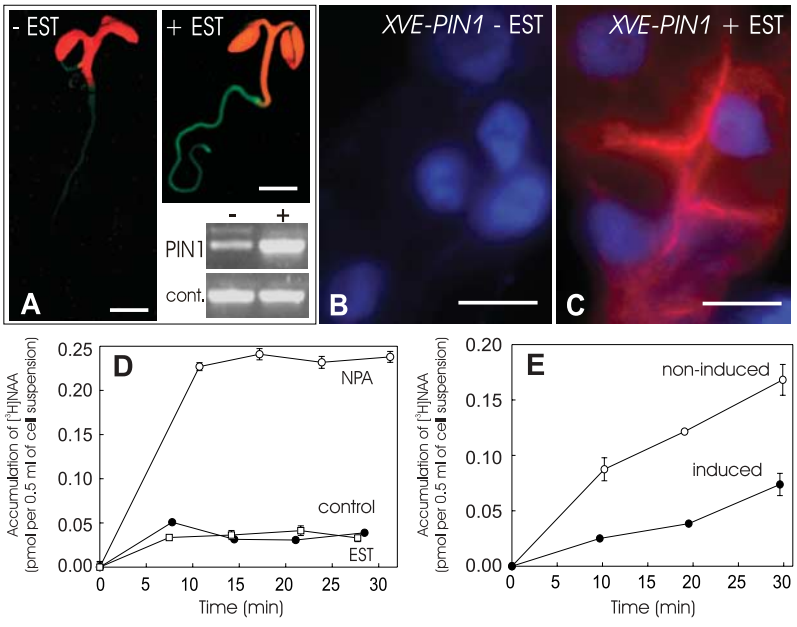


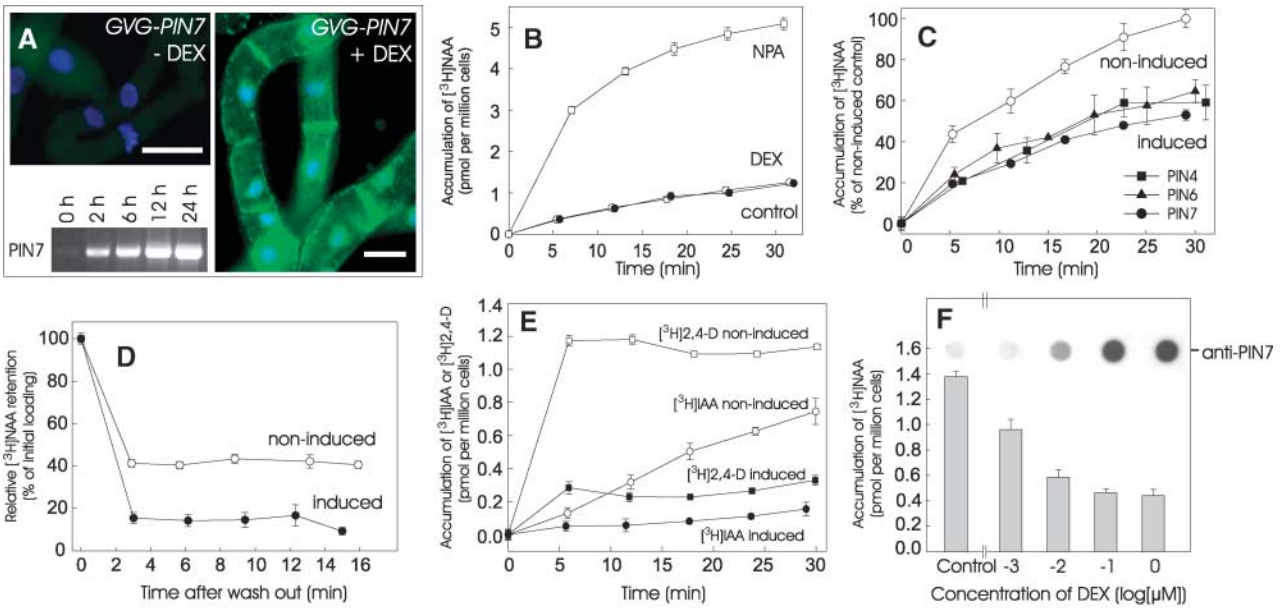
Fig. 1. PIN1-dependent auxin efflux in *Arabidopsis* cultured cells. (**A**) Up-regulation of PIN1 expression in *XVE-PIN1 Arabidopsis* seedlings after estradiol (EST) treatment (1 μM, 4 hours). The expression of coupled GFP reporter (green) and RT-PCR of *PIN1* [PGP19 expression was used as a control (cont.)] are shown. Scale bars, 3 mm. (**B** and **C**) Anti-PIN1 immunostaining (red) at the PM of *XVE-PIN1* cultured cells after EST treatment (1 μM, 24 hours) (C). There was no signal in the untreated control (B). Nuclear counterstain is shown in blue. Scale bars, 10 μm. (**D**) Auxin accumulation in *Arabidopsis* wild-type cells. NPA (10 μM) increased [^{3}H]NAA accumulation inside cells, demonstrating inhibition of auxin efflux. EST treatment (1 μM, 24 hours) had no effect on [^{3}H]NAA accumulation. (**E**) [^{3}H]NAA accumulation kinetics in *XVE-PIN1* cells, demonstrating PIN1-dependent stimulation of NAA efflux after PIN1 overexpression. Error bars show SEM (n = 4); where error bars are not shown, the error was smaller than the symbols.

The evidence from cultured cells shows that PIN proteins are key rate-limiting factors in cellular auxin efflux. This approach, however, cannot distinguish whether PINs play a catalytic role in auxin efflux or act as positive regulators of endogenous plant auxin efflux catalysts. To address this issue, we used a nonplant system: Human HeLa cells contain neither PIN-related genes nor auxin-related machinery and allow efficient heterologous expression of functional eukaryotic PM proteins (*21*). We transfected HeLa cells with *PIN7* and its more distant homolog *PIN2*. Transfected cells showed strong PIN expression (Fig. 3A), which resulted in a substantial stimulation of net efflux of natural auxin [^{3}H]IAA, compared with empty vector controls (Student's t test: $P < 0.001$) (Fig. 3B). Efflux of [^{3}H]BeA was also stimulated but to a lesser extent. These data show that PIN proteins are capable of stimulating cellular auxin efflux in the heterologous HeLa cell system, albeit with decreased substrate specificity.

To test the role of PIN proteins in another evolutionarily distant nonplant system, we used yeast (*Saccharomyces cerevisiae*). PIN2 and PIN7 were expressed in yeast and showed localization at the PM (Fig. 3A). Kinetics of relative [^{3}H]IAA retention demonstrated that expression of the PINs led to a substantial increase in IAA efflux (Fig. 3C). Efflux assays in conjunction with control experiments, including testing metabolically less active yeast in the stationary phase, or after glucose starvation (Fig. 3D), confirmed an active PIN-dependent export of IAA and, to a lesser extent, of BeA from yeast (Fig. 3C and fig. S4B). To test the requirements of the subcellular localization for PIN2 action in yeast, we performed a mutagenesis of the PIN2 sequence to isolate mistargeted mutants. One of the mutations, which changed serine-97 to glycine (pin2Gly97), led to the localization of pin2Gly97

[1]Institute of Experimental Botany, the Academy of Sciences of the Czech Republic, 165 02 Prague 6, Czech Republic. [2]Department of Plant Physiology, Faculty of Science, Charles University, 128 44 Prague 2, Czech Republic. [3]Center for Plant Molecular Biology (ZMBP), University Tübingen, D-72076 Tübingen, Germany. [4]Zurich-Basel Plant Science Center, University of Zurich, Institute of Plant Biology, CH 8007 Zurich, Switzerland. [5]Department of Horticulture, Purdue University, West Lafayette, IN 47907, USA. [6]Institute for Applied Genetics and Cell Biology, University of Natural Resources and Applied Life Sciences–Universität für Bodenkultur, A-1190 Wien, Austria. [7]Department of Biotechnology, Institute of General and Molecular Biology, 87-100 Toruń, Poland. [8]Institute of Plant Sciences, University of Bern, 3013 Bern, Switzerland. [9]Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142, USA. [10]Masaryk University, Department of Functional Genomics and Proteomics, Laboratory of Molecular Plant Physiology, Kamenice 5, 625 00 Brno, Czech Republic.

*To whom correspondence should be addressed. E-mail: eva.zazim@ueb.cas.cz



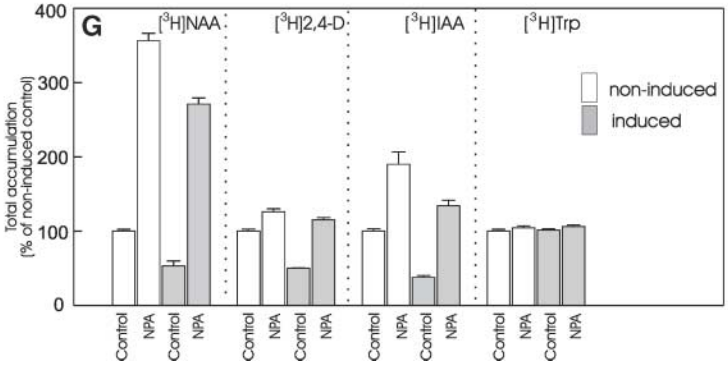


Fig. 2. PIN-dependent auxin efflux in BY-2 tobacco cultured cells. (**A**) Inducible PIN7 expression in *GVG-PIN7* tobacco cells. PIN7 immunostaining (green) is shown at the PM after DEX treatment (24 hours; 1 μM) but not in the untreated control; RT-PCR of *PIN7* was conducted within 24 hours of DEX treatment (1 μM). Nuclear counterstain is shown in blue. Scale bars, 40 μm. (**B**) Auxin accumulation in BY-2 control cells. NPA (10 μM) increased [^{3}H]NAA accumulation inside cells, demonstrating inhibition of auxin efflux. DEX treatment (1 μM, 24 hours) had no effect on [^{3}H]NAA accumulation. (**C**) [^{3}H]NAA accumulation kinetics in *GVG-PIN4*, *GVG-PIN6*, and *GVG-PIN7* cells demonstrating PIN4-, PIN6-, and PIN7-dependent stimulation of NAA efflux. Noninduced control is shown only for PIN7; those for PIN4 and PIN6 were within the range ±8% of the values for PIN7. Data are expressed as a percentage of noninduced control at 30 min after application of labeled [^{3}H]NAA. (**D**) Induced *GVG-PIN7* cells showed decreased retention of [^{3}H]NAA compared with noninduced control. (**E**) Accumulation kinetics in induced *GVG-PIN7* cells revealed PIN7-dependent stimulation of [^{3}H]IAA and [^{3}H]2,4-D efflux. (**F**) Treatments with increasing concentrations of DEX led to gradually higher levels of PIN7 in *GVG-PIN7* cells, as determined by dot blot (top) and to concomitant decrease of [^{3}H]NAA accumulation. (**G**) NPA inhibition of both endogenous and PIN7-dependent efflux of [^{3}H]NAA, [^{3}H]2,4-D, and [^{3}H]IAA. PIN7 overexpression or NPA treatment did not affect accumulation of related compound, [^{3}H]Trp. Open bars, noninduced cells; gray bars, induced cells. For all experiments, error bars show SEM (n = 4); where error bars are not shown, the error was smaller than the symbols.

in intracellular compartments (Fig. 3A). When tested in the [^{3}H]IAA efflux assay (fig. S4A), pin2Gly97 failed to mediate auxin efflux but rather increased [^{3}H]IAA accumulation inside cells (Fig. 3D). This shows that pin2Gly97 is still functional but fails to mediate auxin efflux, suggesting importance of PIN localization at PM. Overall, the results suggest that in yeast as well, PM-localized PIN proteins mediate, although with decreased specificity, a saturable efflux of auxin.

A role in auxin efflux has also been reported recently for PGP1 and, in particular, PGP19 proteins of *Arabidopsis* (*12*). PIN and PGP proteins seem to have a comparable effect on mediating auxin efflux in yeast and HeLa cells, but the genetic interference with their function in *Arabidopsis* has distinctive effects on development. All aspects of the *pin* mutant phenotypes can be mimicked by chemical interference with auxin transport (*4*–*7*, *9*). In contrast, *pgp1*/*pgp19* double mutants show strong but entirely different defects (*10*, *11*), which cannot be phenocopied by auxin transport inhibitors.

To compare the roles of PINs and PGPs in auxin efflux, we constructed the *GVG-PGP19:HA* (hemagglutinin) cell line of BY-2. DEX treatment led to the up-regulation of PGP19:HA protein, which was detected at the PM (Fig. 4A), and to a decrease in [^{3}H]NAA accumulation, similar to that observed in the *GVG-PIN4*, *GVG-PIN6*, and *GVG-PIN7* lines (Fig. 4B, compare with Fig. 2C). BeA did not interfere with [^{3}H]NAA accumulation and [^{3}H]Trp accumulation did not change after DEX treatment. However, compared with PIN-mediated auxin efflux, the PGP19-mediated NAA efflux was notably less sensitive to NPA. Whereas PIN-mediated transport was completely inhibited by NPA, about 20% of PGP19-dependent transport was NPA insensitive (Fig. 4C).

To address whether PIN action in planta requires PGP1 and PGP19 proteins, we analyzed effects of PIN1 overexpression on plant development in *pgp1*/*pgp19* double mutants. PIN1 overexpression in *XVE-PIN1* led to pronounced defects in root gravitropism, which could be detected within 4 hours after estradiol treatment. Quantitative evaluation of reorientation of root growth revealed that PIN1 overexpression in *pgp1*/*pgp19* had the same effects (Fig. 4D). These data show that PIN1 action on plant development does not strictly require function of PGP1 and PGP19 proteins, and they suggest that PINs and PGPs molecularly characterize distinct auxin transport systems. This is also supported by evidence that PIN2 mediates auxin efflux in yeast, which is known to lack homologs to *Arabidopsis* PGP proteins (*21*). It is still unclear whether these two auxin transport machineries act in planta entirely independently or in a coordinated fashion.

Rate-limiting, saturable, and specific action of PIN proteins in mediating auxin movement

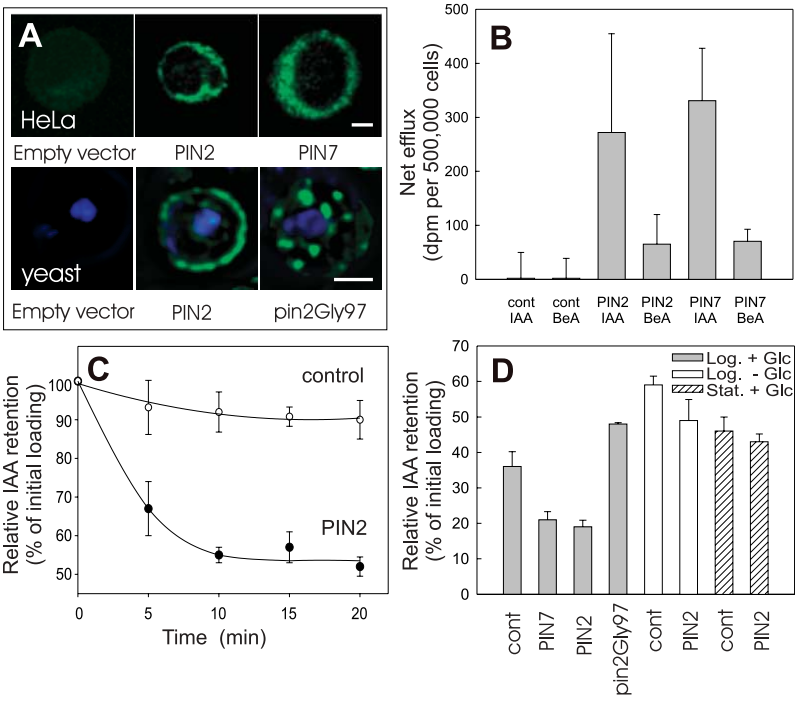


Fig. 3. PIN-dependent auxin efflux in mammalian and yeast cells. (**A**) PIN2:HA and PIN7:HA expression in HeLa and yeast. Anti-HA immunostaining detected PIN2:HA and PIN7:HA at the PM of transfected but not control (empty vector) HeLa cells (top). Anti-PIN2 immunostaining detected PIN2 at the PM and pin2Gly97 in intracellular compartments, compared with empty vector controls (bottom). Scale bars, 2 μm. (**B**) Transfected HeLa cells display PIN2- and PIN7-dependent net efflux of [^{3}H]IAA and to a smaller extent also of [^{3}H]BeA. dpm, disintegration per minute. (**C**) The kinetics of [^{3}H]IAA efflux. PIN2 stimulated saturable [^{3}H]IAA efflux in yeast JK93da strain. (**D**) [^{3}H]IAA retention measured 10 min after loading: PIN2 and PIN7 mediated [^{3}H]IAA efflux; pin2Gly97 failed to mediate efflux but increased [^{3}H]IAA retention. Yeast in stationary phase or without glucose showed much less [^{3}H]IAA efflux. Error bars show SEM (n = 4).

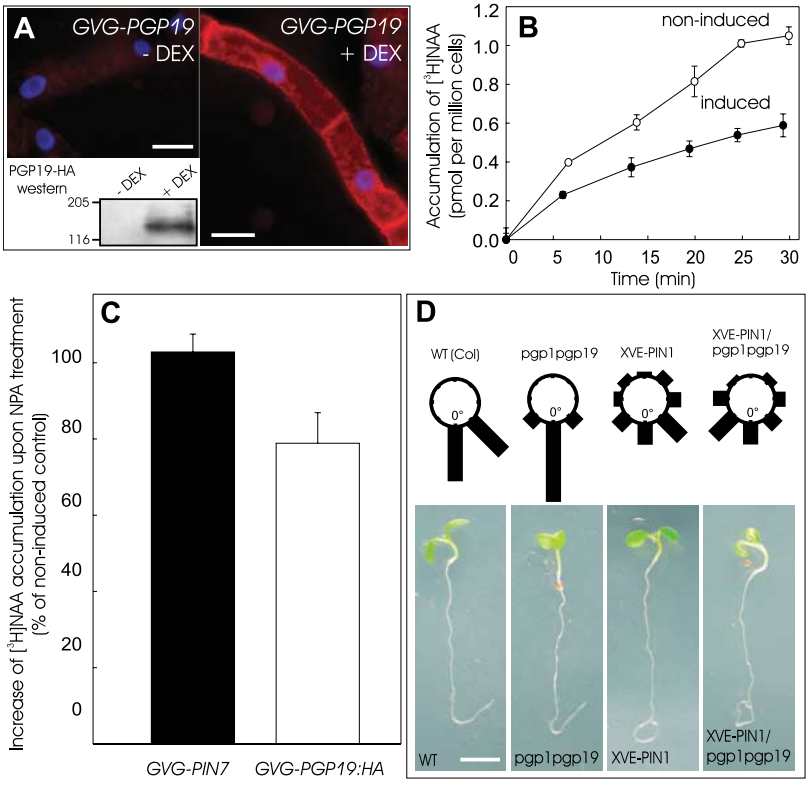


Fig. 4. Requirement of PGP function for PIN role in auxin efflux. (**A**) Inducible PGP19 expression in *GVG-PGP19:HA* tobacco cells. PGP19:HA immunostaining (red) at PM after DEX treatment (24 hours, 1 μM) is shown; no PGP19:HA immunostaining was present in the untreated control. An anti-HA immunoblot was conducted after 24 hours of DEX (1 μM) treatment. Nuclear counterstain is shown in blue. Scale bars, 40 μm. (**B**) [^{3}H]NAA accumulation decreased upon PGP19 expression, revealing function in auxin efflux in BY-2 cells. (**C**) Different sensitivities of PIN7- and PGP19-dependent [^{3}H]NAA efflux to NPA treatment (10 μM, 20 min) in *GVG-PIN7* and *GVG-PGP19:HA* cells (*23*). (**D**) Root gravitropism in *XVE-PIN1* seedlings. PIN1 overexpression (4 hours, 4 μM EST) led to gravitropic defects in *pgp1/pgp19* mutants in contrast to gravitropic growth of EST-treated nontransformed wild-type (WT) and *pgp1/pgp19* seedlings. Root gravitropism was scored 12 hours after gravity stimulation (n > 40). Scale bar, 3 mm. For (B) and (C), error bars show SEM (n = 4); where error bars are not shown, the error was smaller than the symbols.

across the PM out of plant cells largely clarifies a role of PIN proteins in intercellular auxin transport. Furthermore, the polar, subcellular PIN localization provides a vectorial component to the directional auxin flow (*22*). Therefore, transport function of PINs together with their asymmetric subcellular localization defines directional local auxin distribution underlying different developmental processes.

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Oceanographic Basis of the Global Surface Distribution of *Prochlorococcus* Ecotypes

Heather A. Bouman,[1]* Osvaldo Ulloa,[1] David J. Scanlan,[3] Katrin Zwirglmaier,[3] William K. W. Li,[4] Trevor Platt,[4] Venetia Stuart,[5] Ray Barlow,[6] Ole Leth,[2] Lesley Clementson,[7] Vivian Lutz,[8] Masao Fukasawa,[9] Shuichi Watanabe,[9] Shubha Sathyendranath[5]

By using data collected during a continuous circumnavigation of the Southern Hemisphere, we observed clear patterns in the population-genetic structure of *Prochlorococcus*, the most abundant photosynthetic organism on Earth, between and within the three Southern Subtropical Gyres. The same mechanisms that were previously invoked to account for the vertical distribution of ecotypes at local scales accounted for the global (horizontal) patterns we observed. Basin-scale and seasonal variations in the structure and strength of vertical stratification provide a basis for understanding large-scale horizontal distribution in genetic and physiological traits of *Prochlorococcus*, and perhaps of marine microbial communities in general.

Prochlorococcus is the smallest and most abundant phytoplankter in the global ocean and contributes significantly to the primary productivity of tropical and subtropical oceans (*1*). That the genus thrives throughout a wide range of photic zone conditions has been explained by the discovery of genetically and physiologically distinct populations, commonly referred to as high light (HL)– and low light (LL)–adapted ecotypes (*2*). *Prochlorococcus* ecotypes partition themselves according to depth in a stratified water column (*3*); however, the coexistence of multiple ecotypes (*2*) and phylotypes (*4*–*6*) has also been reported and attributed to vertical mixing in response to local physical forcing. But the effect of physical forcing on *Prochlorococcus* ecotypes at the global scale has not been explored. By using data collected during a circumnavigation of the Southern Hemisphere, we investigated whether the genetic structure of *Prochlorococcus* populations changes in response to vertical mixing within and between the major ocean basins of the world.

Samples were collected during the Blue Earth Global Expedition (BEAGLE) (Fig. 1A). The 7-month expedition spanned the southern Pacific (winter), Atlantic (late spring), and Indian (summer) Oceans (*7*); covered several biogeochemical provinces (*8*); and provided a rare opportunity to study physical forcing of phytoplankton at the global scale. We used the depth of the surface mixed layer (z_m) and the strength of the vertical density gradient (N) as indicators of the physical state of the water column (*9*). The three ocean basins differed markedly in these properties (Fig. 1B). The basin-scale variations in the vertical structure of the water column observed in the BEAGLE data are partly due to seasonal and latitudinal differences in the sampling of the three ocean basins. Mixed-layer–depth climatology reveals strong seasonality in z_m, with high values of z_m occurring during the Austral winter in all three ocean basins, and relatively uniform and shallow z_m values in the summer months (fig. S1). However, differences among basins are also found. Thus, spatial differences in vertical mixing as indexed by z_m observed during the BEAGLE have a seasonal as well as a geographical component.

Prochlorococcus cell abundance was determined by flow cytometry, and the concentration of divinyl chlorophyll a (DV Chla), a pigment marker for this genus, was measured by high performance liquid chromatography (HPLC). A clear difference between the geographic patterns of these two indices of abundance was found (Fig. 2A). *Prochlorococcus* abundance has a minimum in the well-mixed, mesotrophic waters of the Western Pacific Basin and a maximum in the strongly stratified oligotrophic waters of the Indian Ocean, a pattern that is consistent with our current understanding of the distribution of this genus (*1*, *10*, *11*). However, the concentration of divinyl chlorophyll a is high in the Pacific Basin (except near 140°W) and low in the Atlantic and Indian Basins. This is perhaps counterintuitive; it can be explained as follows. Because all samples were collected within the top 10 m of the water column, vertical mixing would be an important mechanism altering the growth conditions (light and nutrients) of the phytoplankton cells. Thus, the high divinyl chlorophyll a concentrations in the Pacific may arise from photoacclimatory (physiological) or photoadaptive (genetic) response of the cells to a decrease in mean light intensity. Basin-scale patterns in the intracellular concentration of divinyl chlorophyll a (C_i) for *Prochlorococcus* are evident (Fig. 2B), with low C_i values observed in the strongly stratified Indian Ocean during the summer (averaging 0.14 fg DV Chla per cell), consistent with those found in the surface waters of the subtropical North Atlantic (*12*), and high values observed in the well-mixed Archipelagic Deep Basins Province (*8*) during the winter (averaging 1.00 fg DV Chla per cell), similar to those typically found deeper in the water column in subtropical gyres (*12*).

Because light decreases exponentially with depth, phytoplankton cells mixed deeper in the water column would experience a lower mean daily irradiance than if they remained at the sea surface. Phytoplankton respond to this reduction in irradiance by increasing the concentration of pigment per cell. An inverse relation between C_i

[1]Laboratorio de Procesos Oceanográficos y Clima, Departamento de Oceanografía, and Centro de Investigación Oceanográfica en el Pacífico Sur-Oriental, [2]Departamento de Geofísica, Universidad de Concepción, Casilla 160-C, Concepción, Chile. [3]Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK. [4]Bedford Institute of Oceanography, Dartmouth, Nova Scotia, B2Y 4A2, Canada. [5]Department of Oceanography, Dalhousie University, Halifax, Nova Scotia, B3H 4J1, Canada. [6]Marine and Coastal Management, Private Bag X2, Rogge Bay 8012, Cape Town, South Africa. [7]Commonwealth Scientific and Industrial Research Organization, Marine Research, Post Office Box 1538, Hobart, Tasmania, Australia, 7001. [8]Instituto Nacional de Investigación y Desarrollo Pesquero, Paseo Victoria Ocampo 1, 7600 Mar del Plata, Argentina. [9]Japan Agency for Marine-Earth Science and Technology, 2-15, Natsushima, Yokosuka, 237-0061, Japan.

*To whom correspondence should be addressed. E-mail: heather@profc.udec.cl

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Supporting Online Material for

PIN Proteins Perform a Rate-Limiting Function in Cellular Auxin Efflux

Jan Petrášek, Jozef Mravec, Rodolphe Bouchard, Joshua J. Blakeslee, Melinda Abas, Daniela Seifertová, Justyna Wiśniewska, Zerihun Tadele, Martin Kubeš, Milada Čovanová, Pankaj Dhonukshe, Petr Skůpa, Eva Benková, Lucie Perry, Pavel Křeček, Ok Ran Lee, Gerald R. Fink, Markus Geisler, Angus S. Murphy, Christian Luschnig, Eva Zažímalová,* Jiří Friml

*To whom correspondence should be addressed. E-mail: eva.zazim@ueb.cas.cz

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Materials and Methods

Plant material, gene constructs, transformation and inducible expression

Arabidopsis seedlings were grown at a 16 hours light/8 hours dark cycle at 18-25 °C on 0.5 x MS with sucrose as described (*1*). The *XVE-PIN1* (Col-0) transgenic plants were obtained by introducing the $p_{G10\text{-}90}$*::XVE* activator and the *LexA::PIN1*; *LexA::GFP* reporter constructs (*2, 3*) into *pin1-7* mutant line. This line was crossed with *pgp1pgp19* double mutant (*4*) to generate *XVE-PIN1/pgp1pgp19* line. The *XVE-PIN1* construct was generated using *PIN1* cDNA (GenBank accession number AF089084). *GVG-PIN4,6,7* constructs were generated by inserting the corresponding cDNAs (*PIN4*: AF087016, *PIN6*: AF087819, *PIN7*: AF087820) into the pTA7002 vector (*5*). *GVG-PGP19:HA* construct was generated by introducing the full length genomic fragment of *PGP19* (locus name At3g28860) with C-terminal hemaglutinin tag (HA) into pTA7002 vector (*5*).

Cell suspension from *XVE-PIN1 Arabidopsis* line was established from calli induced on young leaves (*6*) and grown in liquid MS medium containing 1 μM 2,4-D. BY-2 tobacco cells (*Nicotiana tabacum* L., cv. Bright Yellow 2, (*7*)) were grown as described (*8*) and stably transformed by co-cultivation with *Agrobacterium* (*8*). Transgenic tobacco cells and calli were maintained on the media supplemented with 40 μg ml^{-1} hygromycin and 100 μg ml^{-1} cefotaxim. Expression of *PIN* and *PGP* genes in tobacco cells was induced by the addition of dexamethasone (DEX, 1 μM, 24 hours, except for stated otherwise) at the

beginning of the subcultivation period. The same approach was used for *Arabidopsis* cell culture, where 1 μM β-Estradiol (EST) was added. Both DEX and EST were added from stock solutions in DMSO (200 μM), appropriate volume of DMSO was added in controls.

Expression and localization analysis

Tobacco and *Arabidopsis* RNA was isolated using the Plant RNA Qiagen Mini-Prep and RT-PCR performed using Qiagen® OneStep RT-PCR or Invitrogen SuperSriptII kits according to the manufacturer's protocols.

Total protein fraction from *GVG-PGP19* tobacco cells was obtained after homogenization in liquid nitrogen using mortar and pestle. The frozen powder was then mixed with an equal volume of extraction buffer (50 mM Tris-HCl, pH 6.8; 2 % SDS; 36 % w/v urea; 30 % v/v glycerol; 5 % v/v mercaptoethanol; 0,5 % w/v Bromphenol Blue), vortexed for 1 min, boiled for 3 min, and centrifuged at 13,000 rpm and 4°C for 5 min. The supernatant was transferred into a new tube and re-centrifuged at 13,000 rpm and 4°C for 5 min. The resulting supernatant was defined as total protein extract and stored at –20°C until use.

Microsomal protein fraction from *GVG-PIN7* tobacco cells was used for immunoblot analysis of PIN7 protein. Briefly, cells were homogenized by sonication in extraction buffer (50mM Tris pH 6.8; 5% (v/v) glycerol; 1.5% (w/v) insoluble polyvinylpolypyrrolidone; 150mM KCl; 5mM Na EDTA; 5mM Na EGTA; 50mM NaF; 20mM beta-glycerol phosphate; 0.5% (v/v) solubilized casein, 1mM benzamidine; 1mM PMSF; 1μg/ml pepstatin; 1μg/ml leupeptin; 1μg/ml aprotinin; 1 Roche Complete Mini Protease Inhibitor tablet per 10ml). After centrifugation at 3,800 x *g* for 20 minutes, the supernatant was filtered through nylon mesh and spun again at 3,800 x *g*. The supernatant was centrifuged

at 100,000 x *g* for 90 min. The resulting pellet was homogenized and re-suspended in buffer containing 50mM Tris pH 7.5; 20% glycerol; 2mM EGTA; 2mM EDTA; 50-500µM DTE; 10µg/ml solubilized casein and protease inhibitors as in the extraction buffer. Equal amounts of protein (about 10µg) were heated at 60°C for 40 min in sample buffer (3% (w/v) SDS; 40mM DTE; 180mM Tris pH 6.8; 8M urea), and transferred on PVDF membrane using dot-blot (SCIE-PLAS, U.K.) or semi-dry electro-blot. Primary rabbit polyclonal anti-PIN7 antibody (*10*) or mouse monoclonal anti-HA antibody (Sigma) followed by secondary HRP-conjugated anti-rabbit antibody and ECL detection kit (Amersham Biosciences, U.K.) were used for dot or western blot analysis.

Indirect immunofluorescence method was used for immunolocalizations in *Arabidopsis* cell suspension (11) and BY-2 cells (*8*). Briefly, *Arabidopsis* cells were fixed for 30 min at room temperature with 4% (w/v) paraformaldehyde in 0.1 M PIPES, pH 6.8, 5 mM EGTA, 2 mM $MgCl_2$, and 0.4% (w/v) Triton X-100. Cells were then treated with the solution of 0.8% (w/v) macerozyme R-10 and 0.2% (w/v) pectolyase Y-23 in 0.4 M mannitol, 5 mM EGTA, 15 mM MES, pH 5.0, 1 mM PMSF, 10 µg/ml of leupeptin, and 10 µg/ml of pepstatin A. Then the cells were washed in PBS buffer and attached to poly-L-lysine coated coverslips and incubated for 30 min in 1% (w/v) BSA in PBS and incubated for 1 h with primary antibody. The specimens were then washed three times for 10 min in PBS and incubated for 1 h with secondary antibody. Coverslips with cells were carefully washed in PBS, rinsed with water with Hoechst 33258 (0,1µg/ml) and embedded in Mowiol (Polysciences) solution.

Tobacco BY-2 cells were pre-fixed 30 min in 100 µM MBS and 30 min in 3.7% (w/v) PFA in buffer consisting of 50 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$, pH 6.9, at 25°C and

subsequently in 3.7% (w/v) PFA and 1% Triton X-100 (w/v) in stabilizing buffer for 20 minutes. After treatment with an enzyme solution (1% (w/v) macerozyme and 0.2% (w/v) pectinase) for 7 min at 25°C and 20 minutes in ice cold methanol (at -20°C), the cells were attached to poly-L-lysine coated coverslips and treated with 1% (w/v) Triton X-100 in microtubule stabilizing buffer for 20 minutes. Then the cells were treated with 0.5% (w/v) bovine serum albumin in PBS and incubated with primary antibody for 45 minutes at 25°C. After washing with PBS, a secondary antibody in PBS was applied for 1 h at 25°C. Coverslips with cells were carefully washed in PBS, rinsed with water with Hoechst 33258 (0,1μg/ml) and embedded in Mowiol (Polysciences) solution.

The following antibodies and dilutions were used: anti- PIN1 (*13*, 1: 500), anti-PIN7 (*10*, 1:500), anti-HA (Sigma-Aldrich; 1:500), TRITC- (Sigma-Aldrich; 1:200), FITC- (Sigma-Aldrich; 1:200) anti-rabbit secondary antibodies. PIN immunostaining in yeast and HeLa cells was performed as described (*13, 14*).

All preparations were observed using an epifluorescence microscope (Nikon Eclipse E600) equipped with appropriate filter sets, DIC optics, monochrome integrating CCD camera (COHU 4910, USA) or colour digital camera (DVC 1310C, USA).

Quantitative analysis of root gravitropism

5 days old seedlings of WT-Col, *pgp1pgp19*, *XVE-PIN1* and *XVE-PIN 1/pgp1pgp19* lines grown vertically were transferred on new MS plates containing 4 μM β-estradiol for 12 hours. Seedlings were then stretched and plates turned through 135° for additional 12 hour gravity stimulation in dark. The angle of root tips from the vertical plane was measured using ImageJ software (NIH, USA). All gravistimulated roots were assigned to one of the

eight 45° sectors on gravitropism diagram. The length of bars represents the percentage of seedlings showing respective direction of root growth.

Auxin accumulation assays in plant, HeLa and yeast cells

Auxin accumulation experiments in suspension-cultured tobacco BY-2 cells were performed and the integrity of labeled auxins during the assay was checked exactly as described (*15, 8, 12*). The same protocol was used for suspension-cultured *Arabidopsis* cells. Labeled [^{3}H]IAA, [^{3}H]2,4-D and [^{3}H]Trp (specific activities 20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO), and [^{3}H]NAA (specific radioactivity 25 Ci/mmol, Institute of Experimental Botany, Prague, Czech Republic) were used. Briefly, the accumulation was measured in 0.5-mL aliquots of cell suspension. Each cell suspension was filtered, resuspended in uptake buffer (20 mM MES, 40 mM Suc, and 0.5 mM $CaSO_4$, pH adjusted to 5.7 with KOH), and equilibrated for 45 min with continuous orbital shaking. Equilibrated cells were collected by filtration, resuspended in fresh uptake buffer, and incubated on the orbital shaker for 1.5 h in darkness at 25°C. [^{3}H]NAA was added to the cell suspension to give a final concentration of 2 nM. After a timed uptake period, 0.5-mL aliquots of suspension were withdrawn and accumulation of label was terminated by rapid filtration under reduced pressure on 22-mm-diameter cellulose filters. The cell cakes and filters were transferred to scintillation vials, extracted in ethanol for 30 min, and radioactivity was determined by liquid scintillation counting (Packard Tri-Carb 2900TR scintillation counter, Packard Instrument Co., Meriden, CT). Counts were corrected for surface radioactivity by subtracting counts obtained for aliquots of cells collected immediately after the addition of [^{3}H]NAA. Counting efficiency was determined by

automatic external standardization, and counts were corrected automatically. NPA was added as required from ethanolic stock solutions to give the appropriate final concentration. The concentration dependence of auxin accumulation in response to NPA or BFA was determined after a 20-min uptake period. For wash out experiments cells were loaded with [^{3}H]NAA (2 nM) for 30 min. After quick wash out, cells were re-suspended in fresh loading buffer but without [^{3}H]NAA; cell density before and after wash out was maintained the same. Relative NAA retention was measured as a radioactivity retained inside cells at particular time points after wash out and expressed as % of total radioactivity retained inside the cells just before wash out. The accumulation of various auxins or structurally related inactive compound (Trp) after induction of PIN or PGP expression was expressed together with SEs as the percentage of the accumulation of non-induced cells at time 30 min after application of respective labelled compound. If not indicated otherwise, 24 hours treatments with dexamethasone (1 μM) or β-estradiol (1 μM) were performed. Different sensitivities of PIN7- and PGP19-dependent [^{3}H]NAA efflux to NPA treatment (10 μM, 20 min) in *GVG-PIN7* and *GVG-PGP19:HA* cells was determined as the average value from three independent experiments. In each, the accumulation of [^{3}H]NAA was measured in NPA-treated induced and non-induced cells and scored after 20 minutes of incubation. The increase in the accumulation of [^{3}H]NAA upon NPA treatment in non-induced cells was considered as 100% and all other values expressed as the percentage of this increase.

The transient vaccinia expression system was used to transfect HeLa cells with PIN1:HA, PIN2:HA, and PIN7:HA in 6-well plates. The expression was verified by RT-PCR and western blot analysis. Auxin transport assays were performed exactly as described (*16, 14*). 16-24h after transfection cells were washed and incubated 40 min at 37°C, 5% CO_2 with

[^{3}H]IAA (26 Ci/mmol, Amersham Biosciences, Piscataway, NJ), or [^{3}H]BeA (20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO). After incubation, cells were harvested, and retained radiolabeled substrate was quantitated. Net efflux is expressed as dpm/500,000 cells divided by the amount of auxin retained by cells transformed with empty vector minus the amount of auxin retained by cells transformed with gene of interest. Thus, the PIN-dependent decrease in retention is presented as positive efflux value expressed as means (n=3) with standard deviations. Cell viability after treatment was confirmed visually and via cell counting.

For auxin accumulation and growth assays in yeast, PIN2, PIN7 or PIN2:HA were expressed in *S. cerevisiae* strains *gef1* (*13*) and JK93da or *yap1-1* (*17*). The expression was verified by western blot analysis or immunolocalization. Export of [^{3}H]IAA (specific activity 20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) and [^{14}C]BeA (53 mCi/mmol, Moravek Biochemicals, Brea, CA) and growth assays were performed exactly as described (*17, 14*). The effluent species was determined by thin-layer chromatography of aliquots of exported [^{3}H]IAA (Supplementary fig. S4a) and images were taken using a phosphoimager (Cyclone, Packard Instruments) and by UV detection using [^{3}H]IAA as standard. Yeast viability before and after transport experiments was ascertained by light microscopy.

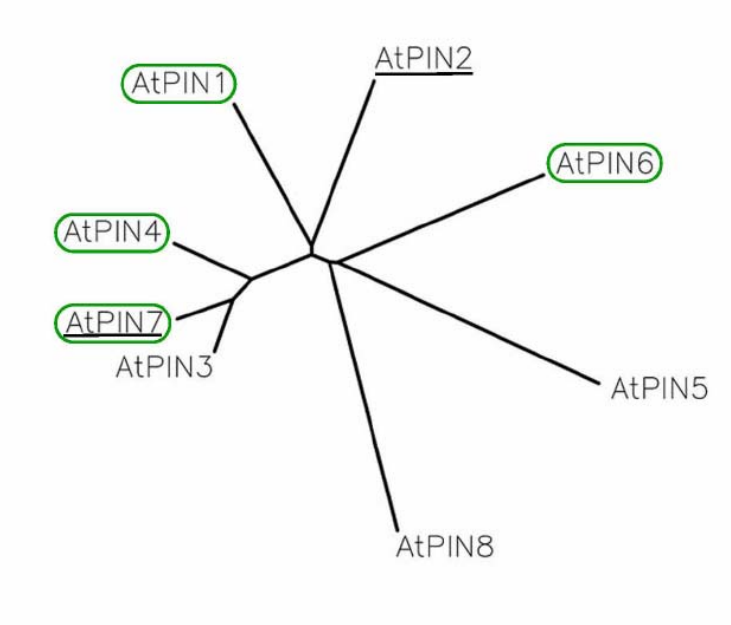

fig. S1 Arabidopsis *PIN* genes family.

Phylogenetic tree of 8 Arabidopsis *PIN* genes. Phenotypes of loss-of-function mutants in *PIN1*, *PIN2*, *PIN3*, *PIN4* and *PIN7* clearly suggest role in polar auxin transport and they all can be phenocopied by inhibitors of auxin transport (*18*). PIN6 remains functionally uncharacterized. PIN5 and PIN8 lack the middle hydrophilic domain and seem to by functionally distinct (*19*). Based on homology, *PIN7* is the most typical member of PIN family forming a distinct homologous subclade with *PIN3* and *PIN4*. *PIN6*, on the other hand, is the least homologous *PIN* from the *PIN1,2,3,4,6,7* subfamily. PIN1, PIN4, PIN6 and PIN7 (respective genes encircled in green) have been shown here to mediate auxin efflux *in planta*. PIN2 and PIN7 (genes underlined) show auxin efflux activity in heterologous systems. Notably, the confirmed expression of PIN1 in HeLa or yeast cells did not result in increased auxin efflux suggesting, in contrast to PIN2 and PIN7, that either PIN1 loses its functionality, when expressed in heterologous system, or distinctively PIN1 requires plant-specific factor(s) to mediate its function in auxin efflux.

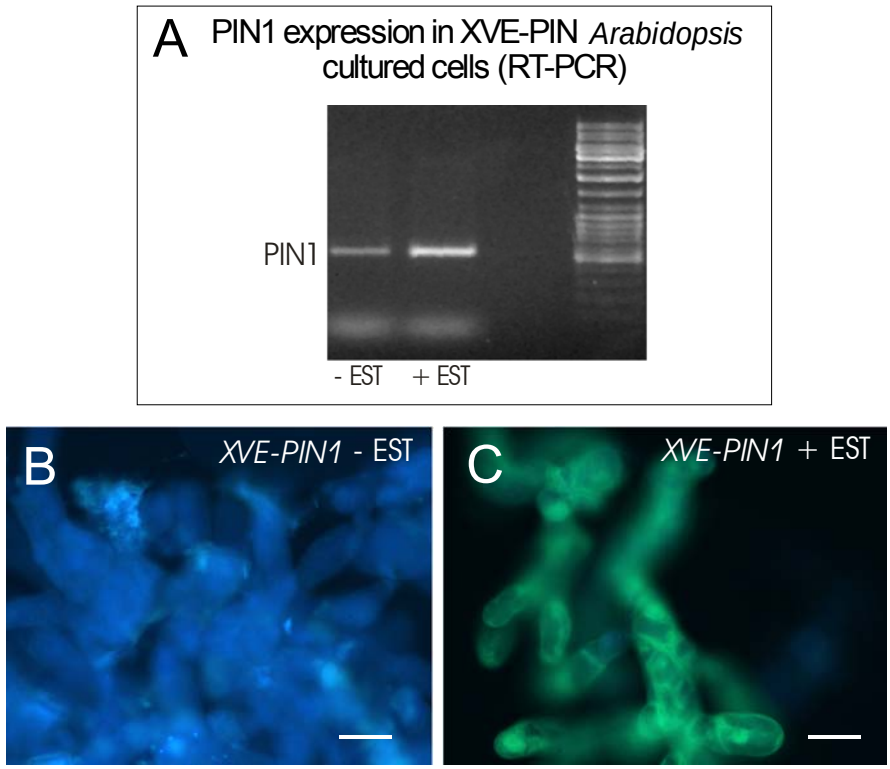

fig. S2 The expression of PIN1 in *XVE-PIN1 Arabidopsis* cultured cells

(A) RT-PCR of PIN1 in non-induced and β-estradiol-induced (1 μM, 24h) cells. (B, C) The activation of expression verified by the fluorescence of co-expressed GFP reporter. Compare the autofluorescence of cell walls in non-induced cells (B) with GFP fluorescence after 24 h incubation in 1 μM β-estradiol (C). Scale bars 30μm.

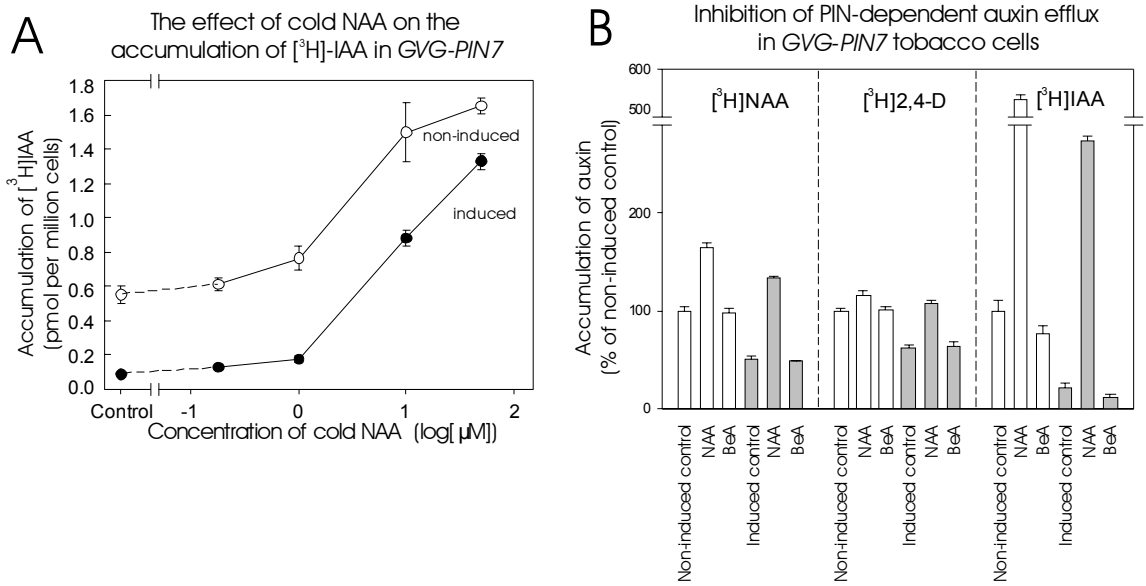

fig. S3 Auxin accumulation in *GVG-PIN7* BY-2 cells

(A) Displacement curve: The competitive inhibitory effect of cold (non-labeled) NAA on the accumulation of [^{3}H]IAA in non-induced and induced *GVG-PIN7* cells. (B) Effects of NAA and benzoic acid (BeA) on efflux of different auxins in DEX-treated (induced, full bars) and non-induced (open bars) *GVG-PIN7* cells. NAA (10 µM), a good substrate for auxin efflux machinery, interferes with both endogenous and PIN7-dependent efflux of [^{3}H]NAA, [^{3}H]2,4-D and [^{3}H]IAA in non-induced and induced *GVG-PIN7* cells, respectively. In contrast, structurally similar but inactive BeA (10 µM) does not have any detectable effect in the same experimental system.

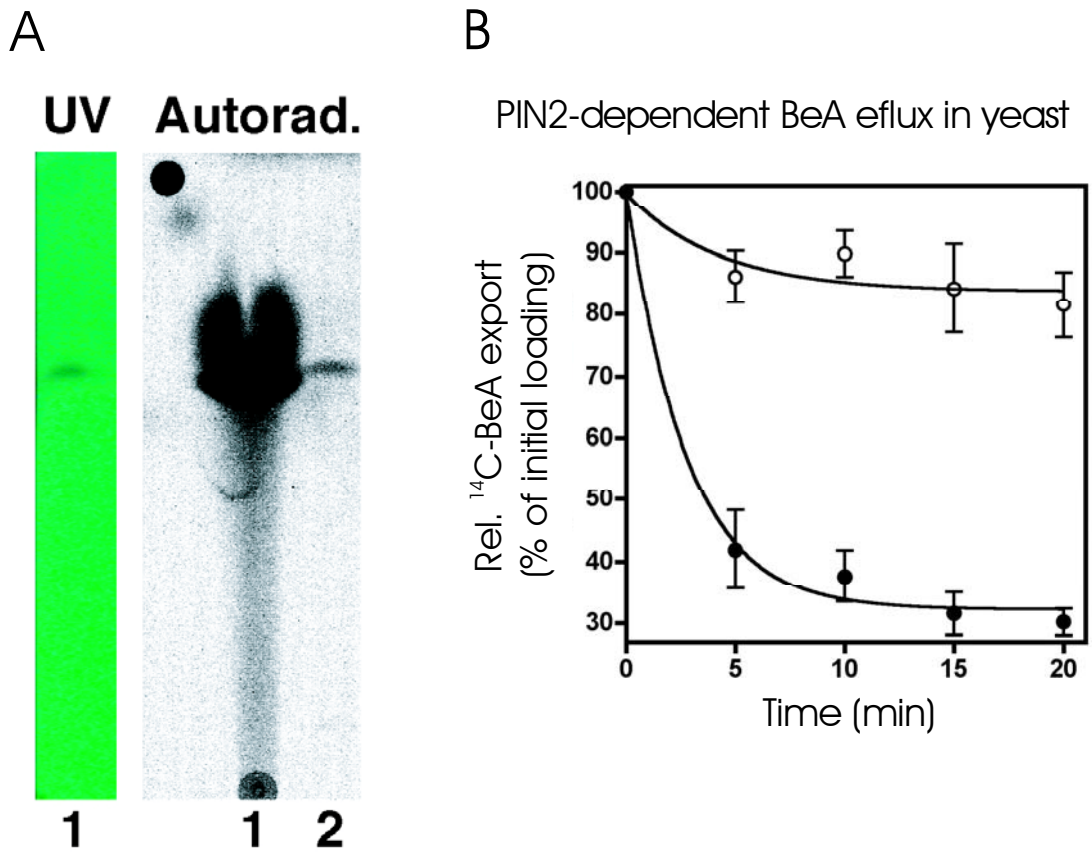

fig. S4 Control experiments for auxin efflux assays in yeast.

(A) The effluent species in yeast were determined to be [^{3}H]IAA by thin layer chromatography (lane 2). Non-exported [^{3}H]IAA was used as the standard which itself was verified by UV detection (lane 1). Images were taken using a phosphoimager (Cyclone, Packard Instruments) and by UV detection using [^{3}H]IAA as the standard. The integrity of exported [^{3}H]IAA in this assay was also proved by MS-MS, as described elsewhere (*14*).

(B) PIN2-expressing yeast show increased net efflux of [^{14}C]benzoic acid ([^{14}C]BeA)

compared to empty vector controls. [^{14}C]BeA (53 mCi/mmol, Moravek Biochemicals, Brea, CA) was used and transport experiments were performed exactly as described (*14*).

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Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development

Jozef Mravec[1,2], Martin Kubeš[3,4], Agnieszka Bielach[1,2], Vassilena Gaykova[2], Jan Petrášek[3,4], Petr Skůpa[3,4], Suresh Chand[2], Eva Benková[1,2], Eva Zažímalová[3] and Jiří Friml[1,2,*]

The signalling molecule auxin controls plant morphogenesis via its activity gradients, which are produced by intercellular auxin transport. Cellular auxin efflux is the rate-limiting step in this process and depends on PIN and phosphoglycoprotein (PGP) auxin transporters. Mutual roles for these proteins in auxin transport are unclear, as is the significance of their interactions for plant development. Here, we have analysed the importance of the functional interaction between PIN- and PGP-dependent auxin transport in development. We show by analysis of inducible overexpression lines that PINs and PGPs define distinct auxin transport mechanisms: both mediate auxin efflux but they play diverse developmental roles. Components of both systems are expressed during embryogenesis, organogenesis and tropisms, and they interact genetically in both synergistic and antagonistic fashions. A concerted action of PIN- and PGP-dependent efflux systems is required for asymmetric auxin distribution during these processes. We propose a model in which PGP-mediated efflux controls auxin levels in auxin channel-forming cells and, thus, auxin availability for PIN-dependent vectorial auxin movement.

KEY WORDS: PGP, PIN, Auxin transport, Embryogenesis, Organogenesis, Tropisms

INTRODUCTION

Directional (polar) transport of the signalling molecule auxin between cells is a plant-specific form of developmental regulation. Transport-based asymmetric auxin distribution within tissues (auxin gradients) plays an important role in many developmental processes, including patterning and tropisms (reviewed by Tanaka et al., 2006). Because the auxin molecule is charged inside cells and, thus, membrane impermeable, its intercellular transport relies on carrier-mediated cellular influx and efflux (reviewed by Kerr and Bennett, 2007; Vieten et al., 2007). Genetic approaches in *Arabidopsis thaliana* have identified two groups of proteins that are involved in auxin export from cells: PIN-FORMED (PIN) proteins and several ABC transporter-like phosphoglycoproteins (PGPs) (reviewed by Bandyopadhyay et al., 2007; Vieten et al., 2007).

The PIN protein family consists of plant-specific integral plasma membrane proteins that have been identified based on mutants defective in organogenesis (*pin-formed1* or *pin1*) (Okada et al., 1991; Gälweiler et al., 1998) and tropism (*pin2/agr1/eir1*) (Luschnig et al., 1998). The *Arabidopsis* genome encodes eight PIN-related sequences, most of which have been already characterized at cellular and developmental levels (reviewed by Vieten et al., 2007; Zažímalová et al., 2007). PIN proteins are expressed in different parts of the plant and are almost universally required for all aspects of auxin-related plant development, including embryogenesis (Friml et al., 2003), organogenesis (Benková et al., 2003; Reinhardt et al., 2003), root meristem patterning and activity (Friml et al., 2002a; Blilou et al., 2005), tissue differentiation and regeneration (Scarpella et al., 2006; Xu et al., 2006; Sauer et al., 2006a), and tropisms (Luschnig et al., 1998; Friml et al., 2002b). Most phenotypic aberrations in *pin* loss-of-function alleles can be phenocopied by external application of auxin efflux inhibitors, such as 1-naphthylphthalamic acid (NPA) (Tanaka et al., 2006). When expressed in plant and non-plant cultured cells, PIN proteins perform a rate-limiting function in cellular auxin efflux (Petrášek et al., 2006). Importantly, PIN proteins show distinct polar subcellular localization that determines auxin flux direction, as predicted by classical models of directional auxin transport (Wiśniewska et al., 2006). The dynamic regulation of the intracellular movement of PINs, their polar targeting and their protein stability provides a means to regulate directional throughput of auxin flow (Friml et al., 2004; Paciorek et al., 2005; Abas et al., 2006; Michniewicz et al., 2007). Moreover, the PIN-dependent auxin distribution network involves redundancy and auxin-mediated crossregulation of PIN expression and PIN targeting (Sauer et al., 2006a; Blilou et al., 2005; Vieten et al., 2005). A crucial role for PIN-dependent auxin efflux in generation of morphogenetic asymmetric auxin distribution has recently been suggested by mathematical modelling (Grieneisen et al., 2007).

Plant orthologues of the mammalian multidrug-resistance proteins (Martinoia et al., 2002; Verrier et al., 2008) PGP1 (ABCB1) and PGP19 (MDR1/ABCB19), similarly to PIN proteins, have been shown to perform cellular auxin efflux in both plant and heterologous systems; accordingly, basipetal auxin transport is decreased in *pgp1* and *pgp19* mutants (Noh et al., 2001; Geisler et al., 2005; Petrášek et al., 2006). In addition, these proteins bind auxin efflux inhibitors, such as NPA (Murphy et al., 2002). Phenotypic defects caused by loss of PGP function are most pronounced in vegetative organs and include dwarfism, curly wrinkled leaves, twisted stems and reduced apical dominance, supporting their role in auxin-based development (reviewed by Bandyopadhyay et al., 2007). However, expression, localization and roles of PGPs in patterning processes are less characterized compared with PINs. The cellular localization of PGPs is mainly apolar, but instances of asymmetric cellular distribution have been reported (Geisler et al., 2005; Blakeslee et al., 2007; Wu et al., 2007).

[1]Department of Plant Systems Biology, VIB, and Department of Molecular Genetics, Ghent University, 9052 Gent, Belgium. [2]Center for Plant Molecular Biology (ZMBP), University of Tübingen, D-72076 Tübingen, Germany. [3]Institute for Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 263, 165 02 Praha 6, Czech Republic. [4]Department of Plant Physiology, Faculty of Science, Charles University, Viničná 5, 128 44 Praha 2, Czech Republic.

*Author for correspondence (e-mail: jiri.friml@psb.ugent.be)

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Important questions in auxin research relate to the roles of these two types of auxin efflux proteins in auxin transport. Do they represent independent mechanisms? What would be the functional requirements for two distinct transport systems? Do they cooperate and how? The only partial colocalization of PINs and PGPs at the plasma membrane and the difference in the corresponding mutant phenotypes favours a scenario in which PGPs and PINs have independent functions. Nevertheless, recent biochemical studies have demonstrated an interaction between PIN and PGP proteins that is functionally relevant in heterologous systems, because it influences the rate of efflux, its substrate specificity and its sensitivity to inhibitors (Blakeslee et al., 2007; Bandyopadhyay et al., 2007). However, the relevance of the interaction between PINs and PGPs in planta and, eventually, in asymmetric auxin distribution, remains unclear.

Here, we present evidence that PINs and PGPs define independent auxin transport mechanisms that cooperate to mediate auxin distribution-mediated development during embryogenesis, organogenesis and root gravitropism. Our data suggest a model for how non-polar auxin efflux mediated by PGPs is linked with vectorial transport driven predominantly by PINs.

MATERIALS AND METHODS

Plant material and DNA constructs

We used wild-type *Arabidopsis thaliana* plants of ecotypes Wassilewskija (Ws) and Columbia (Col-0); mutants *pgp1*, *pgp19*, *pgp1pgp19* (Noh et al., 2001), *pin1pgp1pgp19* (Blakeslee et al., 2007), *pin1-1* (Okada et al., 1991), *pin1-3×DR5rev::GFP* (R ži ka et al., 2007), *rcn1-1* (Garbers et al., 1996), *eir1-3* (Luschnig et al., 1998), *eir1pgp1pgp19* (Blakeslee et al., 2007); and transgenic lines *DR5::GUS* (Sabatini et al., 1999), *DR5rev::GFP* (Friml et al., 2003), *XVE-PIN1* (Petrášek et al., 2006), *pPGP1::PGP1-myc* and *pPGP19::PGP19-HA* (Blakeslee et al., 2007). *pPGP1::PGP1-myc* was generated as previously described for *pPGP19::PGP19-HA* (Blakeslee et al., 2007). For *pPGP19::PGP19-GFP* and *pPGP1::PGP1-GFP*, tag sequences in *pPGP19::PGP19-HA* and *pPGP1::PGP1-myc*, respectively (Blakeslee et al., 2007), were replaced by an enhanced green fluorescent protein (GFP) sequence to create C-terminal fusion constructs and was transformed to *pgp19* or *pgp1* mutants. The pGVG-PGP1-*myc* and pGVG-PGP19-HA plasmids were constructed by cloning the whole genomic coding region of *PGP1* and *PGP19* genes fused with the respective tag by primer extension PCR to pTA7002 (Aoyama and Chua, 1997), and transformed to the *UAS::GUS* (Weijers et al., 2003) line. Fifteen independent transgenic lines were analysed for each construct.

Growth conditions

Arabidopsis plants were grown in a growth chamber under long-day conditions (16 hours light/8 hours dark) at 18-23°C. Seeds were sterilized with chlorine gas or ethanol, and stratified for 2 days at 4°C. Seedlings were grown vertically on half Murashige and Skoog medium with 1% sucrose and supplemented with 5 μmol/l NPA, 4 μmol/l β-estradiol (EST) or 5 μmol/l dexamethasone (DEX). Drugs were purchased from Sigma-Aldrich (St Louis, MO, USA).

BY-2 cell lines

The transgenic lines *GVG-PIN4*, *GVG-PIN6*, *GVG-PIN7*, *GVG-PGP19-HA* and *pPIN1::PIN1-GFP* (Petrášek et al., 2006; Benková et al., 2003) of *Nicotiana tabacum* Bright Yellow-2 (BY-2) cells (Nagata et al., 1992) were grown as described (Petrášek et al., 2006). Expression of PIN7 and PGP19 was induced by the addition of 1 μM DEX at the beginning of subcultivation. For the NPA effect, 10 μM NPA was added together with DEX. For microscopy, an Eclipse E600 microscope (Nikon, Tokyo, Japan) and a colour digital camera 1310C (DVC, Austin, TX, USA) were used. Reciprocal plots of cell size distribution represent individual cell lengths and diameters measured by LUCIA image analysis software (Laboratory Imaging, Prague, Czech Republic). At least 170 cells in total were measured on five optical fields for each variant.

Immunotechniques and microscopy

Arabidopsis embryos and roots were stained immunologically as described (Sauer et al., 2006b). The antibodies used were anti-PIN1 (Paciorek et al., 2005) (1:1000), anti-c-*myc* (1:500) from rabbit and anti-GFP (1:500) from mouse (Roche Diagnostics, Brussels, Belgium) (1:500). Fluorescein isothiocyanate isomer I (FITC) or Cy3-conjugated anti-rabbit or anti-mouse secondary antibodies were purchased from Dianova (Hamburg, Germany) and diluted 1:600. The microscopic analyses were carried out on a SP2 confocal microscope (Leica-Microsystems, Wetzlar, Germany). GFP samples were scanned without fixation.

Phenotype analyses

Plates with grown seedlings (5 or 10 days old) were scanned on a flatbed scanner and measured with ImageJ software (http://rsb.info.nih.gov/ij/). The vertical growth index (VGI) was calculated as described (Grabov et al., 2005). The hypocotyl twisting index was determined as the relation between hypocotyl length and the distance from the root base to the apical hook. For embryo and root tip morphology analyses, we used chloral hydrate clearing (Friml et al., 2003) and microscopy was carried out on an Axiophot microscope (Zeiss, Jena, Germany) equipped with a digital camera. Lateral roots were analysed and GUS staining was performed as described (Benková et al., 2003).

RESULTS

Effects of PIN- and PGP-inducible overexpression in cultured BY-2 cells

For the characterization of PIN- and PGP-mediated auxin efflux at the cellular level, we used BY-2 cells that harbour DEX-inducible PGP19-HA (*GVG-PGP19-HA*) or PIN7 (*GVG-PIN7*) constructs that had already been used to study the ability of corresponding proteins to mediate efflux from plant cells (Petrášek et al., 2006). After induction of *GVG-PGP19-HA* or *GVG-PIN7* constructs, cells accumulated less auxin because of the increased auxin efflux (Petrášek et al., 2006). To study how increased auxin efflux influences cellular behaviour in BY-2 cells, we examined the growth and morphology of DEX-treated and untreated cells in these lines. After induction of PIN7 or PGP19-HA expression, identical phenotypical changes occurred: cells ceased to divide, started to elongate, and formed and accumulated starch granules (Fig. 1A,B,D,E). A similar set of morphological changes was observed in induced *GVG-PIN4* and *GVG-PIN6* lines, and in a line constitutively expressing PIN1-GFP that also showed increased auxin efflux (see Fig. S1A-D,H,I in the supplementary material). Importantly, this cellular behaviour could be mimicked by cultivation of cells in medium with a decreased amount of or no auxin (see Fig. S1E-G in the supplementary material). These experiments imply that the enhanced efflux after induction of either PIN7 or PGP19-HA expression leads to depletion of auxin from cells, resulting in a change in the developmental program reflected by the switch from cell division to cell elongation.

All phenotypic changes induced by overexpression of PIN7 in the *GVG-PIN7* line were completely reversed by application of the auxin efflux inhibitor NPA (Fig. 1C,G; see Fig. S1J,K in the supplementary material). By contrast, after induction of PGP19-HA expression in the *GVG-PGP19-HA* line, NPA treatment was ineffective in rescuing auxin starvation phenotypes (Fig. 1F,G; see Fig. S1J,K in the supplementary material). These observations are in line with previously reported differences in sensitivities of PGP19- and PIN7-mediated auxin efflux to NPA (Petrášek et al., 2006). These data collectively suggest that, although PGP and PIN proteins play similar cellular roles in mediating auxin efflux and inducing the switch from cell division to cell elongation, they define two distinct auxin efflux mechanisms that differ in sensitivity to auxin efflux inhibitors.

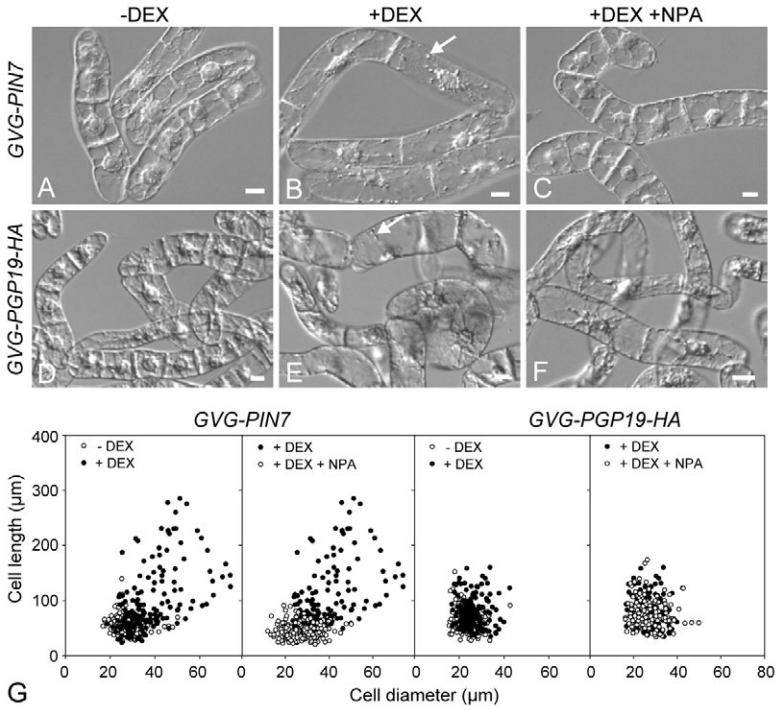

Fig. 1. Identical phenotypes indicative of auxin starvation in DEX-induced expression of *PIN7* or *PGP19* in BY-2 cells. (**A**,**B**,**D**,**E**) Effect of DEX induction in *GVG-PIN7* and *GVG-PGP19-HA* tobacco cell lines. Non-induced *GVG-PIN7* (A) and *GVG-PGP19-HA* line (D). *GVG-PIN7* (B) and *GVG-PGP19-HA* (E) cells after 3 days of cultivation with DEX, showing decrease in cell division, increase in cell elongation and formation of starch-containing amyloplasts (arrows in B,E). (**C**,**F**) Chemical inhibition of auxin transport (10 μM NPA) reversing these defects in DEX-induced *GVG-PIN7* cells (C) but not in DEX-induced *GVG-PGP19-HA* cells (F). Scale bars: 20 μm. (**G**) Depiction (reciprocal plots) of the cell size distribution (cell length and cell diameter) after NPA treatment (10 μM, 3 days) in DEX-induced *GVG-PIN7* and *GVG-PGP19-HA* cells scored at day 3 after inoculation. Non-induced *GVG-PIN7* and *GVG-PGP19-HA* cells were used as a control.

Effect of PIN- and PGP-inducible overexpression in *Arabidopsis* seedlings

To study what effects overexpression of PINs and PGPs may have at the multicellular level, we analysed transgenic lines overexpressing PIN1, PGP1 or PGP19. After induction of PIN1 expression in the estradiol-inducible *XVE-PIN1* line (Petrášek et al., 2006) (Fig. 2A,B), seedlings lost the gravitropic response and had retarded root growth (Fig. 2F,R). This effect was stronger at increased concentrations of oestradiol, supporting the rate-limiting function of PIN proteins (Fig. 2R). Immunolocalization confirmed that ectopically expressed PIN1 was localized predominantly on the basal side of root epidermal cells (Fig. 2A,B), consistent with previous reports (Wiśniewska et al., 2006). In hypocotyls of dark-grown seedlings, we observed a previously uncharacterized phenotype. Unlike the straight-growing hypocotyls of non-induced plants, induced *XVE-PIN1* plants had a twisted growth along the vertical axis. Twisting was usually more pronounced close to the hypocotyl base (Fig. 2I,J). To test for possible changes in auxin distribution after induction of PIN1 expression, we crossed the *XVE-PIN1* line with the *DR5::GUS* reporter line, a widely used auxin response reporter (Hagen and Guilfoyle, 2002). *DR5::GUS* staining of the *XVE-PIN1* line after estradiol treatment suggested a stronger auxin accumulation in the root tip, which rationalizes the root agravitropic phenotype (Fig. 2N,O). In dark-grown seedlings, weak uniform GUS staining along the hypocotyl axis was seen in non-induced controls. This pattern changed after induction of PIN1 expression, and DR5 signal became stronger, with random maxima along the hypocotyl (Fig. 2L,M) reflecting differential cell elongation and hypocotyl twisting. Similar to the situation in BY-2 cells, PIN1-inducible overexpression phenotypes (root elongation and hypocotyl twisting) could be partially rescued by exogenous application of NPA (Fig. 2K,S). Notably, similar phenotypic aberrations and NPA treatment-based rescue were also detected at different levels of induced PIN expression (data not shown) and in other PIN1-overexpressing lines, such as DEX-inducible *GVG-PIN1* and constitutive *35S::PIN1* (data not shown). This confirmed that the phenotypic changes and changes in patterns of DR5 activity observed are due to the overexpression of PIN1 protein.

For comparison, we analysed *GVG-PGP1-myc* and *GVG-PGP19-HA* lines, which conditionally overexpress functional PGP1-*myc* and PG19-HA versions. As confirmed by the activity of the co-regulated GUS reporter construct (see Fig. S2A,B in the supplementary material), RT-PCR (see Fig. S2C,D in the supplementary material) and immunolocalization (Fig. 2C,D), both PGP1-*myc* and PGP19-HA were overexpressed after DEX treatment and localized mostly symmetrically at the plasma membrane (Fig. 2C,D). This confirmed induction of PGP1-*myc* and PGP19-HA expression, however, did not lead to gravitropic defects (data not shown), reduced root growth (Fig. 2G,H,T) or twisting of the dark-grown hypocotyls (data not shown). Instead, *GVG-PGP19-HA* seedling showed reduced outgrowth of cotyledons (Fig. 2G) and slightly shorter dark-grown hypocotyls (Fig. 2T). The *DR5rev::GFP* construct (Friml et al., 2003) introduced into *GVG-PGP19-HA* plants revealed a reduced *DR5* activity in the root tip, contrasting with the increased *DR5* signal after PIN1 overexpression (Fig. 2P,Q). Furthermore the sensitivity to NPA was not visibly altered in these lines (Fig. 2T).

Although other post-transcriptional events may influence the outcome of these overexpression experiments, observations that overexpression of PIN1 when compared with that of PGP1 and PGP19 leads to qualitatively different phenotypes suggest that, unlike in cultured cells, overexpression of PINs and PGPs in planta have different effects on auxin distribution and seedling development. This hypothesis is consistent with a scenario in which PIN and PGP efflux machineries are distinct and might have both overlapping and distinct functions in auxin transport-dependent development.

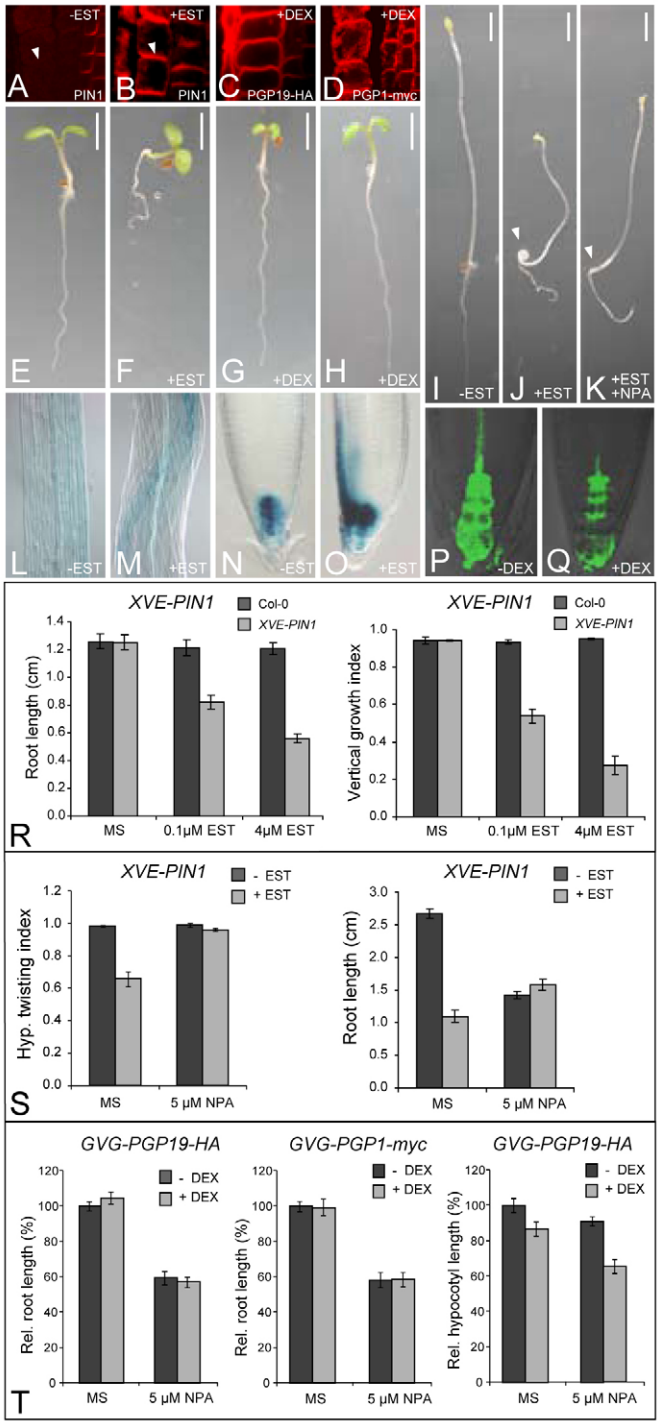

Fig. 2. Differential effect of PIN1 and PGP19 overexpression in *Arabidopsis* seedlings. (**A**,**B**) Immunolocalization of PIN1 in the *XVE-PIN1* line without (A) and with estradiol (B) induction. Ectopically expressed PIN1 localizes to the basal (lower) side of epidermal cells (arrowheads). (**C**,**D**) Immunolocalization of DEX-induced PGP19-HA in *GVG-PGP19-HA* (C) and PGP1-*myc in GVG-PGP1-myc* (D) lines show non-polar localization in epidermal cells. (**E-H**) Differential effects of PIN1, PGP19-HA and PGP1-*myc* overexpression on seedling development. Non-induced control (E); reduced root length and gravitropic response by induced PIN1 expression (F); no dramatic phenotypes caused by induced PGP19-HA (G) and PGP1-*myc* (H) expression, apart from a reduction in cotyledon outgrowth in the DEX-treated *GVG-PGP19-HA* line (G). (**I-K**) PIN1 overexpression phenotypes in dark-grown seedlings: straight hypocotyls in untreated controls (I); hypocotyl twists in estradiol-treated seedlings (J); this phenotype is almost completely reversed by the auxin transport inhibitor NPA (K). (**L-O**) Changes in the *DR5::GUS* auxin response reporter expression after PIN1 overexpression: *DR5::GUS* is weakly and equally expressed in hypocotyls of dark-grown seedlings (L), but shows randomly distributed local maxima that correlate with unequal cell elongation after PIN1 induction (M); GUS signal in the root tip is confined to the columella in the non-induced control (N), but increases and extends to the lateral root cup after PIN1 induction (O). (**P**,**Q**) Reduction of *DR5rev::GFP* signal in the columella after PGP19-HA expression (Q) when compared with untreated controls (P). (**R**) Concentration-dependent effect of estradiol-induced PIN1 overexpression on root elongation and gravitropism (calculated as vertical growth index (VGI) (Grabov et al., 2005). (**S**) Hypocotyl twisting and inhibition of root length following PIN overexpression can be reversed by NPA. (**T**) PGP1-*myc* and PGP19-HA overexpression have no pronounced effects on root growth, hypocotyl growth in the dark or sensitivity to NPA. Scale bars: 3 mm. Error bars represent s.e.m., *n*=20.

PGP proteins are expressed and synergistically interact with PIN1 protein during embryogenesis

To study whether distinct PIN- and PGP-dependent transport mechanisms have common developmental roles, we studied role of PIN- and PGP-dependent transport during different auxin transport-mediated processes. Many data on the developmental roles of various members of the PIN family are available (Tanaka et al., 2006), but comparable information for PGP1 and PGP19 is still largely lacking.

First, we studied the involvement of PIN- and PGP-dependent transport during *Arabidopsis* embryogenesis, which is known to be regulated by PIN-dependent asymmetric auxin distribution (Friml et al., 2003; Weijers et al., 2005). Various PIN proteins are expressed at different stages of embryogenesis in distinct cells, and *PIN* gene mutants (single and double), as well as mutants with defective PIN localization, are defective in embryo patterning (Steinmann et al., 1999; Friml et al., 2003; Friml et al., 2004; Weijers et al., 2005; Michniewicz et al., 2007). However, in *pgp* mutants, no embryo patterning defects have been reported and the function of PGPs in embryogenesis has not been studied so far. To investigate expression and cellular localization of PGP1 and PGP19 during embryogenesis, we used the functional tagged constructs *pPGP1::PGP1-myc* (Blakeslee et al., 2007) and *pPGP19::PGP19-GFP* (see Fig. S3 in the supplementary material). PGP1-*myc* was expressed from the earliest embryo stages onwards in all pro-embryo and suspensor cells. Cellular localization was mainly apolar, but more intense signals were observed between freshly divided cells (Fig. 3A,B). PGP19-GFP was also expressed at early stages, predominantly in the basal cell lineage that forms the suspensor until approximately the octant stage (Fig. 3C). At dermatogen (16-cell) stage, PGP19-GFP expression extended from suspensor to the lower tier cells of the pro-embryo (Fig. 3D). At mid-globular and later stages, PGP19-GFP expression was gradually confined to the outer layers, including protoderm and cells surrounding vascular precursor cells with no expression between initiating cotyledon primordia (Fig. 3E). At later stages, PGP19-GFP expression persisted in cells surrounding the forming vasculature (pericycle, endodermis and protoderm) (Fig. 3H). As for PGP1, no apparent polar localization

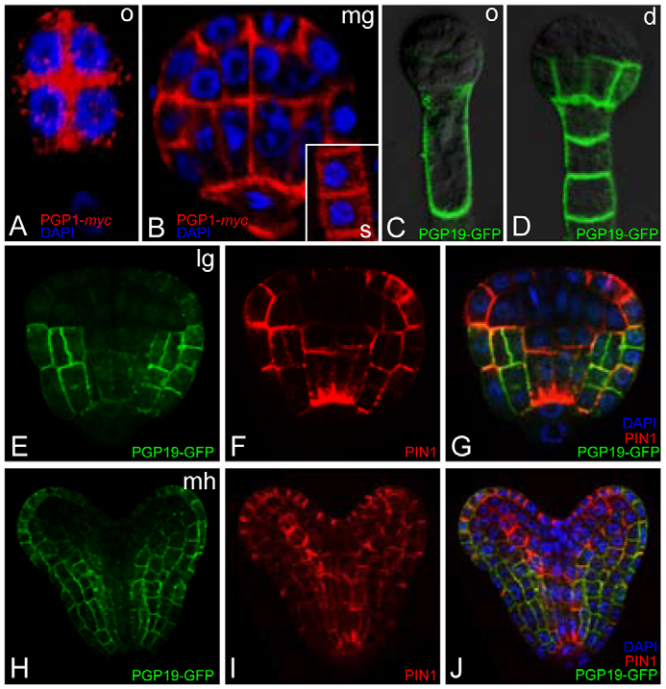

Fig. 3. Expression and localization of PGP1 and PGP19 during *Arabidopsis* embryogenesis. (**A**,**B**) Immunolocalization of PGP1-*myc* in *Arabidopsis* embryos (PGP1 in red, DAPI in blue). Expression of PGP1-*myc* in all cells and non-polar localization to the plasma membrane at octant (o) (A) and mid-globular (mg) (B) stages. Inset shows staining in the suspensor (s). (**C**,**D**) PGP19-GFP localization during early embryogenesis. PGP19-GFP localizes apolarly to the plasma membrane in derivatives of the basal cells at the octant stage (C) and in the suspensor and lower tier cells at the dermatogens (d) stage (D). (**E-J**) Restriction of the expression of PGP19-GFP at later stages of embryogenesis to protoderm and cells surrounding the vascular primordium, which is mainly complementary to PIN1 expression. Immunolocalization at late-globular (lg) (E-G) and mid-heart (mh) stages (H-J) of PGP19-GFP (green) (E,H), PIN1 (red) (F,I). (G,J) Overlay of PIN1, PGP19 and DAPI (blue).

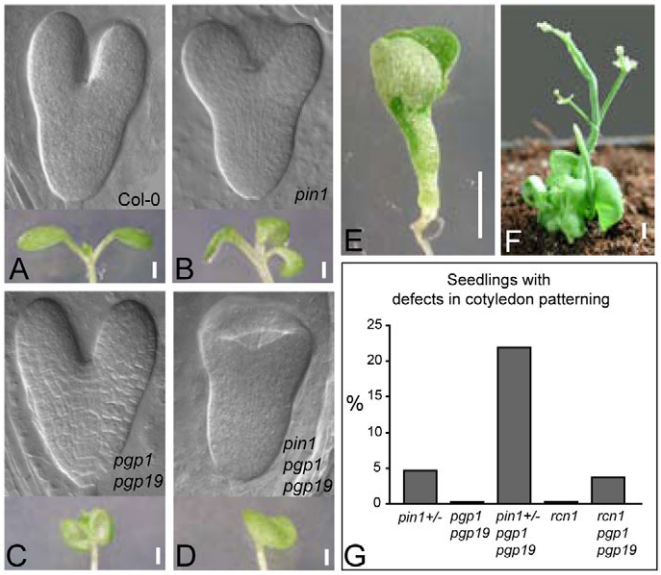

Fig. 4. Genetic interaction of PGPs with PIN1 during embryonic leaf formation. (**A-D**) Synergistic interaction of *pgp1pgp19* and *pin1* during cotyledon formation. Typical defects in cotyledon formation during embryogenesis and their postgermination appearance are shown: wild type (A), *pin1* (B), *pgp1pgp19* (C) and *pin1pgp1pgp19* (D). (**E**) Cup-shaped cotyledons of the *pin1pgp1pgp19* seedling that are rarely seen in the *pin1* mutant. (**F**) Strong enhancement of the *pin1* phenotype in post-embryonal development by the *pgp1pgp19* mutation. An adult, 5-week-old, plant with extremely dwarf appearance, reduced leaf number and apical dominance is shown. (**G**) Quantification of frequencies of cotyledon defects in different mutants and their combinations (n=200). Scale bars: 1 mm in A-D; 5 mm E,F.

was observed. Interestingly, the PGP19-GFP expression pattern at globular and later stages was roughly complementary to that of PIN1 (Fig. 3E-J). PGP19-expressing cells appeared to separate the inner basipetal and outer acropetal auxin streams, which are defined by PIN1 expression and localization (Friml et al., 2003; Vieten et al., 2005) (Fig. 3F,I).

To investigate the function of PGPs and eventually PGP-PIN interactions for embryonic development, we analysed young seedlings of *pin1*, *pgp1pgp19* and *pin1pgp1pgp19* genotypes. No patterning defects were observed in *pgp1pgp19* double mutant seedlings (Fig. 4C,G). In the progeny of *pin1*+/– plants, ~5% of seedlings had a defective cotyledon formation, including tricots and fused cotyledons (Fig. 4B,G), as reported previously (Okada et al., 1991). In the progeny of *pgp1pgp19pin1*+/– plants, almost 25% of seedlings had fused cotyledons and some were cup-shaped, a feature that is extremely rarely seen in *pin1* seedlings (Fig. 4D,E,G). Corresponding mutant phenotypes were also seen during embryogenesis at heart and later stages (Fig. 4A-D). As reported (Blakeslee et al., 2007), later post-embryonic development in *pin1pgp1pgp19* mutants was also very strongly affected (Fig. 4F).

These results reveal a previously unknown role of PGPs during embryogenesis. PGP1 and PGP19 are not strictly required for embryo development, but they act synergistically with PIN1 protein, mainly during cotyledon formation.

PGP genetically interacts with RCN1 in embryogenesis and root development

To test further the functional interaction between PGP- and PIN-dependent auxin transport systems in embryogenesis and patterning, we generated *rcn1pgp1pgp19* mutant that, besides lacking PGP-mediated efflux, is defective in *RCN1* (*Root Curling on NPA*). This gene encodes a subunit of protein phosphatase 2A (PP2A) that has been shown to be involved in various developmental and signalling processes (Garbers et al., 1996; Kwak et al., 2002; Larsen and Cancel, 2003). Importantly, PP2A phosphatase, together with PINOID kinase, regulates PIN polar targeting and, thus, directionality of PIN-dependent auxin transport (Friml et al., 2004; Michniewicz et al., 2007). Some features of the *rcn1* mutant are similar to those of the *pgp1pgp19* mutant, such as wavy root growth, but they do not include embryonic patterning defects (Garbers et al., 1996) (Fig. 4G). Strikingly, *rcn1pgp1pgp19* triple mutants exhibited strong embryonic and post-embryonic auxin-related phenotypes. Some seedlings of the *rcn1pgp1pgp19* mutant were defective in apical-basal patterning (11/97) or cotyledon formation (3/97) (Fig. 5E,F). During embryogenesis, *rcn1pgp1pgp1* exhibited aberrant hypophysis divisions (16/20) at the globular stage (Fig. 5A-D). This spectrum of developmental aberrations is typical for mutants with

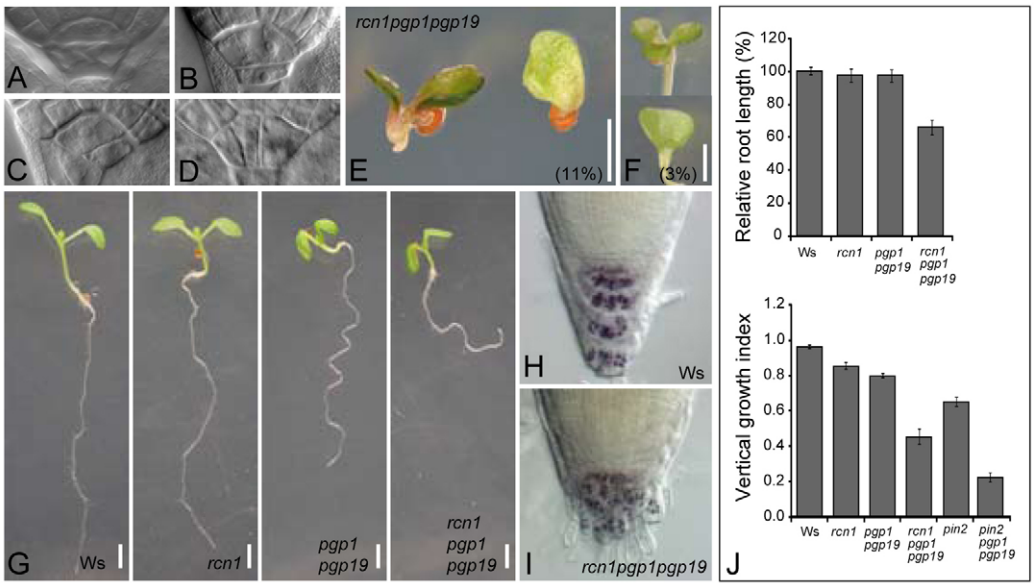

Fig. 5. Genetic interaction of PGPs with RCN1. (**A-D**) Aberrant cell divisions of the hypophysis at the globular stage in *rcn1pgp1pgp19* mutant embryos. The wild-type hypophysis divides into two derivatives: the smaller lens-shaped cells and bigger basal cells (A). Different aberrations in the cell division of the *rcn1pgp1pgp19* mutant (B-D). (**E**,**F**) Rootless (E) and cotyledon patterning (F) defects in *rcn1pgp1pgp19* seedlings (*n*=97). (**G**) Enhanced defects in root elongation and gravitropism in 10-day-old seedlings of *rcn1pgp1pgp19* as compared with controls. (**H**,**I**) Defects in root tip organization, visualized by a lugol staining in *rcn1pgp1pgp19* (I) when compared with wild type (H). (**J**) Quantification of root length and gravitropism phenotypes of the *rcn1pgp1pgp19* mutant. For comparison, *pin2* and *pin2pgp1pgp19* data are also included (*n*=25). Scale bars: 2 mm. Error bars represent s.e.m.

strong defects in auxin transport (for example, *pin1pin3pin4pin7*) (Friml et al., 2003) or auxin signalling (*monopteros* and *bodenlos*) (Hardtke and Berleth, 1998; Hamann et al., 2002). In post-embryonic development, roots of *rcn1pgp1pgp19* seedlings were reduced in length and showed enhanced defects in the gravitropic response and differentiation of columella cells when compared with *rcn1* single or *pgp1pgp19* double mutants (Fig. 5H-J).

This observation further confirms that PGP function significantly contributes to auxin-mediated patterning processes and supports a scenario in which PGP- and PIN-dependent transport systems functionally interact during embryogenesis at the level of the whole transport systems rather than directly through protein interactions.

Diverse functions of PGP1 and PGP19 in lateral root organogenesis

Next, we studied the role of PGP-dependent auxin efflux and its possible interaction with PIN-dependent mechanisms in lateral root initiation and emergence, other processes that involve PIN-dependent auxin transport. Pharmacological or genetic modulation of local transport-dependent auxin distribution inhibits lateral root initiation and its morphogenesis (Benková et al., 2003; Casimiro et al., 2003). The role of PGP1 and PGP19 in lateral root development has already been proposed (Lin and Wang, 2005; Wu et al., 2007), but their precise function and interaction with PINs in initiation and emergence remains unknown.

We determined expression and localization patterns of PGP1 and PGP19 during early post-embryonic development by using *pPGP1::PGP1-GFP* and *pPGP19::PGP19-GFP* constructs. *pPGP1::PGP1-GFP* and *pPGP19::PGP19-GFP* complemented most aspects of the corresponding *pgp1* and *pgp19* mutant phenotypes, such as hypocotyl elongation defect (see Fig. S3A-E in the supplementary material). Similarly to expression during embryogenesis, PGP1-GFP did not exhibit tissue-specific expression pattern and was detected in all cells of hypocotyls and roots (Fig. 6A,C,D), except root-tip columella cells. The expression of PGP19-GFP was also found in hypocotyls and main roots, but, in contrast to PGP1-GFP, exhibited a more tissue-specific pattern, with strongest expression in endodermal and pericycle tissues (Fig. 6B,E,F), in agreement with published data on PGP19-HA (Blakeslee et al., 2007) and MDR1(PGP19)-GFP (Wu et al., 2007). In addition, PGP19-GFP expression was detected also in root tip epidermal cells, which is not supported by the published pattern of MDR1(PGP19)-GFP (Wu et al., 2007). Both proteins are also expressed during all stages of lateral root development and persisted after lateral root emergence as shown previously (Geisler et al., 2005; Wu et al., 2007). PGP1-GFP expression was observed from the first stage of lateral root primordium organogenesis on (Fig. 6G). During developmental stage I, PGP1-GFP was localized to anticlinal membranes of short initials, and, later, when lateral root primordia are formed, apolar membrane localization was detected in all cells of primordia. Expression and membrane localization of PGP19-GFP at developmental stage I fully overlapped with PGP1, but at later developmental stages PGP19-GFP expression was more restricted to endodermis and pericycle. In emerged lateral roots, PGP19-GFP was detected also in cortical and epidermal cells, similar to its expression in primary root tips (Fig. 6H).

Next, we investigated the consequence of PGP1 and PGP19 loss in lateral root initiation and emergence, and their functional interaction with PIN1 in this process. Both *pgp* single mutants initiated fewer lateral roots (Fig. 6I), whereas, interestingly, the combination of *pgp1* and *pgp19* mutations almost completely rescued the effect of single mutations (Fig. 6I). This genetic complementation of *pgp1* and *pgp19* mutations was also observed during lateral root emergence (Fig. 6J). Both single mutants had a reduced progression rate through consecutive stages of lateral root development, but this defect was largely recovered in *pgp1pgp19*

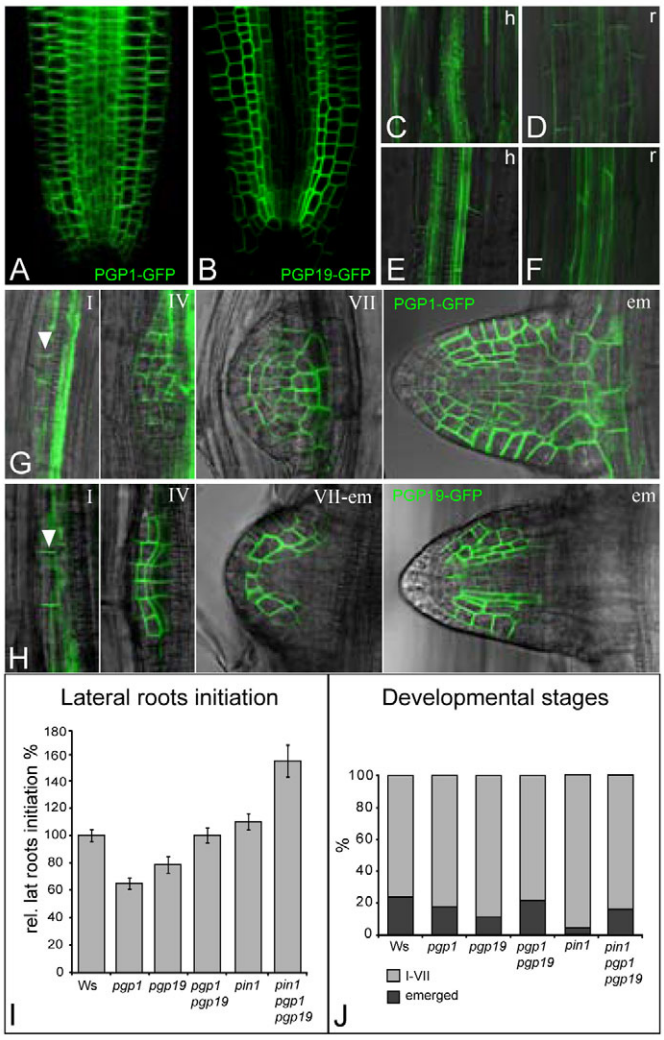

Fig. 6. Post-embryonic expression and the role of PGP1/PGP19 in lateral root development. (**A**,**B**) Expression and localization of PGP1-GFP and PGP19-GFP in root tips of 5-day-old seedlings. PGP1-GFP is expressed in all cells, except the columella (A); PGP19-GFP expression is more restricted to endodermal and pericycle cells (B). (**C-F**) Expression of PGP1-GFP and PGP19-GFP in hypocotyls and main root. PGP1-GFP is expressed in all cells of hypocotyls (C) and main root (D), whereas PGP19-GFP expression is more restricted to cells surrounding vascular tissues in hypocotyls (E) and main root (F). h, hypocotyl; r, root. (**G**,**H**) Expression of PGP1-GFP and PGP19-GFP during lateral root development. PGP1-GFP expression is detected in all cells during all stages (G) (indicated) and that of PGP19-GFP is more confined at later stages (indicated) to the new forming endodermal and pericycle cells (H). Arrowheads indicate the localization of PGP1/PGP19-GFP on anticlinal membranes at stage I. (**I**) Initiation and (**J**) emergence phenotypes of *pgp1*, *pgp19*, *pin1*, *pgp1pgp19* and *pin1pgp1pgp19* mutants (*n*=40).

double mutants (Fig. 6J). Addition of *pin1* to the *pgp1pgp19* mutant surprisingly led to rescue of *pin1* phenotype, which is characterized by delayed lateral root primordium development (Benková et al., 2003), and even slightly increased lateral root initiation above wild-type level (Fig. 6I,J).

These analyses show that functions of PGP1, PGP19 and PIN1 are required for lateral root formation and suggest a complex interaction of these proteins at multiple stages of the process.

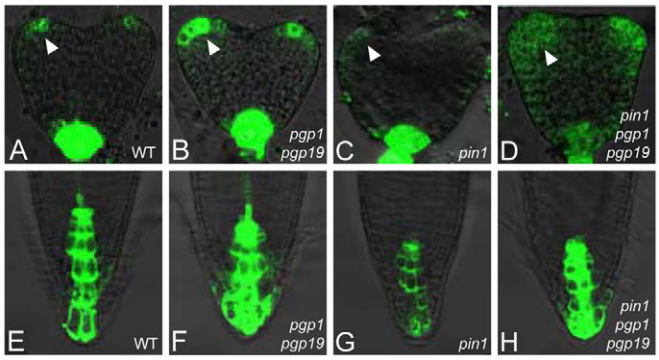

Fig. 7. Role of PGPs and PINs in the regulation of the spatial distribution of the auxin response. (**A-H**) Roles of PGP1/PGP19 and PIN1 in auxin response distribution (as visualized by *DR5rev::GFP*) in heart-stage embryos (A-D) and root tips (E-H). Wild type (A,E). Increased signal in *pgp1pgp19* (B,F), decreased signal in *pin1* (C,G) and pronounced defects in the distribution of the DR5 signal in the *pin1pgp1pgp19* embryos (D) are seen, but restoration occurs in roots (H). At least five roots or embryos from all mutant combination were simultaneously analysed in two independent experiments (for *pin1* and *pin1pgp1pgp19* mutants, only embryos with visible phenotypes were analysed). Arrowheads in A-D indicate auxin maxima in cotyledon primordia.

Antagonistic and synergistic effects of PIN- and PGP-dependent transport on the spatial pattern of auxin responses

To gain further insights into how PGP- and PIN-mediated transport mechanisms together regulate auxin-dependent plant development, we analysed changes in auxin distribution, as visualized indirectly by the *DR5rev::GFP* auxin response reporter (Friml et al., 2003). During embryogenesis, spatial distribution of *DR5rev::GFP* signal did not dramatically change in *pgp1pgp19* mutants, but auxin response maxima in cotyledon primordia and at the root pole were enhanced (Fig. 7A,B). Conversely, *pin1* mutant embryos had a reduced *DR5* signal at the root pole and cotyledon primordia (Fig. 7A,C). When *pin1* and *pgp1pgp19* mutations were combined, the spatial pattern of *DR5* activity distribution in embryos was strongly distorted. The *DR5* activity maxima were less well defined and *DR5* signal was more diffuse (Fig. 7D). These results clearly show that both PGP- and PIN1-mediated transport systems are required together for the spatial pattern of auxin distribution and formation of well-defined auxin maxima during embryogenesis. This observation also fully explains the observed synergistic genetic interaction between *pin1* and *pgp1pgp19* mutations (Fig. 4A-E,G).

In post-embryonic roots, the situation is somewhat different: maintenance of auxin maxima in the quiescent centre/columella region is crucial for controlling root meristem activity (Sabatini et al., 1999; Friml et al., 2002a; Grieneisen et al., 2007). We detected quantitative changes in *DR5* activity similar to those during embryogenesis, including and increase in *DR5* activity in *pgp1pgp19* mutants, but a reduction in *pin1* mutant roots (Fig. 7E-G). However, when *pgp1pgp19* and *pin1* mutations were combined, the spatial pattern of *DR5* activity was not impaired and the level of activity was roughly restored to that of the wild type (Fig. 7H). This apparent difference between the requirements of PGP and PIN transport systems for spatial patterning of the auxin response in embryos and seedling roots is probably due to pronounced

functional redundancy of PIN proteins for auxin delivery to the central root meristem during post-embryonic development (Blilou et al., 2005; Vieten et al., 2005).

Opposite roles of PIN and PGP transport mechanisms were also observed during auxin redistribution after gravitropic stimulation (Lin and Wang 2005), where auxin redistribution to the lower side of gravistimulated root is more pronounced in *pgp1pgp19* (see Fig. S4A,B in the supplementary material), but inhibited in *pin2* mutants (Luschnig et al., 1998). Contrasting functions of PIN and PGP are also supported by the synergistic effects of PIN1 gain-of-function and PGP loss-of-function alleles. For example, defects in root elongation and hypocotyl twisting were more pronounced after *XVE-PIN1* induction in *pgp1pgp19* mutant than they were after PIN1 induction in wild type (Fig. 8A).

In summary, these data show that even when PGP- and PIN-dependent auxin transport mechanisms have opposite or even antagonistic effects on auxin distribution and development during several developmental processes, both systems are complementary and are required together to maintain a dynamic spatial pattern of auxin distribution and subsequent development.

DISCUSSION

Carrier-mediated auxin efflux is considered to be the crucial step in the intercellular auxin transport and is required for multitude of auxin distribution-dependent developmental processes (Tanaka et al., 2006; Vieten et al., 2007). PGP and PIN proteins from *Arabidopsis* are both involved in auxin efflux (Geisler et al., 2005; Petrášek et al., 2006) and can physically and functionally interact in mediating this process (Blakeslee et al., 2007). However, the developmental relevance of the PGP and PIN interactions is unclear. Here, we have systematically studied the distinct and common roles of both auxin efflux systems in auxin distribution-dependent development and provide new insights into understanding the purpose of these two independent auxin efflux mechanisms for regulating plant development.

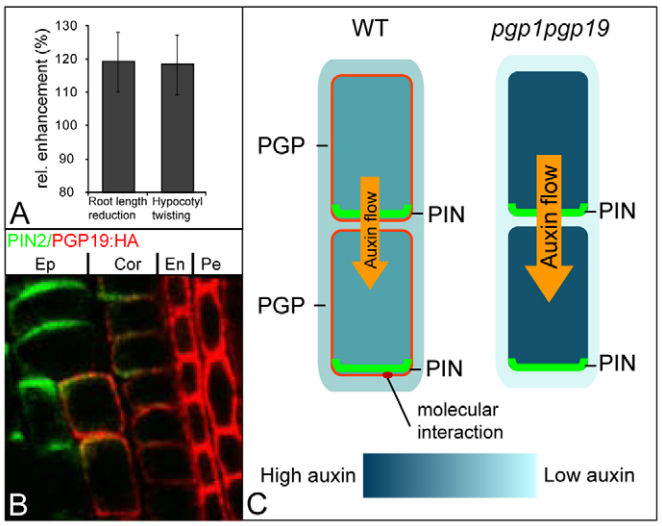

Fig. 8. Model for interaction of PGPs and PINs in the local auxin distribution in meristematic tissues. (**A**) Enhanced effects (~20%) of estradiol-induced *PIN1* overexpression on root length and hypocotyl twisting in the *pgp1pgp19* mutant when compared with wild type, confirming the antagonistic roles of PIN1 and PGP1/PGP19 in seedling development. Error bars represent s.e.m., *n*=20. (**B**) Immunolocalization of PIN2 and PGP19-HA. Polar and non-polar localization of PIN2 and PGP19-HA in the root epidermis, respectively. Expression of PGP19 is higher in the endodermis and the pericycle that form the border between acropetal and basipetal auxin streams. (**C**) Model of PIN and PGP interaction. PGPs and PINs interact intermoleculary at the PIN-containing polar domain, possibly regulating the PIN stability in the plasma membrane. The PGPs remaining in these cells control the cellular auxin pool available for the PIN transport. In *pgp1pgp19*, the cellular auxin concentration is increased and, therefore, the PIN transport is enhanced but less focused.

PGPs and PINs define two distinct auxin efflux systems

Since the identification of both PINs and PGPs as being involved in the same process of cellular auxin efflux (Geisler et al., 2005; Petrášek et al., 2006), an important issue is whether they represent independent transport systems or act together as necessary parts of one transport system. Our results strongly support the scenario in which PGPs and PINs characterise two distinct auxin efflux mechanisms. The earlier observations of the largely non-overlapping phenotypes observed in *pin* and *pgp* loss-of-function mutants (Vieten et al., 2007) indicated a different role for these protein families that could be explained by the distinct expression patterns of the family members. More significantly, when both proteins are overexpressed under comparable general promoters in the present study, the resulting phenotypes, although similar in cultured cells, appear to be distinct in planta, and show different sensitivities to the auxin efflux inhibitor NPA. Phenotypes resulting from PIN overexpression can be reversed by NPA, but similar sensitivities to NPA are not possible to demonstrate in PGP overexpression lines. The different effects of PIN and PGP overexpression, together with the different responses of these transporters to inhibitors, clearly favours the scenario that PIN and PGP protein families define two distinct auxin efflux machineries.

However, the identity of the molecular mechanism that underlies the effect of auxin efflux inhibitors such as NPA still has to be clarified. Previous reports have clearly shown that NPA binds PGP proteins (Noh et al., 2001; Murphy et al., 2002; Rojas-Pierce et al., 2007), that PGP-mediated auxin efflux activity is inhibited by NPA in heterologous systems (Petrášek et al., 2006; Blakeslee et al., 2007) and that NPA inhibits PGP19 action in phototropism (Nagashima et al., 2008). It is possible that NPA and other auxin efflux inhibitors have multiple binding and regulatory sites with different affinities. Moreover, auxin efflux inhibitors might have multiple effects, including modification of actin-based subcellular dynamics (Dhonukshe et al., 2008) or of the PGP-PIN interaction (Blakeslee et al., 2007).

PGP- and PIN-mediated transport are required together for embryogenesis and organogenesis

Another important argument for distinct roles of the PGP- and PIN-dependent transport systems are the divergent phenotypes in *pin* loss-of-function mutants when compared with *pgp1* and *pgp19* mutants. PGP proteins play an important role in determining the plant architecture during vegetative growth (Noh et al., 2001; Lin and Wang, 2005; Wu et al., 2007). For example, agriculturally interesting dwarf mutations in maize and sorghum results from loss of PGP activity and from a reduction in auxin transport (Multani et al., 2003). However, *pgp* mutants do not show clear patterning defects, whereas *pin* single and multiple mutants show defects in embryogenesis, organogenesis, tissue differentiation and meristem activity (Tanaka et al., 2006; Vieten et al., 2007). We found that an important role of PGP1 and PGP19 in patterning processes can be unmasked if the PIN-

dependent transport is compromised. During embryogenesis, the *pgp1pgp19* mutation enhances greatly the effect of the *pin1* mutation on the formation of embryonic leaves. Furthermore, analysis of mutant combinations of *pgp1pgp19* and *rcn1* [a gene encoding a regulatory subunit of PP2A phosphatase that does not influence the PIN function directly, but through the phosphorylation-dependent the PIN polar subcellular targeting (Michniewicz et al., 2007)] revealed very strong embryo and seedling patterning phenotypes that are not observed in any of the single combinations. The phenotypes are reminiscent of those found in mutants that are strongly defective in auxin transport, such as *gnom*/*emb30* (Steinman et al., 1999) and *pin1pin3pin4pin7* (Friml et al., 2003) mutants. These results, together with expression and localization patterns of PGP1 and PGP19 during embryogenesis, reveal a previously unknown role of the PGP-dependent auxin transport in development and support the notion that the common functions of these two transport mechanisms contribute to patterning processes.

Concerted action of PGP- and PIN-mediated transports is required for auxin distribution

Auxin transport executes its effect on plant development largely by generating asymmetric auxin distribution, which is often indirectly monitored using the auxin response. The effects of gain- and loss-of-function mutations in PGPs and PIN proteins on the quantity of local auxin response and resulting development are, at times, seemingly opposite. For example, in root tip, PIN1 overexpression, but not *pgp1pgp19* loss of function, enhances auxin accumulation in central root meristem. Conversely, *pin1* loss of function and PGP19 overexpression both produce the opposite effect: a decrease in auxin response in the root-tip region. Similarly, during embryogenesis, the *pin1* mutation decreases auxin response maxima, but *pgp1pgp19* increases them. These results show that PGP- and PIN-dependent transport systems play, in some instances, opposing roles in mediating auxin accumulation in specific cells.

The opposite actions of PGP and PIN proteins can also be observed at the developmental level. Some features of PIN1 overexpression, such as twisting hypocotyls, can also be seen in the *pgp1pgp19* mutant (Noh et al., 2001) or in the mutant of the apparent activator of the PGP function – immunophilin-like protein TWISTED/DWARF (TWD) (Geisler et al., 2003). Furthermore, hypergravitropic root growth in *pgp1pgp19* seedlings (Lin and Wang, 2005) (see Fig. S4 in the supplementary material) contrasts with agravitropic growth of *pin2* or *pin3* seedlings. Significant also are the additive effects of *pgp1pgp19* mutations on the PIN1 overexpression-induced phenotypes. Interestingly, despite these opposite roles for PGPs and PINs in mediating the quantitative distribution of the auxin response, both transport systems are required together for generating proper spatial patterns of auxin distribution. This effect was observed for auxin distribution in *pin1pgp1pgp19* mutant embryos (Fig. 7H) or in *pin2pgp1pgp19* seedlings during the gravitropic response (Blakeslee et al., 2007) (Fig. 5J). Thus, both the PGP- and PIN-dependent auxin transport mechanisms play distinct, sometimes opposite, roles in auxin distribution, but both transport systems cooperate to generate and maintain the spatial pattern of auxin distribution that is necessary for patterning and tropisms.

Model of the PGP and PIN interaction in local auxin distribution in meristematic tissues

Based on previous and novel findings, we propose a model for the functional interaction between PGP- and PIN-dependent auxin transport mechanisms in embryos and root meristems (Fig. 8C). We take into account the prevalent non-polar cellular localization of PGP1 and PGP19 (Fig. 3, Fig. 6A-H, Fig. 8B) as well as the related loss-of function and overexpression phenotypes. In cells where PINs and PGPs are co-expressed, two types of interactions might take place. A direct interaction between PIN and PGP, which takes place at PIN polar membrane domains, contributes to the specificity and modulation of auxin efflux rate (Blakeslee et al., 2007). The proportion of PGPs that do not colocalize with PINs act multilaterally in auxin efflux and, thus, regulate the effective cellular auxin concentration available for PIN-mediated transport. This combined action of PIN and PGP action determines how much auxin flows through auxin channels. Observation of a higher cellular auxin concentration in *pgp* mutants (Bouchard et al., 2006) that might enhance PIN-mediated transport directly supports this scenario. However, establishment of such a specific auxin concentration by enhanced PGP19 expression in cells, where there is no direct PIN1-PGP19 colocalization (such as basal protoderm and endodermal initials in embryos), additionally focuses auxin flow. It is likely that for long-distance transport, e.g. in stems, another mode of PGP and PIN interaction applies, as suggested by strong auxin transport defects in *pgp* mutant stems (Noh et al., 2001; Geisler et al., 2005).

It is important to note that different internal or external cues, such as light, can influence the extent and mode of PIN-PGP interactions, for instance at the level of functional pairing of PINs and PGPs (Blakeslee et al., 2007) or by producing distinct effects on either PIN or PGP functions. Moreover, the activity of previously uncharacterized PGPs may also significantly contribute to auxin transport. In summary, our model could provide an explanation of the existence of two auxin transport mechanisms that ensure precise and proper formation of spatial and temporal auxin distribution in plants.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/20/3345/DC1

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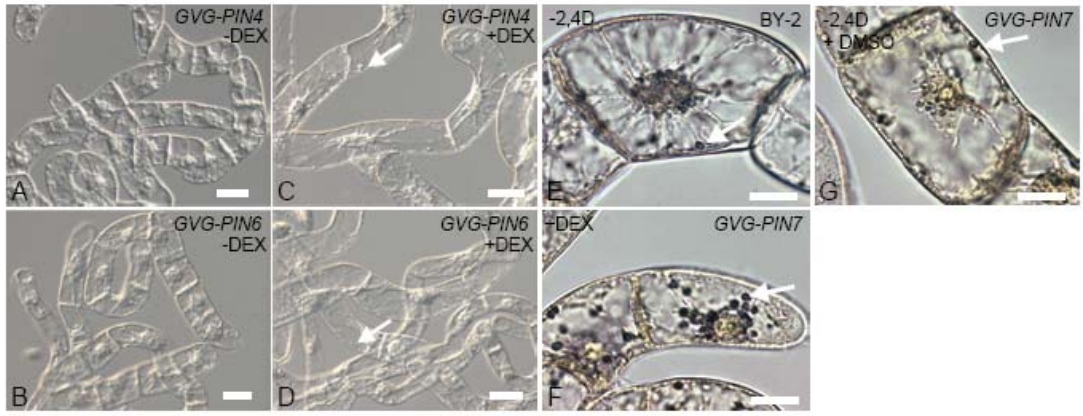

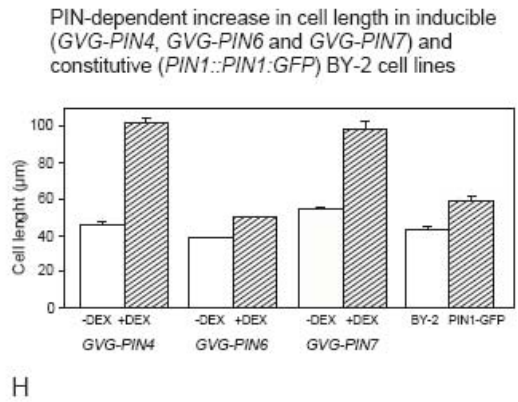

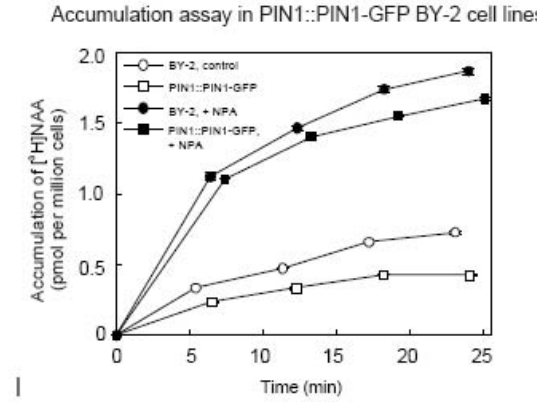

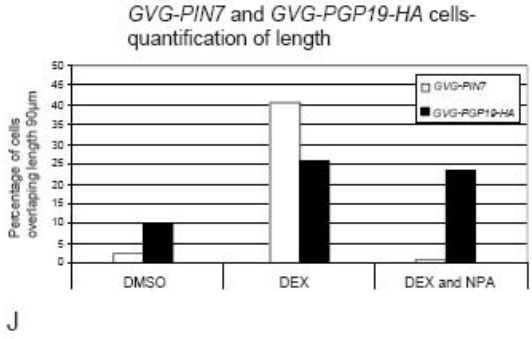

GVG-PIN7 and GVG-PGP19-HA cells
Arithmetic means of lengths and diameters

GVG-PIN7	DMSO		DEX		DEX and NPA	
	length	diameter	length	diameter	length	diameter
aritmetic mean± standard error	54.6 ±1.2	27.6 ±0.41	98.0 ±4.5	36.0 ±1.0	44.3 ±1.1	26.9 ±0.6

GVG-PGP19-HA	DMSO		DEX		DEX and NPA	
	length	diameter	length	diameter	length	diameter
aritmetic mean± standard error	63.8±1.6	23.3 ±0.3	78.0 ± 2.0	25.9 ± 0.4	75.8 ± 1.9	26.8 ± 0.5

K

Fig. S1. Effects of PIN and PGP overexpression in BY-2 cells. (**A-D**) DEX-induced expression of PIN4 and PIN6, resulting in similar phenotypes in *GVG-PIN4* and *GVG-PIN6* BY-2 cells. The phenotype of non-induced *GVG-PIN4* (A) and *GVG-PIN6* cells (B) after 3-day-long cultivation. DEX induction (1 µM) resulted in the stimulated cell elongation and amyloplasts formation (arrows) in both *GVG-PIN4* (C) and *GVG-PIN6* (D) cells. Scale bars: 40 µm. (**E-G**) Induction of BY-2 cell elongation and starch accumulation by auxin starvation. BY-2 cells cultivated in the medium without 2,4-D (E) show defects similar to those after induction of PIN7 or PGP19 expression in *GVG-PIN7* and *GVG-PGP19-HA* lines, namely inhibition of cell division, enhanced elongation and accumulation of starch granules. Non-induced (F) and DEX-induced (G) *GVG-PIN7* cells. Identity of the starch granules (marked by arrows) was confirmed by lugol staining. Scale bars: 20 µm. (**H**) Quantification of increased cell lengths in DEX-induced (+) and non-induced (−) *GVG-PIN4*, *GVG-PIN6* and *GVG-PIN7* cell lines (1 µM DEX, 3 days). Similar increase in cell length was observed in cells constitutively overexpressing PIN-GFP in *PIN1::PIN1-GFP* line at day 3 of the subculture interval. Constitutive expression of PIN1 under natural promoter results in overall prolongation of subculture interval accompanied with decrease in cell division activity (data not shown). Error bars represent standard errors of the mean (n=400). (**I**) Decreased accumulation of ^{3}HNAA in *PIN1::PIN1:GFP* cells (Benková et al., 2003; Zazímalová et al., 2007) reflects increased auxin efflux in

comparison with control BY-2 cells. Interestingly, decrease in the ^{3}HNAA accumulation by ~35% corresponds well with the already reported decrease following inducible expression of *Arabidopsis* PIN 4, 6 and 7 in BY-2 cells (Petrasek et al., 2006). Error bars represent s.e.m. (n=4); where invisible, they are covered by the symbols. (**J**,**K**) Quantification of the effect of DEX and DEX/NPA treatment in *GVG-PIN7* and *GVG-PGP19-HA* lines. (J) For better comprehensibility of our results (Fig. 1G), upper limit (90 μm) for the length of all variants were determined. After DEX induction, the percentage of oversized cells increased in both *GVG-PIN7* and *GVG-PGP19-HA* lines. However, the effect of the NPA treatment was clearly different between *GVG-PIN7* and *GVG-PGP19-HA* lines. Although in the *GVG-PIN7* line, the number of cells exceeding the determined limits was restored to levels nearly the same as in the non-induced variant, increased length and diameter of *GVG-PGP19-HA* was not restored with NPA. (K) The table shows the arithmetic means of all measured cell lengths and diameters expressed with standard error (170 cells in each sample).

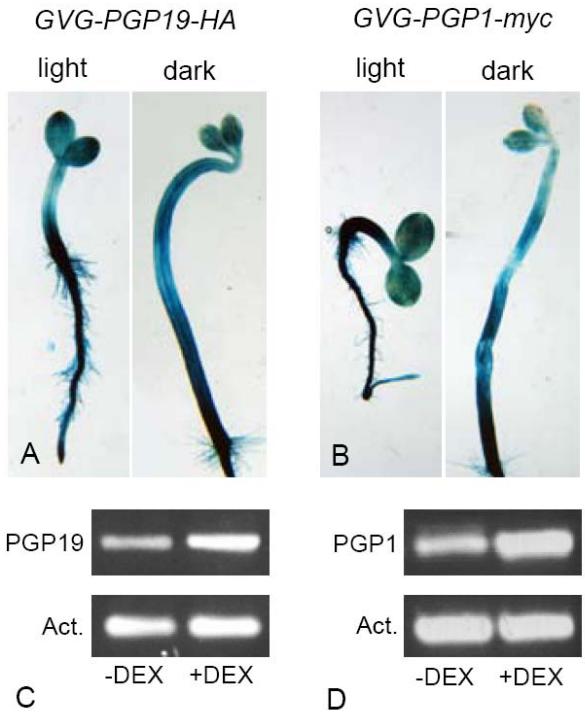

Fig. S2. Analysis of inducible expression of PGP1-*myc* and PGP19-HA in *GVG-PGP lines.* (**A-D**) Inducibility of PGP1 and PGP19 in *GVG-PGP19-HA* (A,C) and *GVG-PGP1-myc* (B,D) lines confirmed by GUS staining of co-regulated *UAS::GUS* reporter construct (A,B) and by RT-PCR (C,D). Strong GUS staining was observed in seedlings germinated on DEX plates either in the light or in darkness.

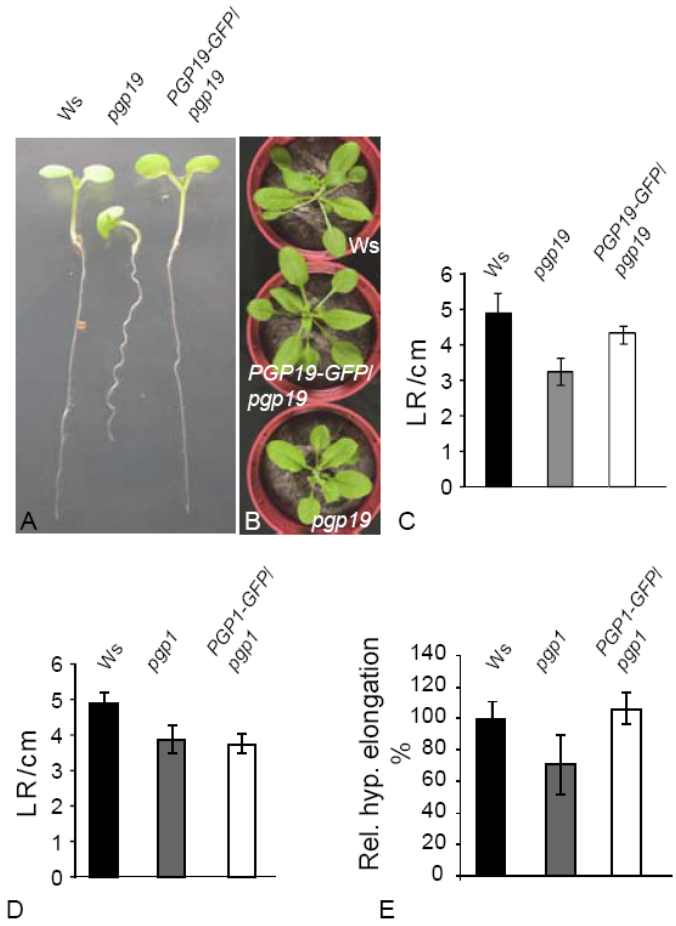

Fig. S3. Complementation of the *pgp19* and *pgp1* mutant phenotypes by the *pPGP19::PGP19-GFP* and *pPGP1::PGP1-GFP* constructs. (**A-C**) Morphological phenotype of *pgp19* mutant (epinastic cotyledons and wavy root growth) in seedlings (A); leaf phenotype in adult plants (B) and partially lateral root formation phenotype (C) could be complemented by the *pPGP19::PGP19-GFP* construct (PGP19-GFP). (**D**,**E**) Lateral root initiation phenotype of *pgp1* mutant could not be complemented by the *pPGP1::PGP1-GFP* (*PGP1-GFP*) construct (D), whereas the hypocotyl elongation phenotype of *pgp1* could be complemented by the *pPGP1::PGP1-GFP* construct (E).

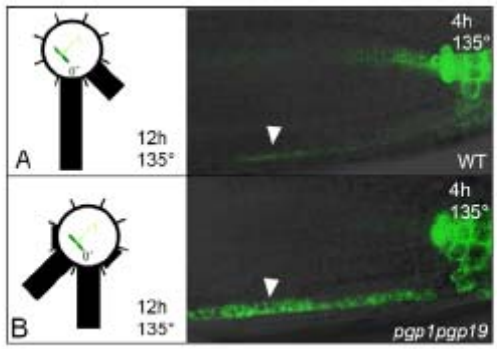

Fig. S4. Opposite effect of *pgp1pgp19* and *pin2* mutations on root gravitropism. (**A**) Normal bending and relocation of the *DR5::GFP* signal in root tip epidermis after 5 hours of gravistimulation in wild type. (**B**) Enhanced relocation of *DR5::GFP* signal in *pgp1pgp19* reflects the enhanced gravitropic bending. The gravitropism diagram was created as described (Petrásek et al., 2006) (12 hours of gravity stimulation, *n*=40).

6.2 Short PINs: Non-canonical role of non-canonical PINs

Mravec J, **Skůpa P**, Bailly A, Hoyerová K, Křeček P, Bielach A, Petrášek J, Zhang J, Gaykova V, Stierhof Y-, Dobrev PI, Schwarzerová K, Rolčík J, Seifertová D, Luschnig C, Benková E, Zažímalová E, Geisler M, Friml J. Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. NATURE 2009;459(7250):1136-40 (IF 2010: 36.101)

This paper is focused predominantly on *At*PIN5 (AT5G16530), one of the three members of the PIN family that were not characterized until then. PIN5 differs significantly from the canonical members of the PIN family, both in gene sequence and in tissue-specific expression. PIN5 almost lacks the large hydrophilic loop, which is rather well conserved in other PM-related PIN auxin efflux transporters and which is localized between two big transmembrane domains of PIN molecules. Together with results obtained on Arabidopsis plants overexpressing the PIN5 protein, these features suggested properties of PIN5 different from those of canonical PINs.

The paper demonstrated that *At*PIN5 localizes unexpectedly to the membranes of the endomembrane system, namely to the membrane of the ER. Based on the ectopic overexpression in yeast cells it was proved that the protein can execute the auxin transport function towards the lumen of ER.

To determine the cause for the observed reduction of DR5 signal and decreased internal level of free IAA after *At*PIN5 overexpression in *Arabidopsis* plants, in our laboratory we used high-performance liquid chromatography (HPLC) to monitor products of metabolism of tritiated IAA in the conditions of BY-2 cells expressing *At*PIN5 under the control of glucocorticoid-inducible system. We demonstrated that change in expression of *At*PIN5 protein affects the intracellular homeostasis of auxin and its metabolic fate, presumably due to the auxin deposition by the PIN5 transporter to the compartment with different enzymatic system (from cytosol to the lumen of ER). HPLC profiling of ^{3}H-IAA metabolites from BY-2 cell suspension culture, allowed us to monitor metabolic changes associated with auxin transport into subcellular compartments and the findings pointed at significant role of PIN5 in the establishment and maintenance of cellular auxin homeostasis.

Moreover the work has suggested, that PIN5 belongs to the new, functionally distinct subfamily within the PIN proteins that is characteristic with short inner loop,

and showed that also other members of that group, *At*PIN6 and *At*PIN8 localize on the endomembranes.

My contribution to this paper consisted of transformation, deriving and verifying the *GVG-PIN*5 BY-2 cell line and of determination of subcellular localization of *At*PIN5 in it. I designed the above-mentioned experimental setup to obtain necessary (indirect) evidence of a functional transport of auxin into the ER in plant cells. I also participated in and coordinated the determination of metabolic profiles of IAA associated with the PIN5-related activity and I contributed to writing the manuscript.

LETTERS

Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter

Jozef Mravec[1,2], Petr Skůpa[3], Aurélien Bailly[4], Klára Hoyerová[3], Pavel Křeček[3], Agnieszka Bielach[1], Jan Petrášek[3,5], Jing Zhang[1,2], Vassilena Gaykova[2], York-Dieter Stierhof[2], Petre I. Dobrev[3], Kateřina Schwarzerová[5], Jakub Rolčík[6], Daniela Seifertová[3], Christian Luschnig[7], Eva Benková[1], Eva Zažímalová[3], Markus Geisler[4] & Jiří Friml[1,8]

The plant signalling molecule auxin provides positional information in a variety of developmental processes by means of its differential distribution (gradients) within plant tissues[1]. Thus, cellular auxin levels often determine the developmental output of auxin signalling. Conceptually, transmembrane transport and metabolic processes regulate the steady-state levels of auxin in any given cell[2,3]. In particular, PIN auxin-efflux-carrier-mediated, directional transport between cells is crucial for generating auxin gradients[2,4,5]. Here we show that *Arabidopsis thaliana PIN5*, an atypical member of the *PIN* gene family, encodes a functional auxin transporter that is required for auxin-mediated development. PIN5 does not have a direct role in cell-to-cell transport but regulates intracellular auxin homeostasis and metabolism. PIN5 localizes, unlike other characterized plasma membrane PIN proteins, to endoplasmic reticulum (ER), presumably mediating auxin flow from the cytosol to the lumen of the ER. The ER localization of other PIN5-like transporters (including the moss PIN) indicates that the diversification of PIN protein functions in mediating auxin homeostasis at the ER, and cell-to-cell auxin transport at the plasma membrane, represent an ancient event during the evolution of land plants.

PIN proteins are plant-specific integral membrane proteins directly involved in auxin efflux from cells[6], the asymmetric localization of which at the plasma membrane determines the directionality of auxin flow[7] (Supplementary Fig. 1). The *Arabidopsis* genome encodes eight PIN-related sequences, five of which have already been characterized in terms of their function in cellular auxin efflux and role in many aspects of auxin-related development[2], including embryogenesis[8], organogenesis[9,10], root meristem patterning[11,12], vascular tissue differentiation and regeneration[13–15], and tropisms[16,17]. PIN5 (At5g16530) belongs to the functionally uncharacterized subclade of PIN proteins formed by *Arabidopsis* PIN5, PIN6 and PIN8 (Fig. 1a) and that also includes PIN5-like proteins from other plant species (Supplementary Fig. 2). The typical feature of these proteins is reduction of the central hydrophilic loop that has been found in all PIN proteins characterized so far (Fig. 1b).

To test whether PIN5 functions, similarly to the conventional PIN proteins, in auxin efflux, we performed an auxin transport assay in a heterologous yeast system. PIN5 tagged with a haemagglutinin epitope (PIN5–HA) was targeted predominantly to the plasma membrane, as was confirmed by sucrose gradient fractionation (Supplementary Fig. 3a). Similarly to what was shown for representative members of the PIN family[6], PIN5 expression in yeast led to increased auxin efflux, as indicated by decreased retention of the radioactively labelled auxins indole-3-acetic acid (IAA) and α-naphthaleneacetic acid (NAA; Fig. 1c). In contrast, transport experiments using yeast microsomes demonstrated that PIN5 did not mediate the transport of the auxin conjugate IAA–aspartate (IAA–Asp; Supplementary Fig. 3b). These results show that, despite the differences in the overall protein structure and the reduced hydrophilic loop, PIN5 can mediate, as other PIN proteins[6], specific auxin export through the plasma membrane.

To analyse the PIN5 expression *in planta*, we fused a *PIN5* genomic fragment with the β-glucuronidase (*GUS*) reporter gene. GUS staining revealed increased PIN5 expression in elongating parts of hypocotyl, cotyledon vasculature and guard cells as well as weaker expression in root pericycle and root tip and at later developmental stages in leaves, stems and flowers (Supplementary Fig. 4a–f). The

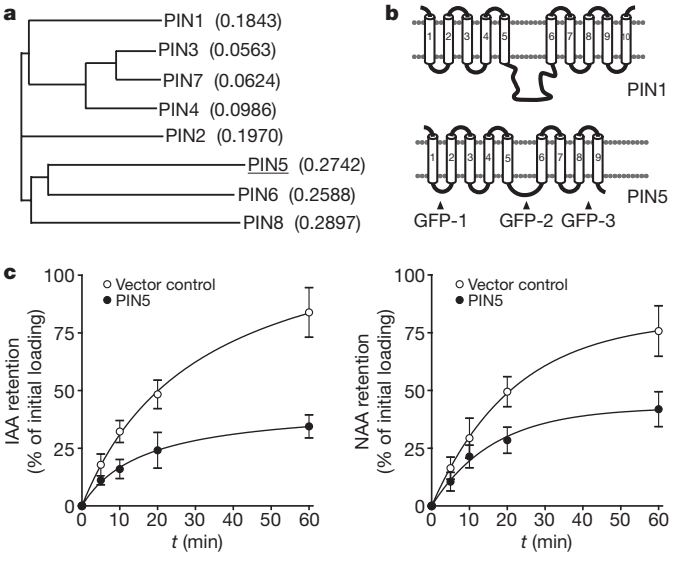

Figure 1 | PIN5 auxin transporter. a, Phylogenetic tree of the *Arabidopsis* PIN protein family with indicated distances. PIN5, PIN8 and PIN6 represent the distant subclade. **b**, Predicted organization of transmembrane domains in PIN5 reveals the reduction of the middle hydrophilic loop that is the hallmark of PIN1-type proteins. Positions of GFP insertions are indicated. **c**, In the yeast auxin efflux assay, decreased retention of IAA and NAA in the PIN5-expressing yeast implies an auxin-export function for PIN5. Error bars represent s.e.m.; $n=5$.

[1]Department of Plant Systems Biology, VIB and Department of Plant Biotechnology and Genetics, Ghent University, 9052 Gent, Belgium. [2]Center for Plant Molecular Biology (ZMBP), University Tübingen, D-72076 Tübingen, Germany. [3]Institute of Experimental Botany, ASCR, 165 02 Praha 6, Czech Republic. [4]Zürich-Basel Plant Science Center, University of Zurich, Institute of Plant Biology, Molecular Plant Physiology, CH-8008 Zurich, Switzerland. [5]Department of Plant Physiology, Faculty of Science, Charles University, 128 44 Praha 2, Czech Republic. [6]Laboratory of Growth Regulators of the Institute of Experimental Botany, ASCR and Faculty of Science, Palacký University, 783 71 Olomouc, Czech Republic. [7]Department for Applied Genetics and Cell Biology, University of Natural Resources and Applied Life Sciences - BOKU, A-1190 Wien, Austria. [8]Department of Functional Genomics and Proteomics, Faculty of Science, Masaryk University, 625 00 Brno, Czech Republic.

PIN5 expression pattern was confirmed by *in situ* hybridization and quantitative reverse-transcription polymerase chain reaction (qRT–PCR; Supplementary Fig. 4g, h) and is consistent with publicly available microarray data (https://www.genevestigator.ethz.ch/at/). In contrast to other tested PINs, which are typically upregulated in response to auxin[18], PIN5 was transcriptionaly downregulated after IAA treatment (Supplementary Fig. 4i). Together, these findings indicate that PIN5 acts as an auxin-regulated broadly expressed auxin transport protein.

To analyse the role of PIN5 in plant development, we characterized three T-DNA insertion mutants in which the *PIN5* locus was fully disrupted (Fig. 2a, b). All *pin5* mutant alleles showed pronounced defects in lateral root initiation as well as in root and hypocotyl growth (Fig. 2c, d). Furthermore, *pin5* root growth was less sensitive to low concentrations of IAA, whereas its sensitivity to auxin analogues and auxin transport inhibitors was unchanged (Fig. 2e and Supplementary Fig. 5a). To get further insights into the role of PIN5, we generated gain-of-function alleles. We used a two-component UAS/GAL4-based system, driving the PIN5-*myc* overexpression by a strong embryonic and meristematic promoter in *RPS5A* ≫ *PIN5-myc* (*PIN5 OX*). *PIN5 OX* seedlings were defective in root and hypocotyl growth; cotyledons did not fully expand and were epinastic (Fig. 2f and Supplementary Fig. 5b). The less affected *PIN5 OX* adult plants formed narrow, wrinkled leaves and bushy inflorescences (Fig. 2g, h), whereas the more affected primary transformants had small filamentous leaves and failed to flower (Fig. 2g, inset). Initiation of lateral roots occurred normally, but the development of primordia progressed more slowly during the later stages than in the control activator line (Supplementary Fig. 5c, d). *PIN5 OX* embryos that overexpressed PIN5 from early embryogenesis onwards were also defective in the establishment of the root pole ($n = 8$ out of 62; Fig. 2i) and postembryonically in differentiation and organization of columella ($n = 7$ out of 25; Fig. 2j). In summary, analysis of the *pin5* loss- and gain-of-function mutant phenotypes indicates a role for PIN5 in a broad range of developmental processes, mainly in those that are regulated by auxin.

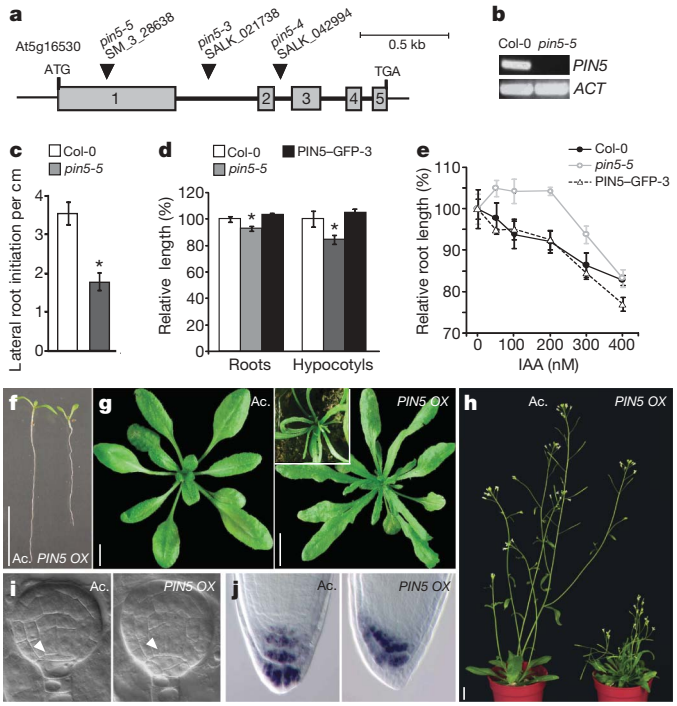

Figure 2 | Phenotypes of *pin5* knockout and gain-of-function mutants. **a**, Structure of the *PIN5* gene with five exons (numbered) and depiction of three T-DNA insertions characterizing the *pin5* loss-of-function mutations. **b**, *PIN5* mRNA is not detected in the *pin5-5* mutant by RT–PCR. Actin (ACT) gene was used as a reference control. **c**–**e**, Developmental defects in *pin5* mutants. *pin5-5* mutants initiate fewer lateral roots (**c**), show defects in hypocotyl and root elongation (**d**), and root growth is resistant to lower concentrations of IAA (**e**). Error bars represent s.e.m., $n = 20$, $^*P < 0.05$. **f**–**j**, Developmental defects of the *PIN5 OX* line in comparison to the *RPS5A:GAL4* activator line (Ac.). *PIN5 OX* seedlings show epinastic, not fully opened cotyledons, shorter roots (**f**) and defects in organization of root meristem as visualized by lugol staining (**j**); adult plants have wrinkled and narrow rosette leaves (inset shows a strongly affected line with filamentous leaves; **g**) and dwarfed and bushy inflorescences (**h**); embryos show aberrations in hypophysis division (indicated by arrowheads; **i**). Scale bars, 1 cm.

To study the involvement of PIN5 in auxin-mediated development further, we examined the activity of the synthetic auxin-responsive reporter *DR5rev::GFP* in *pin5* mutants. The spatial pattern of the DR5 activity did not change markedly but the overall DR5 activity in the root meristems was increased in *pin5* and decreased in *PIN OX* (Supplementary Fig. 6). These results do not support a role for PIN5 in intercellular auxin transport and generation of auxin gradients as shown for the other PIN proteins[2], but instead indicate defects in auxin signalling or homeostasis. To address this issue, we directly measured auxin levels in the *pin5* mutants. Consistently, extraction and gas chromatography/mass spectrometry detection of IAA in root tips and rosette leaves showed that loss-of-function *pin5* mutants had higher levels of endogenous free auxin than the controls whereas the root tips or rosette leaves of *PIN OX* plants had decreased free IAA levels (Fig. 3a). Furthermore, the *pin5* loss-of-function allele showed an unusual genetic interaction with the *yucca* gain-of-function mutant, which overexpresses a rate-limiting enzyme in auxin biosynthesis and has increased auxin levels[19]. The additive effects of these mutations in root and the enhanced effects in hypocotyls (Fig. 3b) are not easy to interpret but further hint at an auxin-homeostasis-related role of PIN5.

To investigate how the loss and gain of the PIN5 function might influence cellular auxin levels, we measured the auxin efflux from mesophyll protoplasts isolated from rosette leaves. *pin5* protoplasts showed an increased, whereas *PIN5 OX* protoplasts had a decreased, efflux of IAA (Fig. 3c). Given the demonstrated auxin export function of PIN5 in yeast (see Fig. 1c), these observations were unexpected because, as shown for other PINs[6], loss of function of a plasma-membrane-localized auxin exporter would be expected to result in reduced export and vice versa. Thus, these data indicate that PIN5 mediates auxin homeostasis by a mechanism that is distinct from the known auxin efflux carriers.

Next we addressed the role of PIN5 in IAA metabolism. We generated cultured tobacco BY-2 cells stably transformed with a dexamethasone (DEX)-inducible PIN5 (as has been done for other PIN proteins). The induction of PIN5 expression led to rapid and notable change in the IAA metabolic profile as demonstrated by the absence of prominent peaks and the appearance of additional ones in the high-performance liquid chromatography (HPLC) profile (Fig. 3d and Supplementary Fig. 7). On the basis of the retention times, we identified the most prominent peaks as free IAA and IAA–glucosyl ester (IAA–Glc), which almost completely disappeared after PIN5 induction (Fig. 3d). Direct analysis by liquid chromatography/mass spectrometry of IAA and selected IAA conjugates confirmed the overall significant changes in rate of IAA metabolism after PIN5 induction, including a pronounced decrease in free IAA levels and an increased capacity to produce amino acid conjugates such as IAA–Asp or IAA–Glu (Fig. 3e). This unexpected finding that PIN5 regulates auxin homeostasis by modulating IAA metabolism further highlights a different mode of action for auxin transporters of the PIN5 subclade than those described for the PIN proteins characterized so far.

We then examined the subcellular localization of PIN5. We fused the *PIN5* genomic fragment with green fluorescent protein (GFP) and constitutively expressed it under the control of the 35S promoter. To minimize possible interference with targeting and protein folding, we placed GFP at three different positions within the PIN5-coding region (Fig. 1b). The expression of these constructs as exemplified by

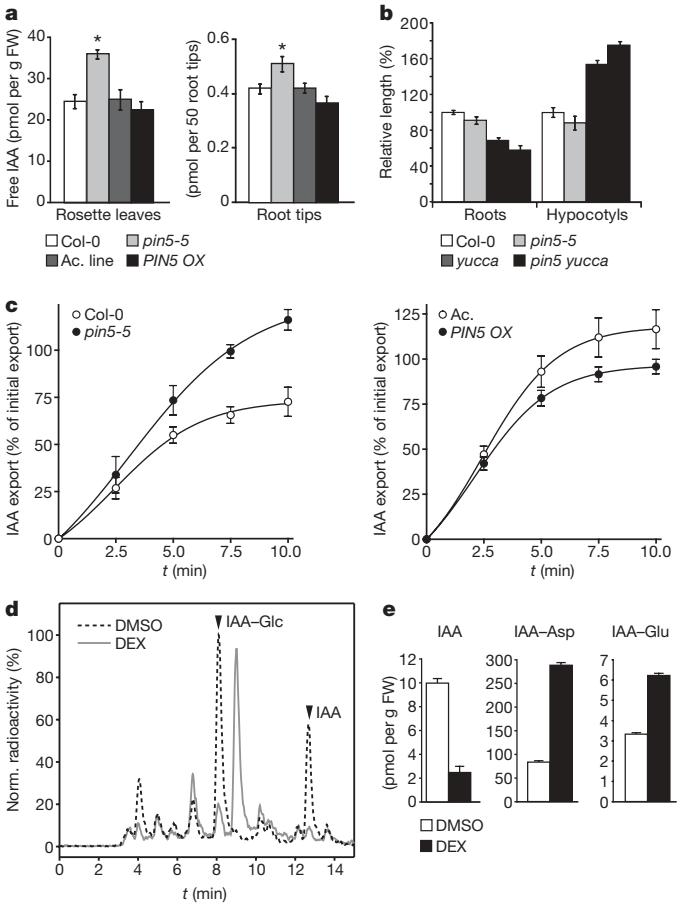

Figure 3 | PIN5 in regulation of auxin homeostasis. **a**, Changes in free IAA levels in *pin5-5* and *PIN5 OX* in rosette leaves and root tips (FW, fresh weight). Error bars represent s.e.m., $n = 3$, $^*P < 0.05$. **b**, Genetic interaction between *pin5* and *yucca*. *pin5* enhances the *yucca* phenotype as shown for root and hypocotyl elongation. **c**, Protoplasts of *pin5-5* mutant exhibit a higher, whereas *PIN5 OX* a lower, rate of IAA export. Error bars represent s.e.m., $n = 5$. **d**, The HPLC chromatogram of IAA metabolic profile changes noticeably after induction of PIN5 expression in tobacco BY-2 cells (24 h of DEX induction, 2.5 h incubation with ^{3}H-IAA). **e**, Liquid chromatography/mass spectrometry detected lower levels of free IAA and strongly increased capacity to conjugate IAA to amino acids (IAA–Asp, IAA–Glu) after induction of PIN5 expression in BY-2 cells (24 h of DEX induction). Error bars represent s.e.m.

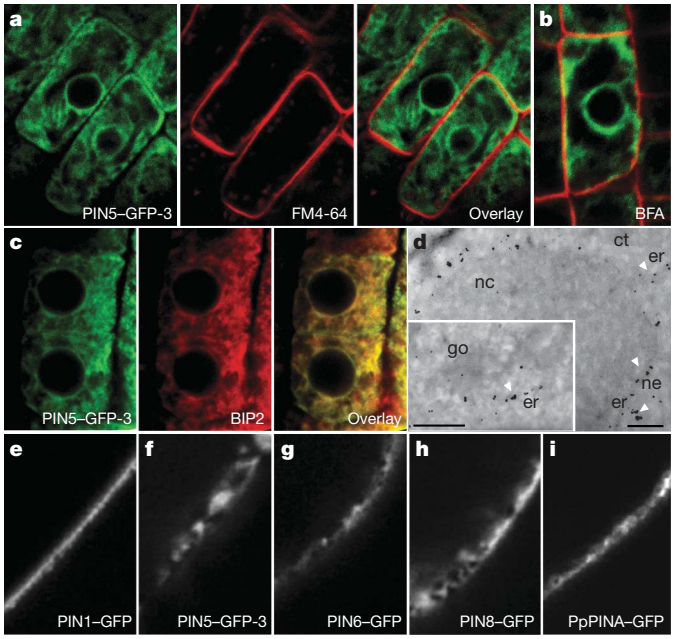

Figure 4 | PIN5 localization at the ER. **a**, PIN5–GFP-3 (green) localizes not to the plasma membrane (briefly stained by FM4-64, red) but to intracellular structures. **b**, PIN5 localization is insensitive to trafficking inhibitor brefeldin A (BFA). **c**, Complete co-localization of PIN5–GFP-3 (green) and the ER marker BIP2 (red) by immunocytochemistry. **d**, Anti-GFP-immunogold labelling on ultrathin cryosections, confirming the presence of PIN5–GFP-3 at the ER (marked by arrowheads). Abbreviations: ct, cytoplasm; er, endoplasmic reticulum; go, Golgi network; n, nucleus; ne, nuclear envelope. Scale bars, 0.5 μm. **e**–**i**, Localization of different PIN–GFP fusion proteins in tobacco BY-2 cells. Magnification of the plasma membrane regions is shown. PIN1–GFP at the plasma membrane (**e**). No plasma membrane but predominant ER localization of PIN5 (**f**), PIN6 (**g**), PIN8 (**h**) and PpPINA (**i**).

PIN5–GFP-3 fully complemented all aspects of *pin5* knockout phenotypes (Fig. 2d, e and Supplementary Fig. 5c). In contrast to the localization of other PIN proteins[2], all three PIN5–GFP variants as well as the PIN5-*myc* in the *PIN5 OX* plants did not show any plasma membrane but a strong intracellular signal (Fig. 4a and Supplementary Fig. 8a, b) that was, unlike that of other PIN proteins[17,20,21], insensitive to treatment with the vesicle trafficking inhibitor brefeldin A (Fig. 4b). Reliable co-localization with different ER markers such as BIP2 (spinach), Sec12 (*Arabidopsis*) and ER tracker dye (Fig. 4c and Supplementary Fig. 8c–f) as well as immunogold labelling on ultrathin cryosections (Fig. 4d) showed that PIN5 localizes to the ER. Taken together, these data indicate that the ER membrane is the site of the PIN5 action and not the plasma membrane as was shown for other members of the PIN family[2]. This also indicates that the function of PIN5 in export of auxin from the yeast cells (Fig. 1c) is due to ectopic localization of PIN5 at the plasma membrane in the heterologous system. Given the export function at the plasma membrane in yeast, the necessary inward transport orientation of the protein at ER membranes *in planta* implies a role for PIN5 in transporting auxin from the cytosol into the lumen of the ER (see model in Supplementary Fig. 1). Such PIN5-dependent transport would limit the availability of cytosolic auxin for auxin exporters at the plasma membrane and increase the IAA amount in the ER pool, where some enzymes in IAA metabolism are compartmentalized[3,22]. The defect in this mechanism in the *pin5* mutant would lead to higher levels of free IAA and increased auxin efflux, whereas PIN5 gain-of-function at the ER would have opposite effects, such as lower free IAA levels and decreased cellular auxin efflux, exactly as observed (see Fig. 3a, c, e).

Interestingly, the localization analysis in tobacco BY-2 cells showed that all *Arabidopsis* PIN5-like proteins (PIN5, PIN6 and PIN8) do not localize to the plasma membrane (as do PIN1-type proteins) but localize to the ER (Fig. 4e–h and Supplementary Fig. 9). These observations indicate that the PIN5-type proteins found throughout all land plant genomes presumably have a function at the ER. The relatively high sequence homology between ER- and plasma-membrane-localized PIN proteins indicates that only small motif changes were involved during diversification of these two classes, as shown, for example, for similar diversification of the ER- and plasma-membrane-based subclasses of plant aquaporins, which differ in a diacidic motif known to be required for exit from the ER[23]. Within the PIN family, the sequence around a putative tyrosine motif, which is a signal for recruitment into the clathrin-coated vesicles[24], consistently differs between the PIN1- and PIN5-like subclasses and might be involved in targeting diversification (Supplementary Fig. 10).

The localization data together with phylogenetic analyses revealed that the PIN protein family comprises two distinct subclasses, the plasma-membrane-localized PIN1-type and the ER-localized PIN5-type, that got separated during evolution well before the establishment of gymnosperms and angiosperms (Supplementary Fig. 2). To address which of these PIN types is ancestral, we analysed the most ancestral PIN-like sequences in the genome of the moss *Physcomitrella patens*.

The *Physcomitrella* genome[25] contains three closely related PIN sequences that cannot be conspicuously allocated to either of the two PIN subclasses based on the protein structure or sequence similarities. Nonetheless, GFP-tagged *P. patens* PINA (XP_001753612), the typical member of the *P. patens* PIN family, localizes to ER in BY-2 cells (Fig. 4i and Supplementary Fig. 9g). This indicates that *Physcomitrella* PINs are functionally related to the ER-localized PIN5-like proteins of higher plants. Thus, the ER-based role of the PIN proteins appeared very early in the evolution of land plants and might represent the ancestral function of this family of auxin transporters.

Our analysis of PIN5, an ER-localized member of the PIN auxin efflux protein family, hints at the previously unknown regulation of the cellular homeostasis of the plant signalling molecule auxin by subcellular compartmentalization (Supplementary Fig. 1). The localization as well as genetic and physiological data support a view in which PIN5 transports auxin intracellularly, from the cytoplasm into the lumen of the ER, thus reducing the auxin availability for plasma-membrane-based auxin efflux and possibly also for nucleus-located TIR1-based auxin perception[26,27]. Importantly, manipulation of the PIN5-dependent cytosol-to-ER auxin transport can markedly change the metabolic fate of IAA, suggesting a subcellular compartmentalization of auxin metabolic pathways. The importance of the ER in auxin biology has already been highlighted by the presence of components of auxin metabolism and signalling such as several deconjugation enzymes[3,22] and the auxin-binding protein ABP1 that is essential for plant growth[28,29]. Thus, PIN5-mediated auxin uptake into the ER lumen might have a relevant role in the control of the ER-resident auxin metabolism, as well as ABP1-mediated auxin perception in this compartment. Moreover, this mode of PIN action at the ER seems to be evolutionarily ancestral as signified by the ER-localized PIN proteins in moss. Auxin as a signalling molecule and thus its homeostasis regulation has been implicated already in unicellular algae, but intercellular polar auxin transport was detected only in the moss sporophyte and evolutionary younger species[30]. Thus, it is conceivable that the ER-based PIN function in regulating the auxin homeostasis is evolutionarily older and that the plasma-membrane-based PIN function in cell-to-cell transport has been acquired later with the evolution of auxin-distribution-based patterning of multicellular plants.

METHODS SUMMARY

Material and growth conditions. For all experiments, we used *Arabidopsis thaliana* of ecotype Columbia 0 (Col-0). Insertion mutant lines were *pin5-3* (SALK_021738), *pin5-4* (SALK_042994) and *pin5-5* (SM_3_28638). Insertion sites were verified, homozygous lines selected and the absence of the *PIN5* transcript shown by RT–PCR. For detailed description of mutant, transgenic lines and DNA constructs, see Methods. Seeds were sterilized with chlorine gas and stratified at 4 °C for 2 days in the dark. Seedlings were grown vertically on half Murashige and Skoog medium supplemented with 1% sucrose and the respective drugs. Plants were grown under the stable long-day (16 h light/8 h dark) conditions at 20–22 °C.

Phenotype analyses, GUS staining and auxin measurements. Plates were scanned on a flat-bed scanner and root and hypocotyl lengths were measured with the ImageJ (http://rsb.info.nih.gov/ij/) software. Lateral root initiation, developmental stage progression analyses and GUS staining were performed as described[9]. Embryos were analysed as described[8]. Details of the free IAA measurements, metabolic profiling, yeast and protoplast transport assays and analyses on BY-2 cells are described in Methods.

Microscopy. Details regarding immunological staining, antibodies and dilutions can be found in Methods. Immunogold labelling on ultrathin cryosections was performed as described[20]. GFP samples were scanned without fixation. Confocal imaging was performed on a Leica SP2 and Zeiss LSM 5 DUO confocal microscopes.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions J.F. and J.M. designed the research. J.M. cloned the constructs, established the mutants and performed the expression and localization analyses, P.S. designed and performed metabolic profiling, and established transgenic tobacco lines, A.Ba. and M.G. performed the transport assays in yeast

and protoplasts, K.H. performed the free IAA measurements, P.I.D. and J.R. ran HPLC and MS auxin analyses, J.P. and K.S. analysed localization of PINs in BY-2 tobacco cells, D.S. prepared PIN6-GFP construct, A.Bi. and E.B. performed the analysis of the lateral root phenotypes, J.Z. performed the site-directed mutagenesis, V.G. performed the phenotype analyses, Y.-D.S. performed the electron microscopy, C.L. originally identified the *PIN5* gene, isolated the *PIN5* cDNA and generated overexpression lines, J.F. performed the *in situ* hybridization, P.K. constructed the cladogram, and J.M., E.Z. and J.F. wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to J.F. (jiri.friml@psb.vib-ugent.be).

METHODS

DNA constructs, transformation and plant material. SALK lines were obtained from the Nottingham *Arabidopsis* Stock Center. The EXOTIC line SM_3_28638 was a gift from J. Clarke. The pPIN5:PIN5-GUS was generated by amplification of the *PIN5* genomic fragment and cloning to the BamHI and NcoI sites of pCAMBIA1391Z (http://www.cambia.org/daisy/cambia/-materials/vectors/585.html#dsy585_table) and transformed to Col-0 plants. For the *PIN5–GFP* constructs, *eGFP* was introduced into genomic *PIN5* at the following positions of the predicted coding region: GFP-1, 37 (RD/QC), GFP-2, 164 (NI/SD), GFP-3, 319 (YG/LH). For *PIN8−GFP*, *eGFP* was positioned at amino acid 203 (TR/SV). *PIN6−GFP* was generated by insertion of *GFP* into the whole *PIN6* genomic fragment (nucleotides −1,876 to 4,194 from ATG) after amino acid position 401. The resulting fusions were cloned to pGREENII-35S-tNos[31] (www.pgreen.ac.uk/).

For GFP fusion constructs with *Physcomitrella* proteins, the corresponding coding genomic fragments were amplified and inserted with the Gateway recombination, between the 35S promoter and *GFP* in the vector p7FWG2 (ref. 32). Mutated PIN1–GFP was generated by replacing NPNTY with NSLSL using the Invitrogen QuikChange II XL kit. The resulting constructs were transformed to *pin5-5* or Col-0 plants. For PIN5 overexpression, a myc-tagged *PIN5* genomic fragment was cloned into pTA7002 (ref. 33) to create pUAS:PIN5-*myc*. This plasmid was used to transform the *RPS5A: GAL4* activator line[34] to create the *RPS5A* ≫ *PIN5-myc* line (*PIN5 OX*). For heterologous expression in yeast, the *PIN5* cDNA was fused to the HA by primer-extension PCR and cloned to the pNEV yeast expression vector, and transformed to *Saccharomyces cerevisiae* strains JK93 (ref. 35). The standard floral dip method was used for all *Arabidopsis* transformations.

BY-2 cell lines and biolistic transformation. The *GVG-PIN5* BY-2 line was created by stable transformation of pUAS:PIN5-myc (in pTA7002) to tobacco Bright Yellow-2 (BY-2) cells[36]. The methods for stable transformation, growth conditions and analyses were as described[6]. Three to five-day-old BY-2 cells were used for biolistic transformation (Helios Gene Gun, Bio-Rad) according the manufacturer's instructions. After bombardment, transformed cells were cultivated at 27 °C in the dark and analysed after 10–16 h.

RNA extraction and real-time qRT–PCR. Whole RNA of 7-day-old seedlings and different plant organs was extracted with the Qiagen RNA extraction kit and used for cDNA synthesis (SuperscriptII, Stratagene). The whole coding region of the *PIN5* gene was amplified by standard PCR (QP5_FW1: TAATCTT AGGCTATGGCTCTG and QP5_REV1: GACCGTGACTATGATGACC). Primers specific for tubulin or actin were used as a control. For auxin induction assays, 7-day-old seedlings were transferred to fresh plates with and without IAA (5 μM) and incubated for 5 h before RNA extraction. qPCR analysis was done on an iCycler (Bio-Rad, USA) with the Platinum SYBR Green qPCR Super-UDG kit (Invitrogen) as described[18]. Transcript levels were normalized to expression of MYB88A (At2g02820).

IAA determination, protoplast and yeast auxin transport assays. To determine internal levels of endogenous auxin in *Arabidopsis* leaves, free IAA was extracted by methanol/formic acid/water (15/1/4, v/v/v) from leaf rosettes of 23-day-old *Arabidopsis* plants homogenized in liquid nitrogen. The extract was purified with the dual-mode solid-phase extraction method as described[37]. Free IAA was purified on two different HPLC columns and determined on Finnigan Polaris Q GC-MS/MS. Internal levels of endogenous auxin in *Arabidopsis* root tips were determined by collecting root tips (3–5 mm) of 8-day-old *Arabidopsis* seedlings in a 1.5% sucrose solution. For extraction of free IAA from the tips, two volumes of methanol were added to one volume of the solution. After disintegration by sonication for 5 min, root tips were separated by centrifugation. Free IAA released to the solution was determined on the Finnigan Polaris Q GC-MS/MS. For measurement of IAA and its conjugates, the homogenate from BY-2 cells was purified by solid-phase extraction followed by immunoaffinity extraction. IAA and its conjugates were then quantified by HPLC (UPLC Acquity, Waters) coupled to tandem mass detection (Quatro micro API tandem quadrupole mass spectrometer; Waters). Auxin protoplast efflux and yeast loading assays were performed as described[35].

HPLC metabolic profiling. Twenty-four hours after induction, cells were prepared for the experiment by equilibration in uptake buffer without auxin as described[6]. Experiments were done in the same buffer at room temperature and in otherwise standard culture conditions. Cells were incubated with addition of 20 nM ^{3}H-IAA (specific activity 20 Ci mmol^{-1}, American Radiolabeled Chemicals) for a period of 2.5 h. Extraction and purification of auxin metabolites were performed as described[37]. The radioactive metabolites of ^{3}H-IAA were separated on HPLC using two methods. The first one was a pilot method with lower resolution. For this method was used the column Luna C8(2), 50 × 4.6 mm, 3 μm (Phenomenex) and mobile phase A: 40 mM $HCOONH_4$, pH 3.0. The second method was to a higher resolution with column Luna C18(2), 150 × 4.6 mm, 3 μm and mobile phase A: 40 mM CH_3COONH_4, pH 4.0. Mobile phase B was CH_3CN/CH_3OH, 1/1, v/v. Flow rate was 0.6 ml min^{-1} with linear gradient 30–50% B for 10 min, 50–100% B for 1 min, 100% B for 2 min, 100–30% B for 1 min. The column eluate was monitored by a Ramona 2000 flow-through radioactivity detector (Raytest) after online mixing with three volumes (1.8 ml min^{-1}) of liquid scintillation cocktail (Flo-Scint III, Packard BioScience Co.). The radioactive metabolites of ^{3}H-IAA were identified on the basis of comparison of their retention times with authentic standards.

IAA-conjugate uptake into membrane vesicles. Yeast microsomal fractions were isolated from enzymatically prepared spheroblasts from *S. cerevisiae* strain JK93da and from strains expressing pNEV (vector control) and pNEV-PIN5-HA (PIN5–HA), and uptake of [^{3}H]N-(3-IAA)-DL-aspartate (IAA–Asp; Moravek Biochemicals (1 Ci mmol^{-1})) and [14,15,19,20-^{3}H(N)]–Leukotriene C4 (LTC4; NEN Radiochemicals (131 Ci mmol^{-1})) was measured at 30 °C by using an ATP-regenerating system and the rapid filtration technique as described[38]. Uptake rates were calculated by subtracting the radioactivity measured after 1 min of incubation from corresponding 10-min values. Conditions were repeated with three independent microsome preparations, each performed in triplicate.

Yeast membrane fractionation and western blot. Total microsomes of yeast expressing PIN5–HA were separated via continuous sucrose gradient centrifugation[39], subjected to 4–20% PAGE (Long Life Gels, Life Therapeutics), and western blots were immunoprobed with anti-HA (Roche) and the plasma membrane marker anti-H^+-ATPase[39].

Whole-mount *in situ* hybridization, immunolocalization and lifetime confocal microscopy. Whole-mount *in situ* hybridization of *PIN5* in seedlings was performed as described[40]. As a probe, digoxigenin-labelled antisense *PIN5* cDNA was used. Whole-mount immunological staining on 4-day-old seedlings was done in an Intavis robot[41]. Antibodies were used at the following dilutions: anti-BIP2 (Hsc70), 1:200 (Stressgen Bioreagents); rabbit anti-Sec12 (ref. 42), 1:200; mouse anti-GFP, 1:600 (Roche); rabbit anti-myc, 1:600 (Sigma-Aldrich). Anti-rabbit and anti-mouse antibodies conjugated with Cy3 or fluorescein isothiocyanate (FITC; Dianova, Germany) were used at 1:600 dilutions. For FM4-64 and ER tracker dye labelling, PIN5–GFP seedlings were incubated for 3 min in the medium with a 1:1,000 dilution of FM4-64 or ER tracker dye (Invitrogen). For BFA treatment, we incubated seedlings in liquid Murashige and Skoog medium supplemented with 50 μM brefeldin A (Molecular Probes) for 1 h, after wash out and incubation for 3 min in FM4-64 to stain the plasma membrane. The intensity correlation analysis was done with ImageJ plugin as described[43,44].

Phylogenetic analyses. Protein sequences of PIN proteins were retrieved from the NCBI database with the BLAST program; the sequences from *P. patens* and *Selaginella moellendorffii* were predicted from the preliminary releases of the genomic sequences released by DOE Joint Genome Institute (www.phytozome.net). Sequences were aligned with the program MAFFT, version 5.664 (ref. 45), and the central region that contained extensive gaps was edited. Phylogenetic relationships for each protein were assessed via Bayesian inference by the computer program MrBayes version 3.1 (ref. 46). We performed Metropolis-coupled Markov chain Monte Carlo analysis of the PIN protein evolution using the following parameters: JTT+I+Γ substitution model with six categories of gamma rate, four independent models of four chains (one cold and three heated with temperature 0.2). The analysis was run for 2,000,000 generations, sampling every 100 generations; 2,000 burn-in samples were discarded. The consensus tree was deduced from the sampled trees and rooted according to the evolutionary relationships of the organisms.

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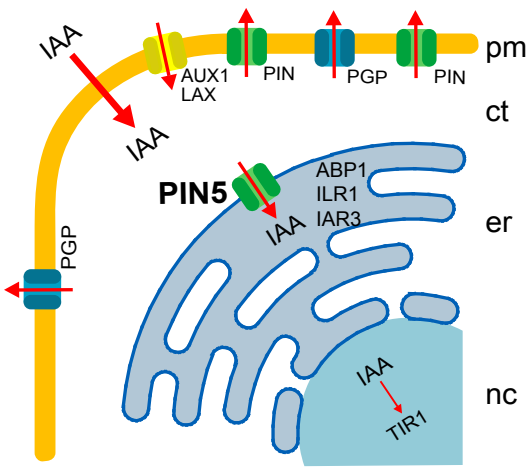
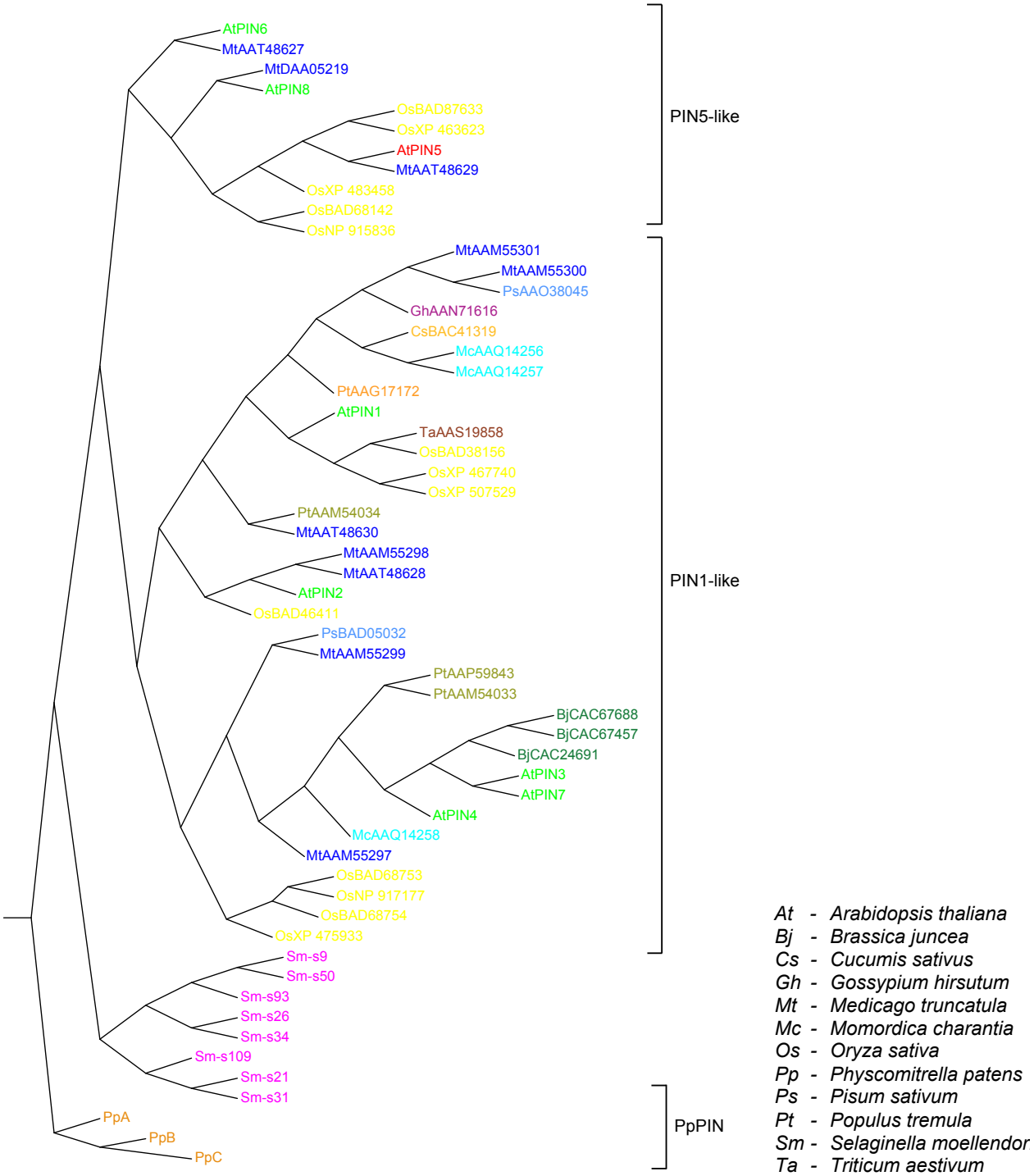


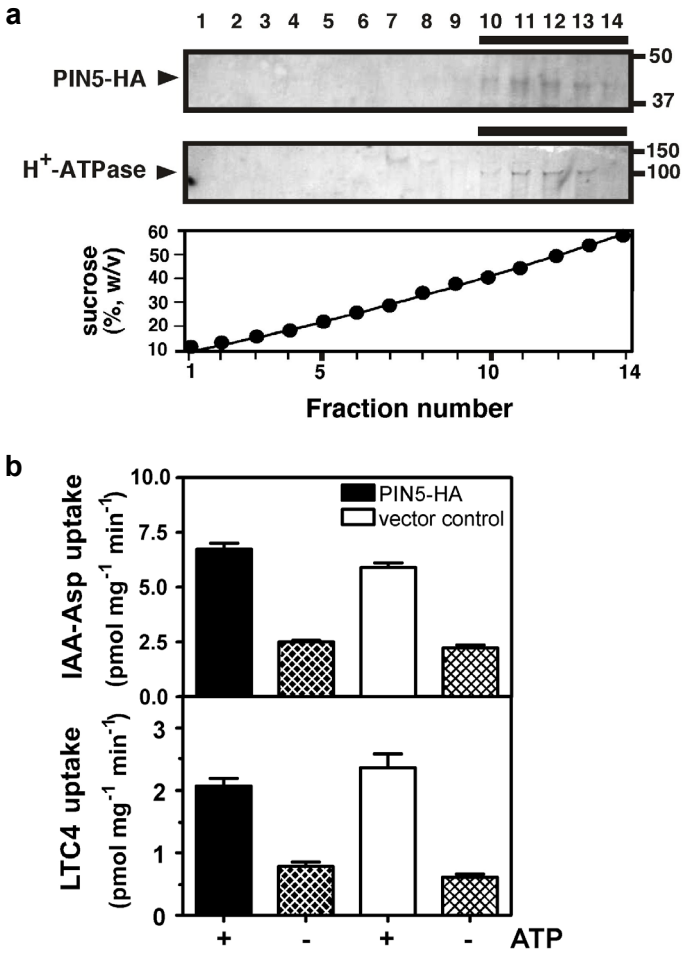
Supplementary Figure 1 Model for cellular auxin transport

Polar, intercellular auxin transport is mediated by the plasma membrane localized auxin efflux (PIN1-type and PGP-type) and influx (AUX1/LAX) carriers[3-5,47-48]. Intracellular auxin is compartmentalized into two pools. Cytosolic auxin can diffuse to the nucleus where it triggers the TIR1 receptor-mediated signalling[1,26,27]. Part of cytosolic auxin is transported to the ER by PIN5-like transporters and thus become not available for plasma membrane-based auxin efflux. This mode of action together with auxin-induced decrease in PIN5 transcription might be important for modulation of intercellular auxin transport. ER pool of auxin might be relevant for ER-residential signalling and metabolism. The Auxin Binding Protein1 (ABP1) that binds auxin with high affinity localizes to large extent to the ER[28,29]. ABP1 mediates auxin-dependent cell expansion and division and the full loss-of-function *abp1* allele is embryo lethal[29,49-51]. Additionally, several IAA metabolic enzymes were found or predicted to be ER localized. ILR1 and IAR3 encode IAA-amino acid hydrolases which can release free IAA from IAA-amino acid conjugates[22,52]. Interestingly another IAA-conjugate resistant mutant *irl2* is a predicted modulator of the ER membrane based metal transporter[53]. The auxin conjugation to amino acids is catalyzed by GH3-like IAA-amido synthetases[54] and the esterification of IAA to glucose and formation of IAA-Glc is catalyzed by UGT84B1 protein (indole-3-acetate beta-glucosyltransferase)[55]. The localization of these enzymes or other auxin metabolic proteins has not been determined yet but already the available information highlights importance of ER subcellular compartment for IAA metabolism.



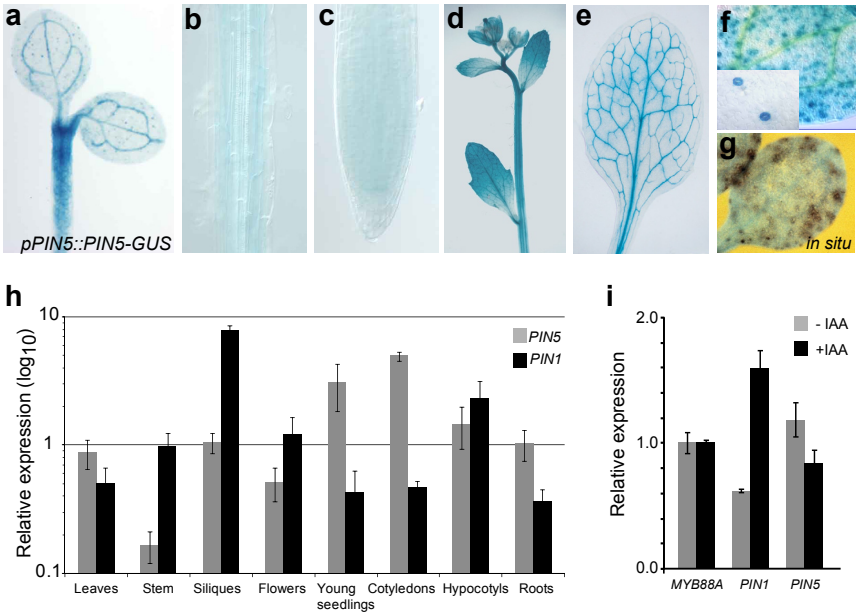
Supplementary Figure 2 Cladogram of PIN proteins

The PIN proteins from *A. thaliana* have their common names; the others are marked with their accession numbers. PIN proteins form two subclasses: Plasma membrane-localized (PIN1-like) and ER-localized (PIN5-like). The allocation of evolutionary more archaic *Physcomitrella* and *Selaginella* PINs to one of these groups is unclear. No PIN-like genes were found in available sequences from uni- or multicellular algae. Most archaic available PIN sequences are found in *Physcomitrella* and encompass three closely related sequences PpPINA, PpPINB and PpPINC. More distantly related *Physcomitrella* sequence (XP_001765763) is the acquisition by a recent horizontal gene transfer event and its localization is cytosolic (see Supplementary Fig. 9h); therefore was not included in the cladogram. PpPINA (XP_001753612) is a typical member of the *Physcomitrella* PIN group and localizes to ER suggesting that ER-localized PINs are evolutionary older than the PM-localized PINs functioning in intercellular auxin transport.



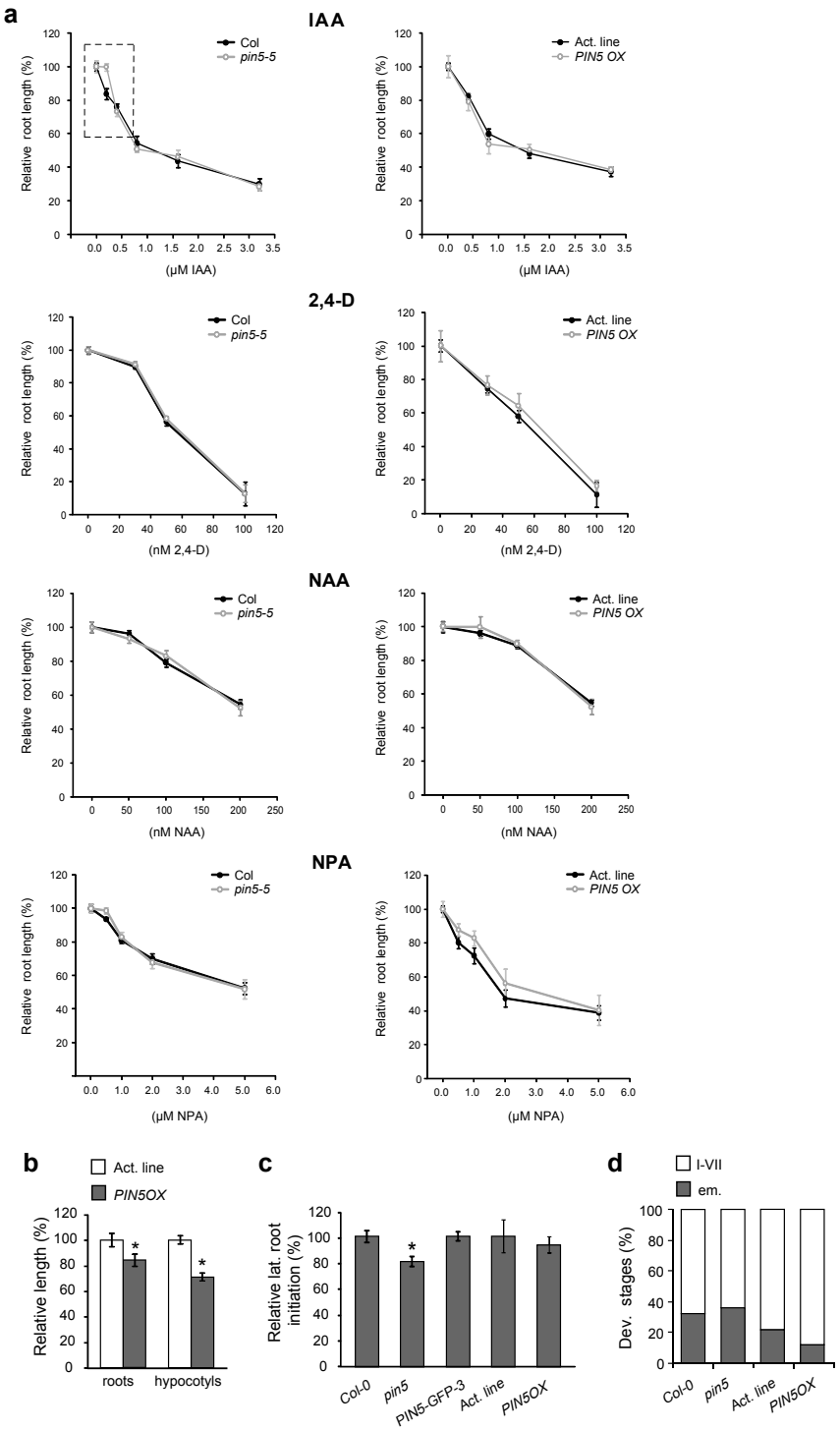
Supplementary Figure 3 PIN5 functionality in yeast

a,Confirmation of the PIN5-HA localization at yeast plasma membrane by continuous sucrose gradient fractionation. Anti-HA was used to detect PIN5-HA and H^+-ATPase antibodies were employed as a typical plasma membrane marker. **b**, PIN5 does not mediate ATP-independent or ATP-dependent transport of the auxin conjugate IAA-aspartate (IAA-Asp) in yeast. The uptake of radioactively labeled IAA-Asp into PIN5-HA yeast vesicles was not significantly different from vector control. Error bars represent s.e.m, n=3; transport competence of microsomes was verified by quantifying ATP-dependent uptake of the ABCC-type transporter substrate, leucotriene C4 (LTC4).

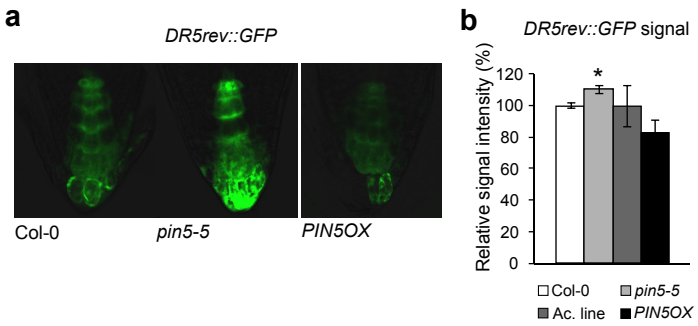


Supplementary Figure 4 PIN5 expression pattern

a-f, Expression of the *pPIN5::PIN5-GUS* reporter construct in different plant organs. PIN5 is expressed in (**a**) light grown hypocotyls, cotyledon vasculature, (**b**) pericycle and endodermis of main root and (**c**) weakly in the root tip. In adult plants the expression was observed (**d**) in the whole inflorescence and (**e**) rosette leaves- mainly in the vasculature and moderately in mesophyl cells. (**f**) The distinct staining of guard cells can be observed in cotyledons. **g**, The GUS staining was confirmed by whole mount *in situ* hybridization in seedling cotyledons. **h**, Broad expression pattern of PIN5 was also confirmed by qRT-PCR. PIN5 expression did not correlate with the PIN1 expression. **i**, Auxin regulation of PIN5 expression: Unlike auxin-inducible PIN1 (and PIN2, PIN3, PIN4, PIN6 and PIN7, not shown), PIN5 is downregulated following auxin treatment. The qRT-PCR experiment was performed in 2 biological and 3 technical replicates. Error bars represent s.e.m.

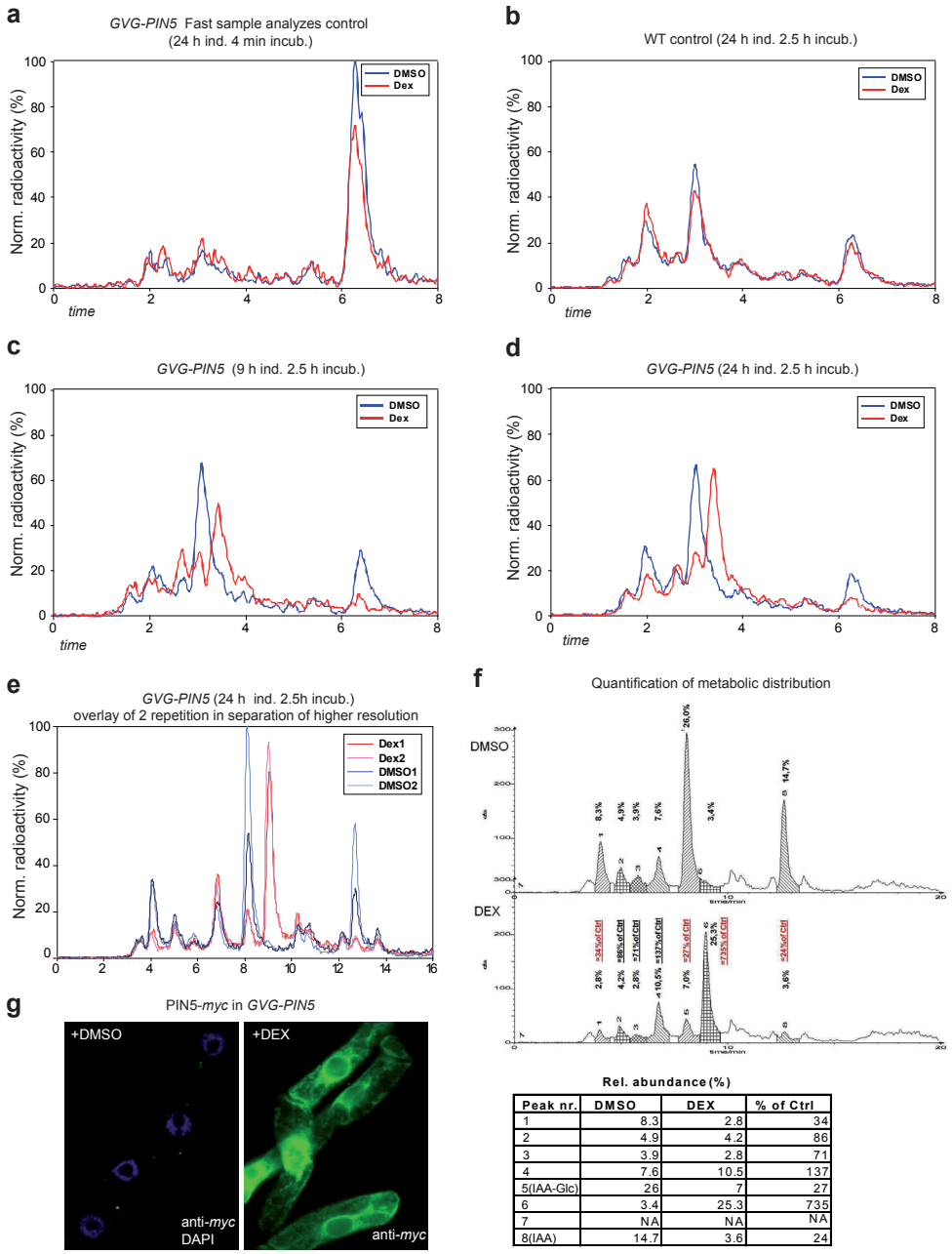


Supplementary Figure 5 Quantitative analysis of *pin5* and *PIN5 OX* phenotypes
a, Sensitivity of the *pin5-5* mutant and the *PIN5 OX* line to the natural auxin indole acetic acid (IAA), auxin analogs: 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthaleneacetic (NAA) acid) and an auxin transport inhibitor N-1-naphthylphthalamic acid (NPA). Root growth inhibition of 9-day-old seedlings grown on plates supplemented with the respective drugs. For IAA measurements, the plates were covered with UV-protective foil to prevent IAA degradation. Col-0 was used as a control for the *pin5-5* mutant and the *RPS5A::GAL4* activator line for the *PIN5 OX* line. The higher resolution of marked area is provided in Fig. 2. **b-d**, Quantification of *PIN5 OX* seedlings phenotypes: (**b**) root and hypocotyl length, (**c**) relative lateral root initiation and (**d**) progression through developmental stages. em, emerged; I-VII, all stages before emergence. Error bars represent s.e.m, n=20, * $P<0.05$.



Supplementary Figure 6 *DR5rev::GFP* expression in *pin5* mutants

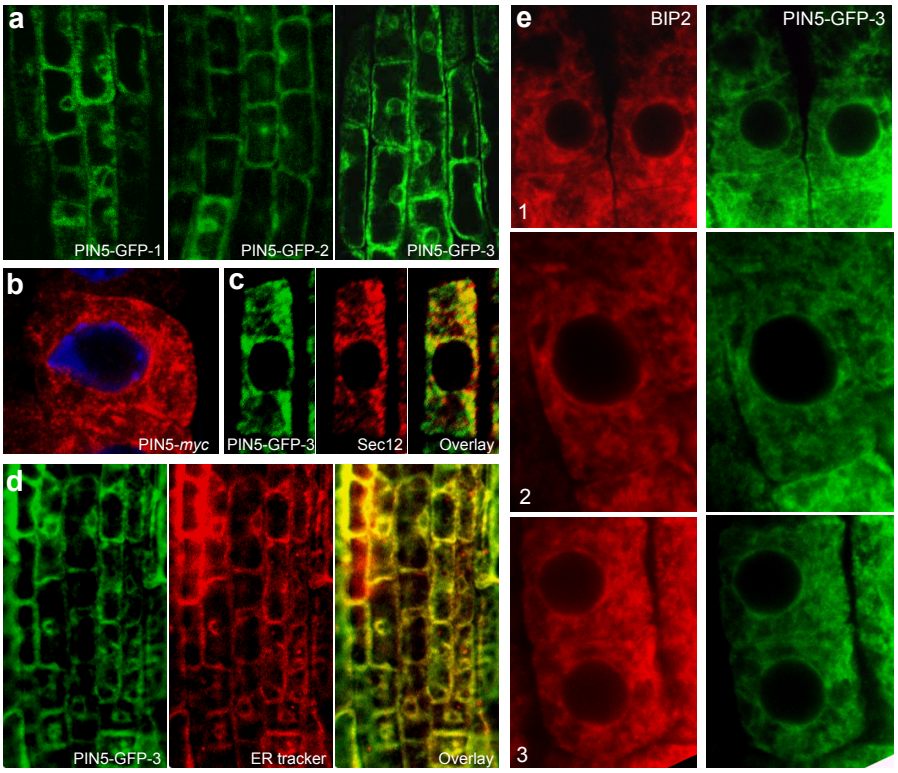
a, Analyses of auxin responsive reporter *DR5rev::GFP* expression in *pin5* mutants by confocal microscopy. The representative images are presented. **b**, Quantification of signal intensity of *DR5rev::GFP* reporter in *pin5* mutants. Error bars represent s.e.m, n=8, * $P<0.05$.



Rel. abundance (%)

Peak nr.	DMSO	DEX	% of Ctrl
1	8.3	2.8	34
2	4.9	4.2	86
3	3.9	2.8	71
4	7.6	10.5	137
5(IAA-Glc)	26	7	27
6	3.4	25.3	735
7	NA	NA	NA
8(IAA)	14.7	3.6	24

Supplementary Figure 7 PIN5 action in regulating auxin metabolism and homeostasis
a-f, HPLC-based metabolic profiling of ^{3}H-IAA derivatives in BY-2 tobacco cells. (**a**, **b**) Control analyzes. (**a**) Fast sample analyses control; after 24 h long induction the *GVG-PIN5* cells were incubated for 4 min with ^{3}H-IAA. IAA fraction represents the highest peak. No differences between DEX and DMSO treated cells were observed. (**b**) Wt control; wt BY-2 cells were incubated for 24 h with DMSO or DEX following 2.5 h incubation with ^{3}H-IAA and analyzed. No changes in DMSO and DEX treated cells were observed. (**c**, **d**) Analyses of ^{3}H-IAA metabolism in *GVG-PIN5* line induced with DMSO or DEX for (**c**) 9 h or (**d**) 24 h and incubated with ^{3}H-IAA for 2.5 h. The metabolic profiles of DMSO and DEX lines strongly differ also in most abundant products. (**e**) Overlay of metabolic profiles of ^{3}H-IAA metabolites with improved resolution of peak separation. The ^{3}H-IAA products correspond to the metabolite distribution of the first separation, but with peaks of interests being more distinctly separated. This together with reliable overlay of two repetitions for each variant denotes reproducibility of specific change in auxin metabolism after PIN5 overexpression. (**f**) Quantification of ^{3}H-IAA metabolites in HPLC profile. Each delimited peak of metabolite was numbered, its area integrated and expressed as percentual part of corresponding plot area. Differences in corresponding peak pairs were evaluated. The results are summarized in the table. **g**, Immunological staining of PIN5-*myc* in *GVG-PIN5* shows ER-like localization of PIN5 after induction by 1μM DEX.

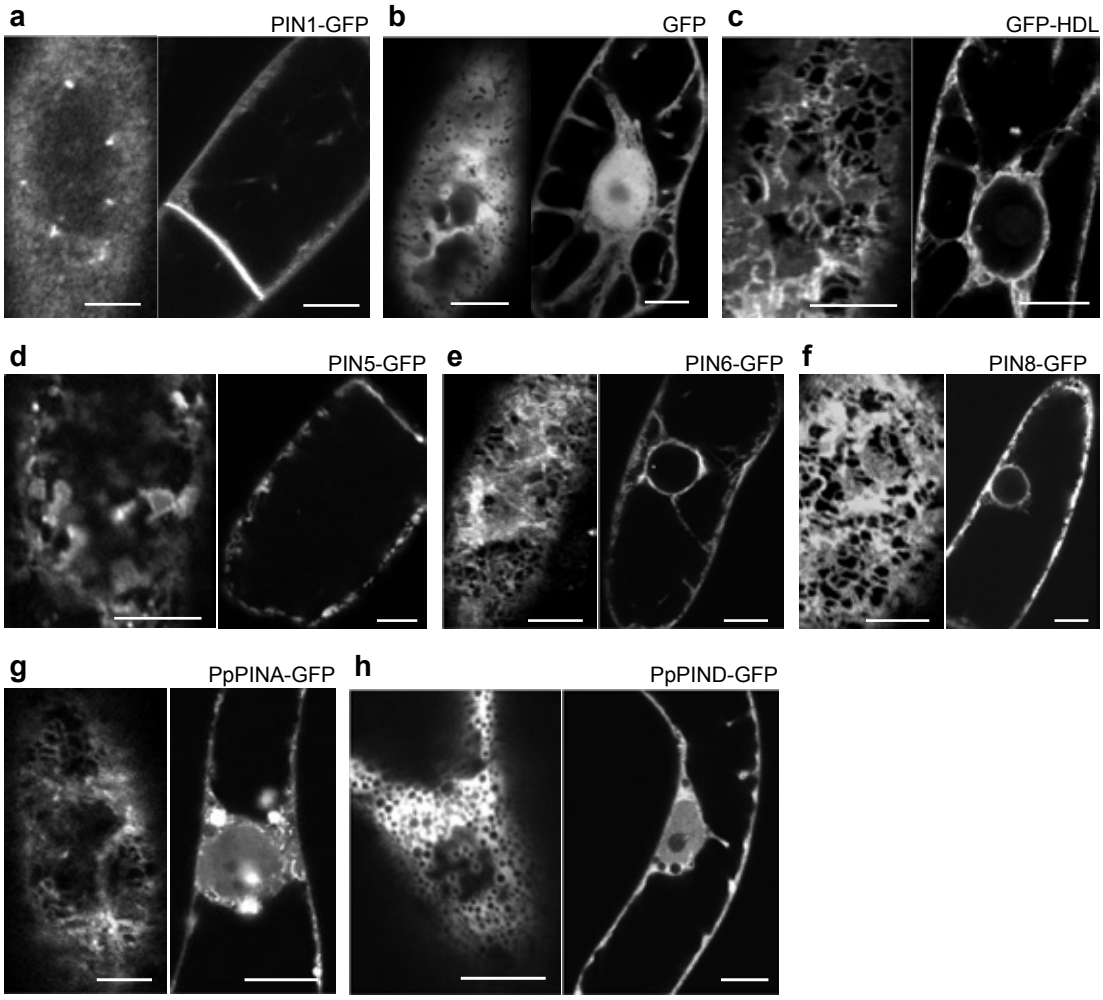


f

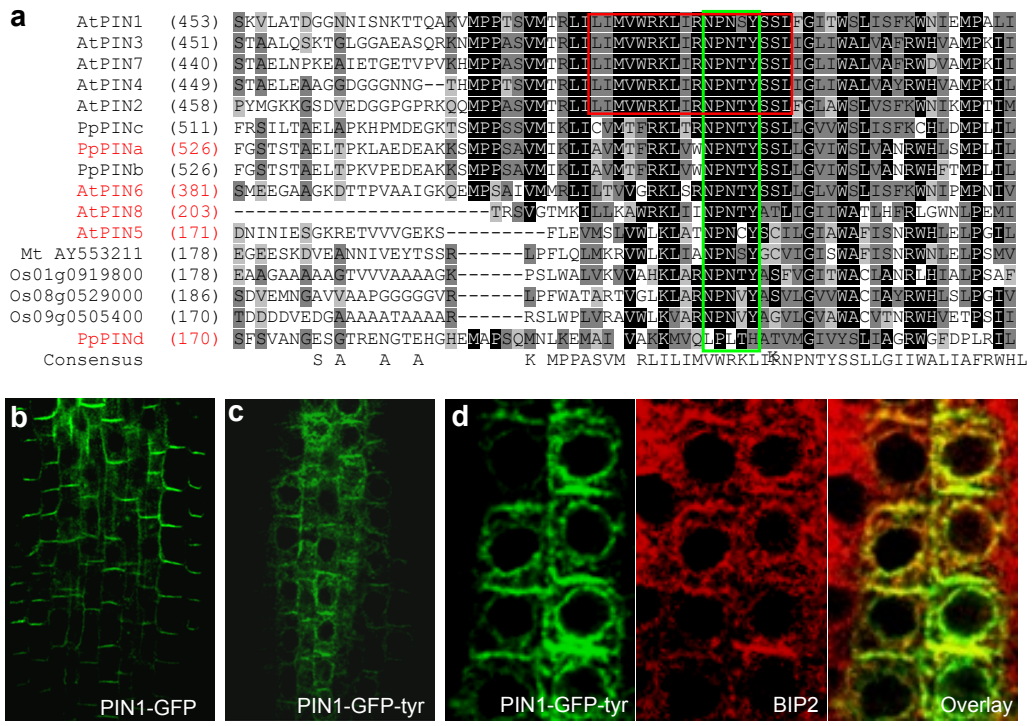
Image	Rr	R	ch1:ch2	M1	M2	N+ve	Ntotal	ICQ
1	0.16	0.63	1.004	0.995	1	297082	498455	0.096
2	0.079	0.631	1.069	0.846	1	286028	519526	0.051
3	0.195	0.685	1.067	0.953	0.973	214211	365377	0.086
AV	0.144667	0.648667	1.046667	0.931333	0.991	265773.7	461119.3	0.077667
Stdev	0.059501	0.03147		0.076827	0.015588	44995.32	83581.95	0.023629

Supplementary Figure 8 ER localization of PIN5

a, Localization of three PIN5-GFP constructs. All variants show the similar ER localization pattern. **b**, Immunological staining of PIN5-*myc* in *RPS5A>>PIN5-myc* line epidermal cells with an anti-*myc* antibody also shows ER-localized signal. **c**, Co-localization staining of PIN5-GFP-3 (green) with ER marker Sec12 (red). **d**, Co-localization of PIN5-GFP-3 (green) with the ER-tracker dye (red) (Molecular Probes). **e**, **f**, Intensity Correlation Analysis of PIN5-GFP-3 and BIP2 co-staining of three independent confocal pictures (**e**) using the plug-in for the ImageJ software. The Mander's overlap coeficient (*R)*, the Pearson's correlation coefficient (*Rr*) and the Intensity Correlation Quocient (*ICQ*) were calculated (**f**). In all cases, values show reliable co-localization ($R>0$; $Rr\sim1$; $0<ICQ\leq+0.5$).



Supplementary Figure 9 Localization of PIN proteins in tobacco BY-2 cell
a-c, Localization of control constructs; (**a**) membrane staining of PIN1-GFP, (**b**) cytosolic and nuclear staining of free GFP (note the negative staining of membrane structures) and (**c**) ER staining of GFP-HDEL construct. **d-f,** ER localization of three *Arabidopsis* PIN proteins; (**d**) PIN5-GFP, (**e**) PIN6-GFP and (**f**) PIN8-GFP. Note that the ER structure is significantly modified by PIN5 overexpression and the lace like structures are partially lost, towards more aggregates and cisterns. **g**, **h**, Localization of two *Physcomitrella patens* PIN-related proteins. (**g**) PpPINA localizes to ER, whereas (**h**) PIN-related *Physcomitrella* protein (PpPIND; XP_001765763) shows non specific cytosolic localization pattern. Confocal sections through cortical cytoplasm and central part of the cell are presented. Scale bars, 10μm.



Supplementary Figure 10 Analyses of potential motives involved in ER targeting of PIN5-type PINs. **a**, Amino acid sequence alignment of the region spanning the putative tyrosine motif of selected PIN proteins. The group of plasma membrane-localized proteins contains a highly conserved sequence around the putative tyrosine motive (NPNTY), which is compromised in PIN proteins from PIN5 subclade. **b, c,** Mutational analyzes of putative tyrosine motive NPNTY in PIN1 sequence. (**b**) Localization of "wild type" PIN1-GFP (NPNTY) on plasma membrane, (**c**) localization of mutated PIN1-GFP-tyr (NSLSL) to ER. **d**, The ER localization of PIN1-GFP-tyr (NSLSL) (green) was confirmed by co-localization staining with ER marker BIP2 (red).

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6.3 Two groups of PIN auxin transporters: Diversity within the family as a vehicle for evolution

This chapter encompasses two published papers:

Zažímalová E, Křeček P, **Skůpa P**, Hoyerová K, Petrášek J. Polar transport of the plant hormone auxin - the role of PIN-FORMED (PIN) proteins. CELLULAR AND MOLECULAR LIFE SCIENCES 2007;64(13):1621-37. (IF 2010: 7.047)

Křeček P*, **Skůpa P***, Libus J, Naramoto S, Tejos R, Friml J, Zažímalová E. Protein family review: The PIN-FORMED (PIN) protein family of auxin transporters. - GENOME BIOLOGY 2009, 10:249.1-249.11. (IF 2010: 6.885) * Joint first authorship

Both presented papers focus on characterization of the PIN protein family with emphasis on protein function, its regulation, structure and evolution. We provided detailed overview of results about function and developmental roles of individual PIN family members in *Arabidopsis*. Within the goals of the papers, it was to provide classification of the family members based on structural and evolutionary relationships. Moreover, in the perspective of the reported results, together with relationships found among the proteins, we strived to make a synthesis about the role of PIN proteins in plant development in more general fashion and in respect to its impact on the knowledge about plant evolution.

In both of the presented articles, I was primarily responsible for summarizing the knowledge about diversity of the PIN family, especially in the evolutionary context, and with respect to evolutionary history of the polar auxin transport in general. I outlined a potential importance of the evolutionary context for future research and for further clarification that allows prediction of new research hypotheses for evolution and function of various branches of the gene family. To this end, the work provided both phylogenetic analysis in the form of cladogram of PIN proteins (done by Pavel Křeček) and also **evolutionary cladograms of *Plantae* supergroup with respect to occurrence of polar auxin transport and PIN proteins within respective evolutionary grades (done by myself in parallel to the phylogenetic trees based**

purely on bioinformatics). In both papers I was responsible for preparation of **graphical representation of auxin transport at cellular level and, generally, for writing parts of the text**.

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Cellular and Molecular Life Sciences

Review

Polar transport of the plant hormone auxin – the role of PIN-FORMED (PIN) proteins

E. Zažímalová[a,b,*], P. Křeček[a,b], P. Skůpa[a,b], K. Hoyerová[a] and J. Petrášek[a,b]

[a]Institute of Experimental Botany, The Academy of Sciences of the Czech Republic, Rozvojová 263, 165 02 Prague 6 (Czech Republic), Fax: +420 225 106 429, e-mail: eva.zazim@ueb.cas.cz
[b]Department of Plant Physiology, Faculty of Science, Charles University, Viničná 5, 128 44 Prague 2 (Czech Republic)

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Abstract. The PIN-FORMED (PIN) protein family is a group of plant transmembrane proteins with a predicted function as secondary transporters. PINs have been shown to play a rate-limiting role in the catalysis of efflux of the plant growth regulator auxin from cells, and their asymmetrical cellular localization determines the direction of cell-to-cell auxin flow. There is a functional redundancy of PINs and their biochemical activity is regulated at many levels. PINs constitute a flexible network underlying the directional auxin flux (polar auxin transport) which provides cells in any part of the plant body with particular positional and temporal information. Thus, the PIN network, together with downstream auxin signalling system(s), coordinates plant development. This review summarizes recent progress in the elucidation of the role of PIN proteins in polar auxin transport at the cellular level, with emphasis on their structure and evolution and regulation of their function.

Keywords. Plant hormone, phytohormone, plant growth regulator, auxin, polar auxin transport, auxin efflux carriers, PIN, *pin-formed.*

Introduction

Plant growth and development is regulated by both external and internal factors. Native plant growth regulatory compounds (often called plant hormones or phytohormones) belong to the internal cues that play a crucial role in the control of many processes involved in key developmental events in plants. Auxins were the first group of plant growth regulatory substances discovered [1], with indole-3-acetic acid (IAA) as the first identified native representative of the group. Auxins are known to be involved in the regulation of basic growth processes such as cell division and cell elongation, and, at the level of tissues, organs and the whole plant, they exhibit pleiotropic physiological effects [2–5]. Auxin molecules function as mobile signals between cells, tissues and organs and, as such, they are involved in spatial and temporal coordination of plant morphogenesis and in plant responses to their environment.

However, it is not just the mobility of auxin molecules and their downstream signalling 'potential' which is responsible for all these physiological effects. Many developmental processes seem to be dependent on the local asymmetric distribution of auxin molecules [6].

* Corresponding author.

These include e.g. embryo development and apical-basal axis formation in *Arabidopsis thaliana* [7], pattern formation and root development [8–10], organ formation [11] and, last but not least, changes in the direction of organ growth resulting from differential growth rates on opposite sides of the organ (root, stem) in response to environmental stimuli such as light or gravity – so-called phototropism and gravitropism, respectively [12–14]. Auxin, in other words, is fundamentally involved in "shaping the plant" [13]. Other plant growth regulatory substances, including the native ones, do not exhibit such a broad spectrum of physiological effects, which, although very diverse, seem to be coordinated. So, what makes auxin so special? The answer is a unique phenomenon: so-called polar auxin transport. Certainly, auxin, as well as other native plant growth regulatory compounds, can be transported passively in vascular tissues [reviewed in refs. 15, 16], and in such cases, the direction of its movement is determined by mass flow. In contrast to this, however, there is also an active cell-to-cell auxin flow, in vascular cambium and xylem parenchyma cells, which works in parallel with auxin movement through vascular tissues but which is strictly directional [reviewed in refs. 16, 17]. This directional cell-to-cell auxin movement, i.e. polar auxin transport, has been shown to underlie the above-listed physiological auxin effects.

Since it is the efflux of auxins from cells which seems to represent the crucial point in polar auxin transport, both for the maintenance of the auxin flow itself and for the directionality of the process (see below), understanding both the mechanism of the process and the identification and characterization of the proteins involved deserve high priority. Interestingly, the so-called *pin-formed* mutant of *A. thaliana* was isolated by Kappert as early as in 1959, and its function was related to the possible action of the gibberellin group of plant growth regulatory substances [18]. From the 1990s, when the *pin-formed 1* (*pin1*) mutant of *A. thaliana* was characterized [19, 20], it became clear that PIN proteins play a key role in facilitation of auxin efflux from cells [20], if they are not auxin efflux carriers themselves. There are recent reviews available about the relationships between PINs and the various physiological processes in plants [4, 5, 13, 16, 21]. For this reason, this review focuses more on the biochemical role of PINs at the cellular level and on the mode(s) of regulation of their action.

It must be noted that PINs are not the only candidates for auxin efflux carriers. In plants, as well as in bacteria, fungi and animals, there is a group of ATP-binding cassette transporters (ABC) transporters [22], some of which, namely phosphoglycoproteins (PGPs), have also been shown to be involved in catalyzing auxin efflux [17, 23, 24]. However, PIN function seems to be directly connected with several auxin-specific physiological effects, while the function of PGPs may be more general and may apply predominantly in areas with high auxin concentration. It may also involve some kind of interaction(s), even if these have not been identified as yet, with PINs at the plasma membrane (PM, see below) [17, 24–26].

Physical-chemical background of the cell-to-cell movement of auxin molecules

Auxins, both native ones and their synthetic analogues, are weak organic acids. Therefore, their molecules undergo reversible dissociation, the equilibrium of which is pH dependent. At the pH value (ca. 5.5) at the cell wall and the extracellular space, auxin molecules are partly dissociated (Fig. 1); the degree of their dissociation corresponds to the particular values of dissociation constants (often expressed as the negative decimal logarithm, i.e. pK) for particular auxins. Generally, non-polar non-dissociated forms of auxin molecules can penetrate the PM, while auxin anions arising from the dissociation process can be transported into cells only actively – via a transporter. Outside cells, at a pH of circa 5.5, there is always a significant percentage of auxin molecules that are not dissociated (for IAA ca. 20 % of the total amount [27]) and which can thus enter cells passively on the basis of the concentration gradient (i.e. via passive diffusion). However, once auxin molecules are inside cells, where the cytoplasmic pH is approximately 7.0, their dissociation is almost complete and auxin molecules are in the form of anions. As such, they cannot passively penetrate through the PM; they are therefore trapped inside cells ('anion' or 'acid' trap) and can only be excreted actively from cells via a transporter. From these physical-chemical relations, it is obvious that it is the efflux of auxin anions which represents the 'bottleneck' in the movement of auxin molecules between adjacent cells. If the auxin efflux carriers effecting auxin anion excretion from cells are localized asymmetrically (and always at the same sides (either lower or upper) of cells in the vertical cell file), they would provide the auxin flow with a particular direction (down towards roots, up towards the stem apex, or laterally). These physical-chemical conditions and the postulation of an asymmetric localization of auxin efflux carriers at the PM were incorporated into the so-called 'chemiosmotic polar diffusion model' [28, 29] or 'chemiosmotic hypothesis' [30] explaining the physical-chemical basis of the mechanism of polar auxin transport [discussed in detail in ref. 16].

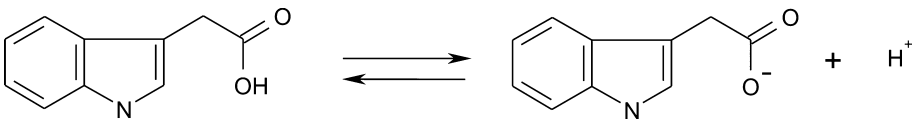

Figure 1. Reversible dissociation of the molecule of native auxin – indole-3-acetic acid (IAA).

Mathematical modelling of auxin flow

In fact, the 'chemiosmotic hypothesis' of intercellular auxin flow was the first step for mathematical modelling of polar auxin transport. The knowledge of the physical-chemical properties of the auxin molecule and a growing body of information about various cell characteristics, for example, the (partial) determination of both auxin carrier kinetics and carrier distribution at the PM, have resulted in various mathematical models of auxin flow in a simplified plant tissue.

The observation that polarized auxin movement in tissues creates 'streams' of auxin that determine future vascular bundles led to the formation of the first mathematical models of auxin flow across tissues.

Thirty years ago [31], it was shown that not only did cells with a flux of auxin higher than their neighbours become specialized in auxin transport and turn into a 'sink' of auxin for surrounding cells, but also that the auxin transport ability of cells increased with auxin flux, resulting in self-enhancement of the flux along auxin paths. The so-called 'canalization hypothesis' [31] was further extended by considering the auxin diffusion coefficient [32] and by the positive feedback regulation between auxin flow and membrane permeability [33]. It was also demonstrated that even a small perturbation in auxin flow led to the formation of an auxin path, which was then preferentially used for auxin transport towards the sink [33].

With respect to the assumption that cells exchanging auxin with adjacent cells increase their PM 'penetrability' for auxin on the side with the larger auxin flux [33], the distribution of auxin efflux carrier proteins (namely PIN1) was included in mathematical models of plant venation [34–36]. In one such model [34], the relationship between the number of auxin efflux carriers on the PM and auxin flux is represented by so-called response functions. These functions were tested in relation to vein formation. The model takes into account auxin carrier protein dynamics and presumes either independent carrier protein regulation or competition between auxin molecules for a limited number of free carriers at the PM region with higher flux. The flux of auxin between two adjacent cells was described as a linear or saturating flux. Various combinations of postulated parameters for both the dynamics of auxin flux and auxin efflux carrier regulation resulted in various patterns of the vascular system [34]. The regulation of carrier proteins, by competition between particular regions of the PM for a limited amount of these proteins, resulted in branching vein patterns which corresponded to native vein formation. The presumption of a limited amount of carrier proteins is in agreement with the observation that the total protein amount in cells changes very slowly [37]. Contrary to a previously presented model [33], the new model [34] revealed a branching pattern with higher auxin level in veins. However, both models used the simplified lattice as an artificial tissue for mathematical modelling. To avoid this limitation, confocal images of plant tissue with PIN1 (visualized using PIN1:GFP expression) were used as a basis for modelling primordial positioning [38]. The model included both the regulation of PIN1 polarity by relative auxin concentrations in neighbouring cells and the mechanics of cell growth, and reflected the situation in real tissue [38].

As shown above, at the tissue level, there is a growing number of mathematical models of auxin transport incorporating several parameters. However, this does not apply at the cellular level. One of the few existing cellular-level models is actually a simplified plant tissue model composed of three types of cells differing in their auxin efflux carrier localization at the PM [39]. In this model, both the lateral PIN localization at the PM of cells at the periphery of auxin-transporting tissues [9, 12, 13] and the ubiquitous PM localization of auxin influx carriers of the AUX/LAX family [40] were considered. Explicit values of auxin concentration and flux, as functions of cell position, were obtained under a variety of assumptions for the expression levels of *PIN* and *AUX/LAX* genes, membrane permeability for auxin and cell length. The impact of different possible strategies for auxin transport was discussed and compared to known localizations of auxin carriers in plant tissues [39].

All these models represent a useful basis for further experimental work because they predict both the quantitative relationships between physical-chemical behaviour of auxin molecules and the activities of the various transporters and their localizations within cells and tissues [for reviews see refs. 27, 41]. These predicted data can be, in turn, further tested and one can assume that detailed determination of the expression patterns for various auxin carriers, their subcellular localization, dynamics, mutual relationships and, last but not least, the function of potential

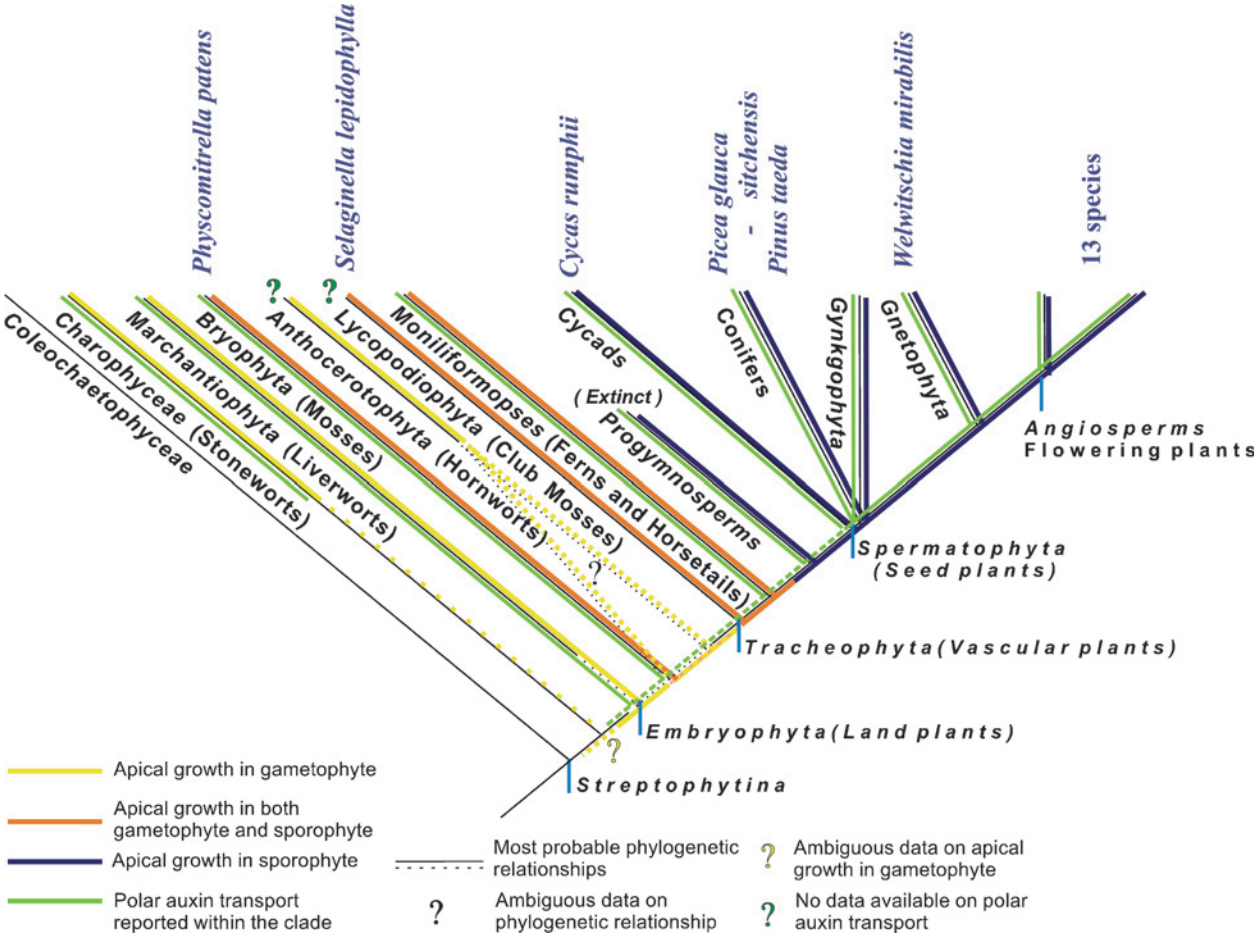

Figure 2. Series of branching events in the plant evolution process with respect to the predictability of *PIN*-like gene distribution. The layout of the groups' relationships and the depicted evolution of apical growth in the diagram were adapted according to Friedman et al. [45] and Friedman and Carmichael [46]. Additional data correlating polar auxin transport with apical growth were included in those cases where polar auxin transport was reported for at least one species within the group (for stoneworts [45, 47, 48], hornworts, liverworts and mosses [48], ferns and lycopsids [indirect evidence in refs. 49–51] and seed plants [5, 16, 46, 52, 53]). Interestingly, a hallmark of polar auxin flow (specific pattern of vascular tissues) was found in 375-million-year-old fossil wood, belonging to the former forest tree *Archaeopteris* genus, an extinct non-seed vascular *Progymnosperm* plant [54]. Except for angiosperms, until now *PIN*-like sequences have only been found in the moss *Physcomitrella patens* [43], and several ESTs were revealed in *Selaginella lepidophylla*, probably one of the oldest of all extant genera of vascular plants [50], in *Cycas rumphii* of the Cycadophyta, in several conifers and in *Welwitschia mirabilis* from the Gnetophyta division (all these species are depicted in blue italics above the relevant clades). Land plants, together with green algae, form the clade Viridiplantae – green plants. Viridiplantae can be divided into two main clades – Chlorophyta and Streptophyta (also Charophyta). The phylum Streptophyta comprises all land plants and six monophyletic groups of charophycean green algae (Mesostigmatales, Chlorokybales, Klebsormidiales, Zygnematales, Coleochaetales, and Charales) [44]. Land plants form a monophyletic group within the Streptophyta clade and Charales are a paraphyletic-sister subgroup to land plants, thus representing the closest living relatives to them [44, 45, 53, 55]. Relationships between PIN sequences of 13 angiosperm species are analysed in Fig. 3a.

regulatory proteins will be further steps for the mathematical modelling of auxin flow in plant cells and tissues.

Auxin and PINs: evolutionary aspects

Sequences homologous to PINs from the model plant, *A. thaliana* have been found in plant genomes and the transcriptomes analysed. Relatively high values of similarity (compared to the closest known bacterial homologues [42]) between individual members of the *PIN* gene family in *Arabidopsis* and other higher plants (ranging from 32 to 85% mutual identity) suggest that evolution of *PIN* genes started from a single ancestral sequence [42]. The report of at least two *PIN*-like genes in the model moss *Physcomitrella patens* [43] indicates that this protein family started to diverge at relatively early phases of the evolution. Representatives of the *PIN* gene family have been found in many angiosperms, but information about *PIN* homologues in evolutionarily more apomorphic plant species and/or most closely related [44] green algae, Charales, remains limited (Fig. 2).

Until sequencing projects reveal new data about gene and protein sequences, the most helpful approach is to study the mode of auxin action and transport in relation to the formation of body plan in extant members of early diverging lineages of multicellular plants [48, 52, 53, 56]. These studies have focused on existing clades of multicellular plants and their closest algal relatives, i.e. on Bryophyta and Charophyta and they have provided a growing body of evidence that polar auxin transport underlie the crucial developmental processes not only in vascular plants, but also in Bryophytes and even in Charophytes. It has been proposed [53] that the evolution of some of those adaptation processes, which are crucial for higher plants, could be related to the new acquisition of polarized growth, supported by polar auxin transport. The ability to produce auxin and control its biosynthesis may be ubiquitous in the land plant lineages [52, 53, 56] and, most probably, auxin operates significantly in the development of several more eukaryotes. Somewhat surprisingly, auxin was found to be synthesized and to be effective in brown algae like *Fucus* [57], which are rather remotely related to plants and which are supposed to have diverged from plant progenitors at very early stages of evolution [57, 58]. An auxin molecule itself is not a specific factor of growth complexity in plants, although the positional information accomplished by an uneven, gradient-like distribution of such signal molecules can have this function. From the beginning of Streptophytes diversification towards the more apomorphic features of their descendants, groups of algae received, among other traits, the competence to create multicellular organisms. Probably, simple adhesive colonies of independent cells showed up first and then filamentous-axial structures might have appeared, having developed, possibly homoplastically, into several independent groups [55]. Clumps of filamentous algae did not necessarily need to depend on the mutual sharing of common information between their cells, and such axiality could be achieved by simple cell divisions following the frame of each cell's internal polarity. Thus multicellular axiality, when cells are growing in one line with a transversal plan of cell division, may be quite a plesiomorphic stage, and the acquisition of polar auxin transport may represent the next crucial innovation for the evolution of plants with a higher degree of body complexity and with their development better regulated.

It must be noted that cell polarity need not necessarily result in tissue polarity [59] and, in polarized tissue, cells have to have positional information. To establish polarity within the tissue, not only must directional information be maintained, but differential gene expression in cells along the axis must proceed to create functionally diverse conditions within the polarized tissue. In relation to their adaptive and flexible development, plants also often have to change cell polarity postembryonically according to demands of the environment and to internal cues [60]. This information is very probably provided by the polar auxin flow and polar auxin transport represents one of the internal cues defining the polarity of the body of higher plants. This idea was confirmed by the demonstration that polarity of mature tissues could be disturbed or even destroyed by explanting them and growing them at the condition of high auxin content [61] and that auxin movement in apolar tissues was primarily diffusive [62]. The latter, together with physical-chemical reasons for the key role of auxin efflux carriers in polar auxin transport, point to the crucial importance of PINs in the establishment/maintenance of the polarity of plant cells, tissues and organs.

While there is a growing body of evidence that polar auxin transport is closely related to apical growth in hornworts, liverworts, mosses [52, 53], ferns [49] and other early diverging plant lineages (Fig. 2), there is almost no information directly focused on carrier-driven auxin flow and its relationship(s) to the establishment of polarity. Nevertheless, it was shown [47] that in *Chlorella vulgaris* Beij. (a simple, non-motile, unicellular alga, distantly related to plants) there was no activity of any auxin carriers. In contrast, in thallus cells of the Charales member *Chara vulgaris,* L. (a multicellular green alga exhibiting polarity and a considerable degree of organ specialization), the activities of both auxin uptake and efflux carriers were revealed. However, *Chara* auxin efflux carriers did not seem to be sensitive to phytotropins, the inhibitors of auxin efflux in higher plants (see below), suggesting the independent evolution of auxin efflux carriers and phytotropin receptors [47].

So, at present there are still not enough data to correlate the appearance of polarity and polar growth with the evolution of the PIN-like auxin efflux carriers. Thus, the question of what type of new developmental advantage in the evolution process of plants is connected with the first appearance of PIN-related regulation of directional auxin flow remains open.

Family of PIN proteins: analysis of sequences

In *A. thaliana*, there are eight sequences assigned to PIN proteins [42]. Until recently [63], the auxin-transporting activity of the PINs had not been proven biochemically. Nevertheless, according to the database PFAM [64], PINs belong to the group

'mem_trans', which is one of several groups of secondary transporters. These transporters derive the energy for transport from the electrochemical gradient across the membrane.

To investigate the structural diversity of the PIN protein family, we searched the public repositories of the sequence data for *A. thaliana* PIN homologues from other plants. The programme BLAST [65] found 57 complete sequences, which were then aligned with the programme MAFFT, method L-INS-i [66].

The alignment was used to construct a cladogram (Fig. 3a) using programmes from the package Phylip [67] based on the relative similarity of proteins. The validation of the phylogenetic tree with the bootstrapping method shows very stable separation of proteins into several groups, but the relations between the groups are not stable. For the orientation of the tree, the sequence of PIN from *P. patens* was used as an outgroup. Based on the structure of the hydrophilic loop, the sequences can be divided into two classes: class I with a very short hydrophilic loop (only domain C1 and variable region V1; see Fig. 3b) and class II with a long hydrophilic loop (domains C1, C2 and C3 and variable regions V1 and V2). The classes can be further divided into smaller groups based on the variable regions. The variable regions are homologous within the groups but, between different groups, variable regions are very dissimilar. According to the data from other secondary transporters [68], the hydrophilic loop is not necessary for the transporting function, but it is important for the regulation of the transporter function and for the proper localization of the protein.

The alignment was also used for the bioinformatic investigation of PIN structural landmarks. Since pattern indications for some of the structures have a low specificity, we considered only the predictions present either in all PINs or in a prevailing part of the PIN protein family. Figure 3b summarizes the predicted structures. All PINs contain two hydrophobic domains (each with five transmembrane helices) separated by a hydrophilic loop. In between the second variable region of the hydrophilic loop and the beginning of the C-terminal hydrophobic domain there is the internalization motif NPXXY [69]. This motif represents a conserved part of the sequence, which can be important for the interaction of the transmembrane protein with the adaptor proteins during clathrin-dependent endocytosis. In the hydrophilic loop, two clusters of motifs important for post-translational modifications were found. Each cluster contains a conserved motif for glycosylation and two motifs for phosphorylation (predicted according to the Prosite database [70]).

Molecular/biochemical function of PINs and the key role of their subcellular localization

The analysis of predicted sequences of PIN proteins showed that they are transmembrane proteins and belong to the group of secondary transporters. *At*PINs also share a limited sequence similarity with some prokaryotic and eukaryotic transporters [20, 21]. However, the PIN sequences themselves do not prove the molecular function(s) of the PIN proteins. From previous reports, there are several indications [16, 21, 71, 72] that PINs have a crucial role in the polar auxin efflux machinery: some *pin* mutants were shown to have serious defects in polar auxin transport [19, 73]; PIN proteins are localized in cells in a polar manner corresponding to the direction of the auxin flow [9, 12, 20, 21, 74]; polar auxin transport inhibitors (see below) can phenocopy loss-of-function *pin* mutations in wild type plants [9, 12, 19, 75]; expression of *At*PIN2 in yeast cells results in lower accumulation of auxin and structurally related compounds [76, 77]. PINs thus became serious candidates for auxin efflux carriers. However, since their biochemical function as auxin efflux carriers was not demonstrated until recently, PINs earned the 'delightfully noncommittal title' [78] of 'auxin transport facilitators'.

To dissect the biochemical function of PINs, the cell culture systems derived from the tobacco BY-2 cell line [79] and *Arabidopsis* cultured cells were used [63]. The heterologous expression of *At*PIN1 protein in translational fusion with GFP demonstrated its preferential localization at transversal PMs [80, 81]. Mild plasmolysis of tobacco cells confirmed the PM localization of PINs and suggested the involvement of interaction(s) between the PM and cell wall in the establishment and/or maintenance of the non-uniform cellular PIN localization (Fig. 4a–c). Moreover, the dynamics of PIN distribution along the PM and in cortical cytoplasm seems to be strong (Fig. 4d–f) as shown by fluorescence recovery after photobleaching (FRAP). Using the inducible overexpression of *At*-PINs in suspension-cultured tobacco cells, the PIN-related kinetics of auxin accumulation, substrate specificity of auxin efflux and sensitivity to polar auxin transport inhibitors were characterized [63]. Auxin-specific efflux was shown to be directly proportional to the degree of PIN expression and this *At*PIN-related auxin efflux was sensitive to polar auxin transport inhibitors, namely 1-naphthylphthalamic acid (NPA, see below). In both yeast cells and mammalian HeLa cells, the heterologous overexpression of *At*PINs also resulted in an increase of auxin efflux, regardless of the fact that these systems do not contain any PIN-related genes nor do they have auxin-related signalling and transport machinery. All these

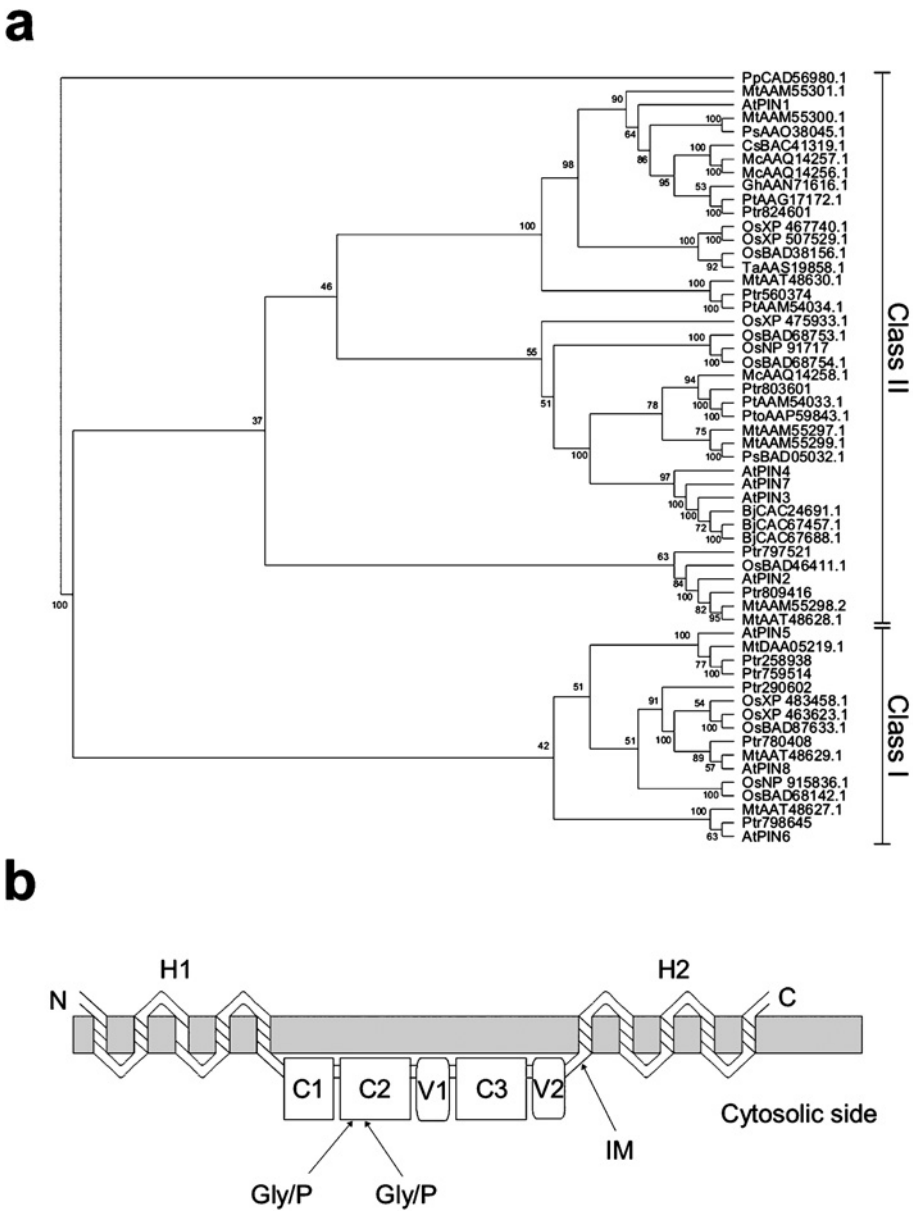

Figure 3. The cladogram and structure of PIN proteins. **(***a***)** The cladogram of PIN proteins: the names of the proteins begin with the first letters of the scientific name: At, *Arabidopsis thaliana*; Bj, *Brassica juncea*; Cs, *Cucumis sativus*; Gh, *Gossypium hirsutum*; Mt, *Medicago truncatula*; Mc, *Momordica charantia*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Ps, *Pisum sativum*; Pto, *Populus tomentosa*; Pt, *Populus tremula* x *Populus tremuloides*; Ptr, *Populus trichocarpa*; Ta, *Triticum aestivum*. The names of Arabidopsis proteins follow the commonly used names (PIN1 – PIN8). The sequences from *Populus trichocarpa* are identified with the codes from the genomic sequence draft. Other proteins are identified with their accession codes in the NCBI database. The cladogram was created with the package Phylip (programmes seqboot, protdist, fitch and consense) [67]. Bootstrapping values for 100 resamplings are shown. **(***b***)** Generalized scheme of the predicted structure of PIN proteins: H1, H2, hydrophobic domains [predicted in refs. 21, 42]; C1, C2, C3, conserved domains of the hydrophilic loop; V1, V2, variable regions of the hydrophilic loop; Gly/P, the cluster of glycosylation and two phosphorylation sites; IM, internalization motif. N, amino-terminus; C, carboxy-terminus.

findings together imply the direct involvement of PIN proteins in catalyzing the efflux of physiologically active auxins from cells and suggest that PINs function as auxin efflux carriers. Similarly, it was also shown that the inducible overexpression of PGP19 resulted in an increase in auxin efflux from cells, albeit with lesser sensitivity to NPA. However, PGP1 and PGP19 did not seem to be required for the action of PIN1 in a plant developmental process, namely the gravitropic response [63].

The evidence for the direct auxin-efflux-catalyzing role of PINs further pointed to their crucial role in polar-auxin-transport-regulated physiological processes. However, even if it was known that asymmetrical PIN localization at the PM corresponded to the direction of auxin flow [13, 16, 17, 21], there was still no direct experimental proof available showing that PINs determine this direction. Recently, variants of *PIN1* and *PIN2* genes were prepared [82], tagged with haemagglutinin (HA) and/or fused with green fluorescent protein (GFP) and they were put under the transcriptional control of the *PIN2* promoter. In the *pin2* mutant, transformed with these constructs, PIN2::PIN2-HA showed a normal PIN2-like polar wild-type-like localization in root cortex and epidermal cells, while PIN2::PIN1-HA was detected in epidermal root cells at the side of cells opposite to that of PIN2. Two PIN2::PIN1-GFP constructs, with the GFP sequence positioned differently within the PIN1-coding sequence, showed opposite localizations

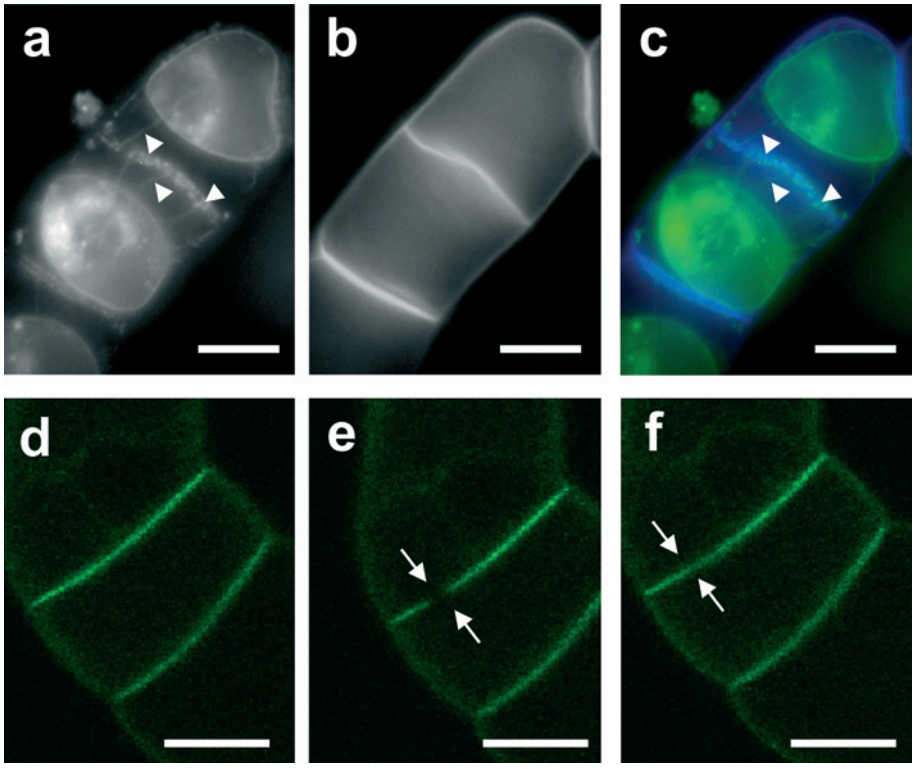

Figure 4. *In vivo* localization (*a–c*) and dynamics (*d–f*) of *Arabidopsis thaliana* PIN1-GFP proteins in stably transformed 2-day-old tobacco BY-2 cells. Cytoplasmic localization of PIN1-GFP after mild plasmolysis with 0.45 M mannitol (5 min) with GFP signal in Hechtian strands and plasma membrane-cell wall attachments (arrowheads). PIN1-GFP (*a*), calcofluor white cell wall staining (*b*) and merged image (*c*); fluorescence microscopy. FRAP (fluorescence recovery after photobleaching) of PIN1-GFP, optical section, confocal microscopy. PIN1-GFP signal in transversal plasma membranes before bleaching experiment (*d*), immediately after bleach (*e*) and after 20 min of FRAP (*f*). Arrows indicate the position of the bleached region of interest. Scale bars, 20 μm.

within epidermal cells and they were used for detailed study of the relationship between PIN1-GFP cellular localization and auxin translocation during a gravity response. Only the PIN1-GFP protein with the correct cellular localization was able to mediate auxin translocation at the lower side of the root after gravity stimulation and to rescue the agravitropic response of the *pin2* mutants. These observations showed convincingly that the polar localization of PIN proteins in competent cells is the primary factor determining the direction of auxin flow [82]. Therefore, the biochemical function of PINs as auxin efflux carriers together with their direction-determining role in auxin cell-to-cell flow imply a central role for PINs in the polar auxin transport machinery and thus in all polar-auxin-transport-dependent physiological processes in plants. Despite the fact that PIN proteins were shown to perform a uniform biochemical function, the individual PIN proteins play a key role in many very diverse physiological processes (see above). They show a tissue-specific or even – as e.g. in the root tip – a cell-type-specific expression pattern [10, 11, 13, 27, 83]. However, surprisingly, the phenotype of most single *pin* mutants (with an important exception of *pin1*) is weak, if observable at all. In contrast, some *pin* quadruple mutations were embryolethal [7] and ectopic expression of PIN proteins was observed in various mutant combinations [10]. Thus, there is a wide functional redundancy among different PIN proteins in various developmental processes [83], implying again the uniformity of their molecular function. If the synergistic interactions of various PIN proteins are to function in various developmental processes, one would expect several well-balanced levels of regulation of PIN activity.

Regulation of activity of PIN proteins

Generally, as for any other protein, there are several possible levels of regulation of PIN activity.
These are gene expression, protein synthesis and maturation, protein trafficking and targeting and regulation of the function of the already 'mature' protein. The latter involves modulation of 'stability' of the protein in the membrane, competitive and non-competitive inhibitions, posttranslational (namely phosphorylation/dephosphorylation) modifications, modulation of the 'environment' of the protein in the membrane and, last but not least, the degradation of the protein itself.

Regulation of PIN expression

The functional redundancy among the members of the PIN family involves cross-regulation of the expression of their genes [83]. This finding together with the

change of expression pattern of PINs in various *pin* mutant combinations [10] suggested a feedback control of PIN gene expression. How is this feedback put into effect? The chemical inhibition of polar auxin transport interfered with PIN gene expression, resembling the situation in *pin* mutants [83]; this finding pointed to the involvement of auxin distribution in tissue-specific modulation of PIN gene expression. Indeed, there are data available about the relationship(s) between auxin itself, its distribution and control of *PIN* gene expression [83–86], and the involvement of the auxin signalling pathway, based on auxin-related F-box protein(s) (AFBs), Aux/IAA repressors and auxin response factor (ARF)-type transcription factors [4], in control of gene expression of PINs was also demonstrated [83, 86].

Vesicle trafficking and PIN targeting to specific domains in the PM

In contrast to the control of *PIN* gene expression, there is nothing known about the regulation of PIN protein synthesis and maturation. However, data are available highlighting the importance of vesicle trafficking and PIN targeting to specific domains in the PM. Once the PIN protein is synthesized and post-translational modifications are finished, the protein seems to be loaded into the vesicle trafficking system. In young *Arabidopsis* globular embryos, both basally localized PIN proteins and activity of GNOM were shown to be needed for apical-to-basal auxin flow, and PIN1 was mislocalized in *gnom* mutants [87]. Moreover, similar phenotype resulted from treatments of *Brassica* embryos either with high doses of auxin or with inhibitors of its polar transport [88]. GNOM codes for a *G*DP/GTP *e*xchange *f*actor for ARF-type small G proteins (ARF-GEF) [89] and this type of protein was shown to be involved in control of vesicle budding and selection of cargo [90]. These observations clearly indicated the importance of GNOM for vesicle trafficking of PINs to the PM and the involvement of GNOM in the establishment and/or maintenance of polar auxin flow. In these studies, inhibitors of protein secretion from eukaryotic cells, such as brefeldin A (BFA), became very potent tools. BFA was shown to inhibit auxin efflux [91–93] and to cause an internalization of PIN proteins in endosomal 'BFA' compartments [37, 94, 95]. Treatments with low concentrations of BFA resulted in growth and developmental defects that pointed to auxin transport inhibition [94]. To dissect the mechanism of action of BFA and the role of GNOM in PIN trafficking and targeting, the BFA-resistant version of GNOM was engineered and plants carrying the fully functional but BFA-resistant GNOM were prepared [94]. In these plants, PIN1 localization as well as polar auxin transport were BFA-insensitive; however, surprisingly, trafficking of several other proteins was still BFA sensitive. These findings, together with the observation that GNOM localized to endosomes and it was needed for their structural integrity, provided evidence for the role of GNOM in trafficking of component(s) of the polar auxin transport machinery via a specific endosomal trafficking pathway.

In eukaryotic cells, the cytoskeleton provides a 'scaffolding' for the cell architecture and serves as an active track for secretory pathways. Thus, it is not surprising that drugs impairing cytoskeletal structures and cytoskeleton functioning also significantly affect PIN distribution. Cytochalasin D and latrunculin B, compounds altering the state of actin polymerization, were shown to reduce polar auxin transport [96] and to decrease polar targeting of PIN1 at the PM and BFA-induced PIN1 internalization and its relocalization after BFA had been washed out [37]. On the basis of such observations and with respect to the possible interactions between inhibitors of auxin efflux (see below) and the actin network, it was suggested that the actin cytoskeleton may fix the auxin efflux carriers in their polar localization on the PM [97]. It was also speculated that the connection ('a bridge') between the actin filaments and a complex of the auxin efflux carrier may be provided by a so far unknown protein which binds inhibitors of polar auxin transport such as NPA (see below) [98, 99]. Perhaps, in a way, this resembles the roles of spectrins and ankyrins in the formation of discrete PM subdomains containing various transporters in some polarized mammalian cells [100]. None of these suggestions have yet been experimentally proven and further studies are necessary. In fact, the inhibitors of polar auxin transport were suggested [37] to block vesicle trafficking and PIN cycling (see below), but the mechanism of their action is also still unclear. It was recently shown that the localization of PIN proteins also depends on local properties of the PM and on its direct environment, namely microtubule arrays, which might provide the crucial positional signal, and that the intact cell wall is needed as well [81].

Nevertheless, there is one protein, which clearly influences polar localization of PINs; it is PINOID (PID), serine-threonine protein kinase. Some aspects of the loss-of-function *pinoid* phenotype resembled those of the *pin1* mutant [101] and, conversely, strong constitutive overexpression of PINOID resulted in other aspects such as hypocotyl and root agravitropy, which can also be related to the impairment of polar auxin transport [102, 103]. Detailed study, focused on PIN1, 2 and 4 [104] showed that PINOID specifically

controls the polarity of PIN localization. PINOID works as a binary switch which, being present at below-threshold levels, directs PINs to a basal localization (towards the root tip), while, at above-threshold levels, PINs are at the opposite, hence apical, position. In accordance with these findings, all developmental defects in both loss-of-function and gain-of-function *PINOID* lines were consistent with the reversed direction of auxin flow. From the experimental data, it is obvious that PINOID-dependent phosphorylation is essential for correct polar PIN targeting [104]. Since *PINOID* gene expression is auxin inducible [103], one can speculate that PINOID may be a part of a feedback loop which operates in order to 'balance' tissue-specific auxin distribution and by which an auxin can control its own polar flow [104]. This speculation was supported by the finding that overexpression of PINOID enhanced auxin efflux from cells and resulted in a decreased intracellular auxin level [105].

Regulation of PIN biochemical activity, inhibitors of polar auxin transport

Even if a particular protein has reached its correct position in the proper subcellular compartment, its activity can still be subject to multiple regulations. In the case of PINs there are several possibilities. One of the possible regulations of PIN biochemical activity, i.e. translocation of auxin molecules out of cells, is the competitive and non-competitive inhibition of their function. This type of regulation is performed by so-called polar auxin transport inhibitors (PATIs). These regulators [106, 107], most of which are synthetic compounds, act at the level of auxin efflux; by inhibiting it, they increase auxin accumulation in cells [80, 93, 108]. Even if they have been known for decades, their molecular mechanism of action is still not clear. It seems that some of them, such as 2,3,5-triiodobenzoic acid (TIBA), are also weak auxins and so can compete with auxins for translocation across the PM [109]. However, there is also another group of PATIs, the so-called phytotropins [106, 107], of which NPA is the most typical representative. Like other PATIs, the mechanism of phytotropin action is not yet completely understood. However, their effects are probably mediated by specific 'NPA-binding protein(s)' [NBP(s)] [107]. More detailed study of the effects of NPA and the protein synthesis inhibitor cycloheximide on carrier-mediated auxin efflux in zucchini hypocotyl segments suggested that the auxin efflux carrier and NBP are two different proteins which may be coupled by a third 'mediator' whose metabolic turnover is rapid [110]. With one exception [111], NBP was believed to be a peripheral membrane protein located at the cytoplasmic face of the PM and connected to actin filaments [16, 98, 99]. Many years before the monitoring of polar localizations of PINs in various tissues, an indirect immunofluorescence technique was used to detect NBP in cells associated with the vascular tissue in pea stem sections [112]. Interestingly, this revealed its polar localization (basal, i.e. towards the root tip), consistent with the predicted localization of auxin efflux carriers in this tissue. The fact that NBPs are present across the entire plant kingdom [71] implied the existence of natural, probably structurally related inhibitors of polar auxin transport. Indeed, such naturally occurring inhibitors were discovered in a screen of phenolic compounds: some flavonoids (e.g. quercetin) were able to increase auxin accumulation in zucchini hypocotyl segments and to compete with NPA for binding to isolated membrane preparations [113]. This finding was later supported by the fact that in the flavonoid-deficient Arabidopsis mutant *tt4* (*transparent testa 4*), cellular efflux of auxin was increased. This behaviour could be rescued by naringenin, the intermediate of the flavonoid biosynthetic pathway [114]. Thus, flavonoids may be the natural equivalents of NPA and other phytotropins in inhibiting the efflux of auxins from the cell and, consequently, in inhibiting polar auxin transport [115, 116].

To identify components of the polar auxin transport machinery, mutant screens were performed to isolate PATI-insensitive mutants [117]. Most of these mutants were demonstrated to have defects in auxin signalling. However, mutant *tir3* (*transport inhibitor response 3*), has many developmental defects, such as decreased apical dominance, reduced elongation of root and inflorescence stalks and reduced lateral root formation, which are directly related to disruption of polar auxin transport. Moreover, both auxin transport and NPA-binding activity were reduced in this mutant, leading to the suggestion that the gene *TIR3* may code for the NBP or another part of the auxin efflux carrier complex. The gene was later characterized [118] and since the corresponding protein is unusually large (560 kDa) it was renamed *BIG*. BIG contains several putative Zn-finger domains and is homologous to the *Drosophila* CALOSSIN/PUSHOVER (CAL/O) protein. This fact suggests a role in vesicle trafficking (see below).

The 'opposite' mutant to *tir3,* named *polar auxin transport inhibitor-sensitive 1* (*pis1*) was isolated in a similar screen [119]. This mutant was hypersensitive to some PATIs and thus it was tempting to suggest that PIS1 functions as a negative regulator of PATI action. Recently, the gain-of-function and loss-of-function mutants *pdr9* have been characterized [120]. PDR9 is

a member of the pleiotropic drug resistance (PDR) family of ABC transporters, and the analysis of *pdr9* mutants suggested its involvement in efflux of the synthetic auxin 2,4-D, but not of the native auxin IAA. The loss-of-function *pdr9–2* mutant was also hypersensitive to NPA, so PDR9 may also be responsible for excretion of PATIs from cells.

NPA-affinity chromatography was used to isolate NBPs [121], and both high- and low-affinity NPA-binding fractions were obtained. The high-affinity fraction contained PGPs (see above), the plant orthologues of mammalian multidrug-resistance (MDR)-like ABC transporters and, also, "twisted dwarf" (TWD1), the glycosylphosphatidylinositol (GPI)-anchored immunophilin, which is known to interact with PGPs and to be necessary for their function [122]. The low-affinity fraction contained typically APM1, the 103-kDa transmembrane aminopeptidase belonging to the gluzincin dual-function aminopeptidase/protein-trafficking family. In mammals, these dual-function proteins are involved in cycling of asymmetrically localized transporters (e.g. the glucose transporter GLUT4 [123]), or they are modulators of sterol influx into cells [124]. So, how do phytotropins act? At the biochemical level, by force of the NBP, they inhibit auxin efflux and hence increase auxin accumulation in cells. However, phytotropins have been shown to also participate in other cellular processes, which are related to polar auxin transport. In tobacco cells cultured *in vitro,* NPA disturbed the polarity of cell division suggesting that NPA may affect the directed traffic of auxin efflux carriers to the specific regions at the PM and, also, that the directed auxin flow may regulate the orientation of cell division [125, 126]. There are also data available implicating phytotropins in modulation of vesicle trafficking and PIN protein cycling (see below).

Constitutive cycling of PINs

Biochemical and physiological experiments [127] suggested that auxin efflux carriers are not statically 'seated' in the PM but undergo rapid cycling between the PM and an internal pool(s) of these proteins. The cycling was independent of simultaneous protein synthesis [127]. This constitutive cycling was later confirmed and demonstrated for PINs [37] and it was shown that high concentrations of phytotropins interfered with vesicle trafficking of several proteins to and from the PM. This action of phytotropins on vesicle trafficking may be related to some of their 'low affinity' sites [121, 128]. However, it cannot be responsible for direct inhibition of auxin efflux by phytotropins, because these compounds inhibit auxin efflux efficiently at concentrations three to four orders of magnitude lower than those needed for inhibition of PIN cycling [93]. Nevertheless, what is the mechanism of PIN proteins cycling and why do they cycle? The mechanism of PIN cycling seems to be based on the specific GNOM-dependent endosomal trafficking pathway (see above) [94]. Even if GNOM belongs to the *G*ea/*G*NOM/*G*BF1 (GGG) subfamily of large ARF-GEFs, which are probably involved in endoplasmic reticulum/Golgi or intra-Golgi traffic in yeast and animals, it did not colocalize with several markers of this secretory pathway in plants. However, it did colocalize with the endocytic tracer FM4–64. The experiments with engineered BFA-resistant GNOM [94] revealed a differential function of GNOM in the trafficking of several proteins. Trafficking of basally localized PIN1 is GNOM dependent, while recycling of apically localized PIN2 (as well as apolarly distributed PM-ATPase and/or the cell-division-specific syntaxin KNOLLE) did not depend on GNOM. The authors [94] suggest the existence of distinct recycling endosomal pathways in plants controlling the recycling of various proteins. Recently, *Arabidopsis* SORTING NEXIN 1 (*At*SNX1)-containing endosomes, distinct from the GNOM-containing ones, have been shown to be involved in trafficking of PIN2 (but not PIN1) [129].

The constitutive cycling of PM proteins consists of two repeated steps: internalization of the protein from the PM into an endosome (endocytosis) and its recycling back to the PM (exocytosis). While the exocytotic step of the constitutive cycling of some proteins is sensitive to BFA (at the level of GNOM), the endocytotic step seems to be sensitive to auxins [95]. This was shown for PIN1, PIN2, PIN3, PIN4, as well as for the PM water channel PIP2 and PM-ATPase. Endocytosis of these proteins was inhibited by auxins and this led to higher incidence of the proteins at the PM. In the case of PINs, this would provide an important feedback mechanism for regulation of internal auxin levels: at higher auxin concentrations, the endocytotic step of PIN cycling is inhibited, resulting in a higher number of PINs at the PM and thus in a higher capacity for auxin efflux. Since in *big* mutants, endocytosis of the above-mentioned proteins was much less sensitive to auxins than in the wild type and, since the effect of auxins on the internalization of the endocytic tracer FM4–64 was reduced in roots of the *big* mutant [95], it was concluded that the effect of auxins on endocytosis requires the activity of the Calossin-like protein BIG (see above).

It is obvious from the above description that PIN proteins are very dynamic at the PM. What is the purpose of this dynamic carrier protein behaviour? Generally, there are three possibilities [discussed in

refs. 16, 72, 130]. First, dynamic cycling can serve as a flexible 'tool' for fast relocation of the carriers and for concomitant changes in the direction of auxin flow. Second, PINs could have a dual carrier/receptor or sensor function [131, 132] and cycling may serve as a part of the signalling pathway and/or as the way to receptor regeneration. Third, in analogy to animal neurotransmitter-like secretion, auxin itself may represent a vesicle cargo and may also be transported by PINs inside cells. In such a case, PINs would represent not only 'auxin channels' at the PM, but they would also be involved in accumulation and/or retention of auxin molecules within specialized endosomal vesicles and in their delivery to the relevant cell pole. Interestingly, the protein BIG, which is implicated in the polar auxin transport machinery and in PIN constitutive cycling (as mentioned above), is homologous to the CALOSSIN/PUSHOVER (CAL/O) protein in *Drosophila*, where it is known to mediate vesicle recycling during synaptic transmission [133]. It must be noted that BIG function has also been implicated in responses to some other plant growth regulatory substances and in some stress reactions, so it is not 'specific' to auxin transport. Recently, immunolocalization of auxin with a new specific antibody and measurements of the kinetics of BFA action strongly supported the neurotransmitter-like secretion of auxin and a corresponding role for PINs in transcellular auxin movement [134].

The possible role of PM microdomains

Recent observations have shown that the ABC transporter PGP19 (see above) stabilized PIN1 on the PM and that, within the PM, both transporters were localized in detergent-resistant membrane microdomains (DRMs) [25, 26]. These sterol-rich microdomains, resembling lipid rafts, were previously described not only in animal but also in plant cells [135–137]. A possible localization of PINs within such specific microdomains in the PM was already implied by the finding that, in the *cephalopod/orc* mutants of *Arabidopsis*, PINs were not localized correctly. Since both membrane fluidity and vesicle trafficking processes seemed to be normal, the effect of mutation was ascribed to impaired docking of the membrane proteins to specific microdomains at the PM [138]. The fact that these mutants suffered from many polarity defects pointed to the importance of balanced membrane sterol composition for both auxin efflux and establishment/maintenance of cell polarity in *Arabidopsis*. Coexpression of both PINs and PGPs in heterologous expression systems revealed that PINs and PGPs may act synergistically [25, 26], so modulation of the composition of transporter-containing microdomains in the PM may represent another level of auxin transport regulation.

The role of phosphorylation

The activity of many proteins is regulated by phosphorylation/dephosphorylation processes, so it is not surprising that results of earlier physiological experiments indicated the involvement of these processes in the control of the activity of some components of the polar auxin transport machinery, namely those involved in the control of auxin efflux from cells. Moreover, analysis of the sequences of PIN proteins revealed the possible phosphorylation sites at the hydrophilic loop (see above and Fig. 3b). The role of a serine-threonine protein kinase PINOID in polar targetting of several PINs has already been mentioned. Its activation was shown to be controlled by phosphorylation with 3-phosphoinositide-dependent protein kinase 1 (PDK1) [139], suggesting that a complex regulatory phosphorylation cascade is involved in the regulation of auxin transport. Moreover, there is at least one more mutant supporting the role of phosphorylation/dephosphorylation in the regulation of auxin efflux – *rcn1* (*roots curl in NPA 1*) – which shows defects typical for disturbance of polar auxin transport (e.g. reduced root and hypocotyl elongation and defects in apical hook formation). The *RCN1* gene codes for a regulatory subunit of protein phosphatase 2A [140]. More detailed studies of the mutant, as well as suspension-cultured tobacco cells, suggested that there are more targets for reversible phosphorylation within the components involved in control of polar auxin transport [92, 97, 141]. The *rcn1* mutant also pointed to the possible involvement of phosphatase activity in the mechanism of action of NPA (and possibly other phytotropins). Recently, the studies of auxin transport, together with hypocotyl gravitropism of this mutant, also demonstrated the role of ethylene (another plant growth regulatory compound) in this process and suggested that RCN1 protein negatively regulated ethylene biosynthesis in dark-grown seedlings [142]. Recent studies on the modulation of expression of MAP kinase kinase 7 (MKK7) [143] pointed to the possibility that some element(s) of the MKK7-dependent signalling cascade down-regulates polar auxin transport and thus causes significant disturbance in growth of *Arabidopsis* plants. However, it is not clear whether this action lies entirely in the control of the cellular auxin efflux (and not auxin influx). Particular loci of phosphorylation/dephosphorylation process(es), as well as the nature of the interaction between the NBP and the auxin efflux

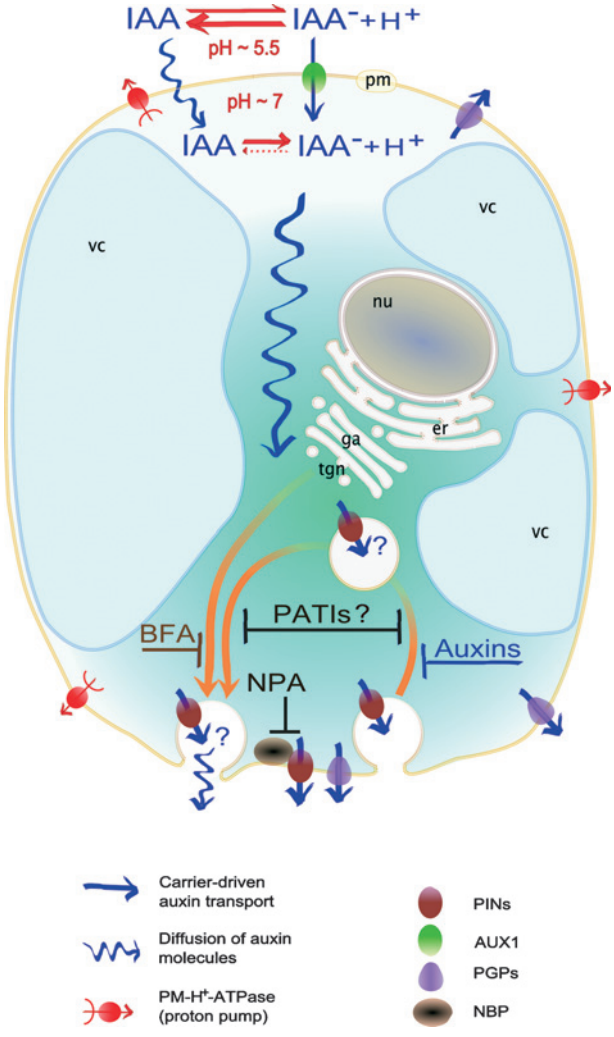

Figure 5. Scheme for the role of PINs in the polar auxin transport machinery in plant cells. Various transporters (auxin influx carrier, i.e. permease AUX1; auxin efflux carriers of the PIN and PGP type) are depicted together with PIN constitutive cycling. Plasma membrane H^+-ATPase, involved in maintenance of the proton gradient across the plasma membrane, is included. The possibility that the vesicle trafficking itself serves as an auxin transport pathway is suggested by the question marks accompanying the arrows representing auxin flow into vesicles. The sites of action of various inhibitors and auxins themselves are shown at the level of auxin efflux. NBP is a hypothetical protein, which binds 1-naphthylphthalamic acid, the non-competitive inhibitor of auxin efflux of the phytotropin type (and possibly also other polar auxin transport inhibitors, PATIs) with high affinity. NBP is believed to be connected with actin filaments and its interaction with auxin efflux carrier(s) might be mediated by another, metabolically very unstable, component. Protein BIG seems to be a part of the endocytic path of constitutive cycling of PINs. PINs may interact with alternative auxin efflux carriers of the PGP type; however, the reason for and the mechanism of such interaction is not known. pm, plasma membrane; vc, vacuole; nu, nucleus; er, endoplasmic reticulum; ga, Golgi apparatus; tgn, trans-Golgi network.

carrier system, are not known, and the elucidation of these processes remains one of the challenges for future research.

Degradation of PINs

Perhaps the last (but not least) level of regulation of protein activity is control of its metabolic stability. There are some data suggesting that auxin can also regulate the degradation of some PIN proteins. This is particularly true for PIN2 [144, 145]. Its post-translational down-regulation required the protein AXR1, which seemed to be involved in ubiquitin-mediated proteolysis [144]. Ubiquitination of PIN2 and the involvement of the proteasome in the control of PIN2 degradation was confirmed later [145]. These findings suggested that ubiquitination may contribute to the control of the proportion between PIN2 molecules recycled back to the PM and those targetted for proteolytic degradation. Studies with transgenic plants [83] revealed that besides time- and auxin-concentration-dependent up-regulation of various *PIN* promoters, as well as auxin-dependent down-regulation of PIN2, there is also a parallel cell-type-specific down-regulation of PIN1 and PIN7 (but not of PIN4) proteins. Thus, at higher auxin concentrations, not only *PIN* gene expression is stimulated, providing a feedback control of intracellular auxin concentrations, but there is also a tissue-specific down-regulation of some PIN proteins. The latter probably provides an additional 'prevention' against overabundance of these auxin efflux carriers and a concomitant depletion of intracellular auxin.

Summary: a flexible PIN network

As suggested by their predicted sequences, PIN proteins are transporters excreting compounds with auxin activity out of cells. There is a functional redundancy between individual members of the PIN family and all PIN proteins seem to be subject to multi-level regulation (Fig. 5). They form a system, consisting of various PIN proteins with tissue/cell-type-specific expression but probably with the same biochemical function. This system can form a very flexible auxin distribution network, which is able to react to many events accompanying development of sessile plants against the 'background' of continually changing environmental conditions. The PIN network underlies the directional auxin flux (polar auxin transport) providing any cell, in any part of the plant body, with particular positional and temporal information. Thus, the PIN network, together with the auxin signalling system(s), coordinates plant development.

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Genome Biology

Protein family review

The PIN-FORMED (PIN) protein family of auxin transporters

Pavel Křeček*#, Petr Skůpa*#, Jiří Libus*, Satoshi Naramoto†, Ricardo Tejos†, Jiří Friml† and Eva Zažímalová*

Addresses: *Institute of Experimental Botany AS CR, Rozvojová 263, CZ-16502 Prague 6, Czech Republic. †Department of Plant Systems Biology, Flanders Institute for Biotechnology (VIB), and Department of Plant Biotechnology and Genetics, Technologiepark 927, Ghent University, 9052 Gent, Belgium.

#These authors contributed equally.

Correspondence: Jiří Friml. Email: jiri.friml@psb.vib-ugent.be

Summary

The PIN-FORMED (PIN) proteins are secondary transporters acting in the efflux of the plant signal molecule auxin from cells. They are asymmetrically localized within cells and their polarity determines the directionality of intercellular auxin flow. *PIN* genes are found exclusively in the genomes of multicellular plants and play an important role in regulating asymmetric auxin distribution in multiple developmental processes, including embryogenesis, organogenesis, tissue differentiation and tropic responses. All PIN proteins have a similar structure with amino- and carboxy-terminal hydrophobic, membrane-spanning domains separated by a central hydrophilic domain. The structure of the hydrophobic domains is well conserved. The hydrophilic domain is more divergent and it determines eight groups within the protein family. The activity of PIN proteins is regulated at multiple levels, including transcription, protein stability, subcellular localization and transport activity. Different endogenous and environmental signals can modulate PIN activity and thus modulate auxin-distribution-dependent development. A large group of PIN proteins, including the most ancient members known from mosses, localize to the endoplasmic reticulum and they regulate the subcellular compartmentalization of auxin and thus auxin metabolism. Further work is needed to establish the physiological importance of this unexpected mode of auxin homeostasis regulation. Furthermore, the evolution of PIN-based transport, PIN protein structure and more detailed biochemical characterization of the transport function are important topics for further studies.

Evolutionary history and gene organization

The PIN-FORMED (PIN) proteins are a plant-specific family of transmembrane proteins that transport the plant signal molecule (phytohormone) auxin as their substrate. Although the limited available data suggest that auxin as a signaling molecule is of an ancient origin in the Plantae supergroup [1,2], the representatives of the *PIN* family have been found only in the genomes of land plants (Figure 1). In land plants, the PIN proteins act as key regulators in multiple developmental events ranging from embryogenesis through morphogenesis and organogenesis to growth responses to environmental stimuli. Most of the PIN proteins characterized are located in the plasma membrane and are restricted to particular faces of the cell; they can therefore mediate directional auxin fluxes within tissues and generate auxin maxima and gradients that influence development [3,4].

The first PIN family members identified and associated with auxin transport were described in the model plant *Arabidopsis thaliana*. The significance and function of *At*PIN1 was discovered through the phenotype generated by the loss-of-function mutation in the gene: mutant plants fail to develop floral organs properly and generate naked, pin-like inflorescences, which gave the name PIN-FORMED (PIN) to the family [5,6]. At the same time, several groups identified the homologous protein *At*PIN2 under different names on the basis of a strong root agravitropic phenotype of the loss-of-function mutant. Independently identified mutant alleles of *PIN2* were *pin2*, *ethylene insensitive root1* (*eir1*), *agravitropic1* (*agr1*), and *wavy6* (*wav6*) [7-10]. Altogether, *Arabidopsis* has eight annotated *PIN* genes, of which six have been functionally characterized up to now: *PIN1* [6], *PIN2* [7-10], *PIN3* [11], *PIN4* [12], *PIN5* [13], and *PIN7* [14]. *PIN6* and *PIN8* are still awaiting characterization.

The eight *Arabidopsis PIN* genes generally can be divided into two broad subfamilies. The prominent feature of the larger subfamily is the distinct central hydrophilic loop separating two hydrophobic domains of about five transmembrane regions each (Figure 2). This subfamily of 'long' PINs encompasses all members of the family that are defined as auxin-efflux carriers localized at the plasma membranes (PIN1-PIN4 and PIN7 as well as their homologs from seed plants - called the canonical PINs) [15,16]. In addition, we include PIN6 also as a member of the long PIN subfamily on the basis of the high sequence similarity in the transmembrane regions and only partial reduction of the hydrophilic loop. The hydrophilic loop is the most divergent part of PIN proteins. On the basis of the sequence of this loop, the long PINs are divided into seven groups (groups 1, 2, and 4-8), such that members of the

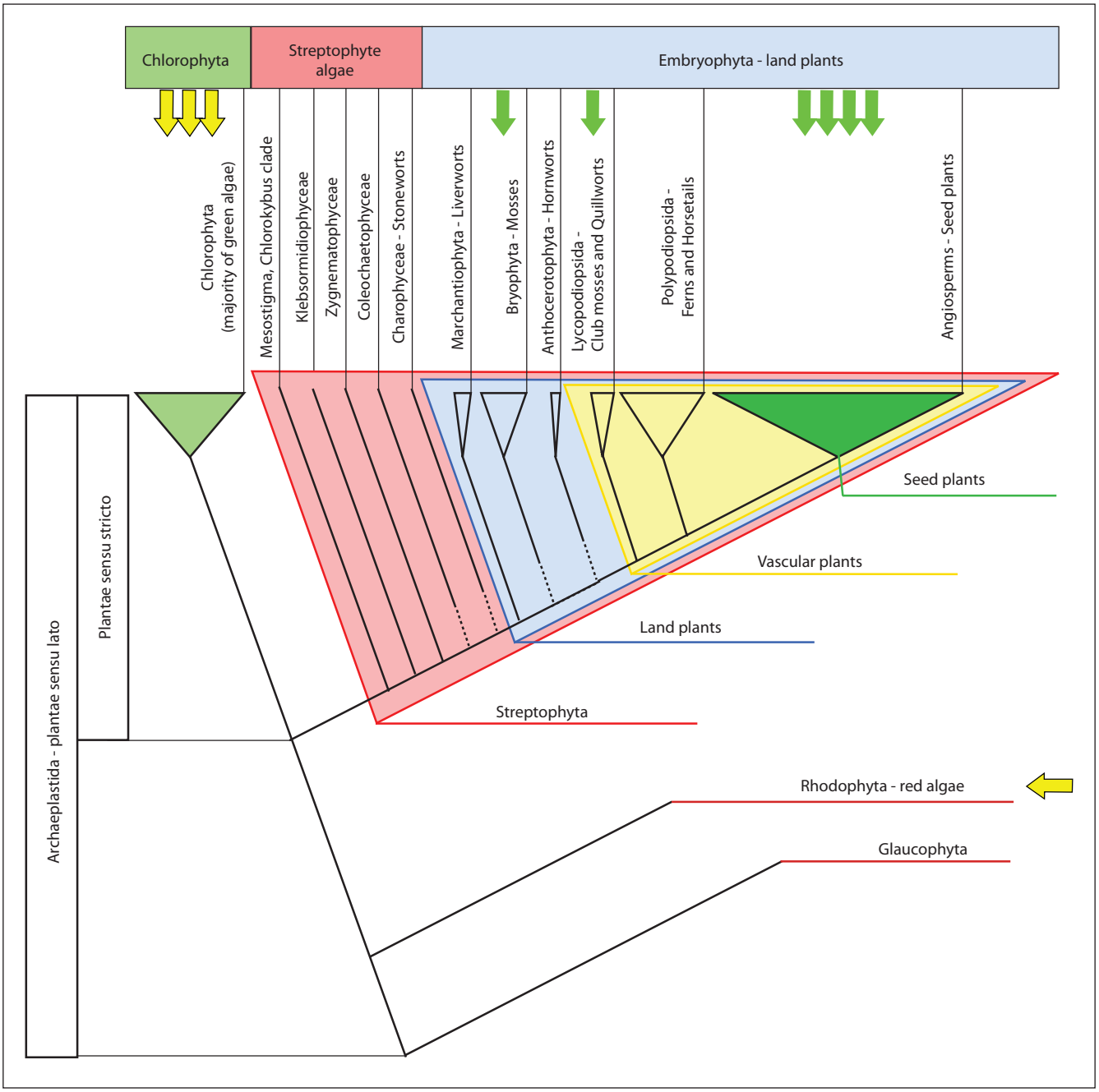

Figure 1

Simplified cladogram of the Plantae supergroup illustrates the distribution of PIN sequences within the group. Species with complete, fully assembled genomes containing PIN sequences are shown as green arrows, and those lacking it as yellow arrows, above their respective lineages. Phylogenetic relationships were revised according to literature (Glaucophyta - red-green algae [57], *Mesostigmatales*/*Chlorokybales* [58], Streptophyte algae [20], bryophytes [59,60], and vascular plants (Embryophyta) [61]). The dotted lines indicate branching events where the consensus about branching order is not well established yet. Arrows indicate the following species. Angiosperms: *Arabidopsis thaliana*; *Oryza sativa*; *Populus trichocarpa*; *Vitis vinifera*. Lycopodiopsida (club mosses): *Selaginella moellendorffii*. Bryophyta (mosses): *Physcomitrella patens*. Chlorophyta (green algae): *Chlamydomonas reinhardtii*; *Ostreococcus tauri*; *Micromonas pusilla*. Rhodophyta (red algae): *Cyanidioschyzon merolae*.

same group share significant homology in their hydrophilic loops. Two of these groups are represented in every sequenced seed plant genome: At*PIN2*- and At*PIN3*-related genes form groups 4 and 7, respectively, as shown

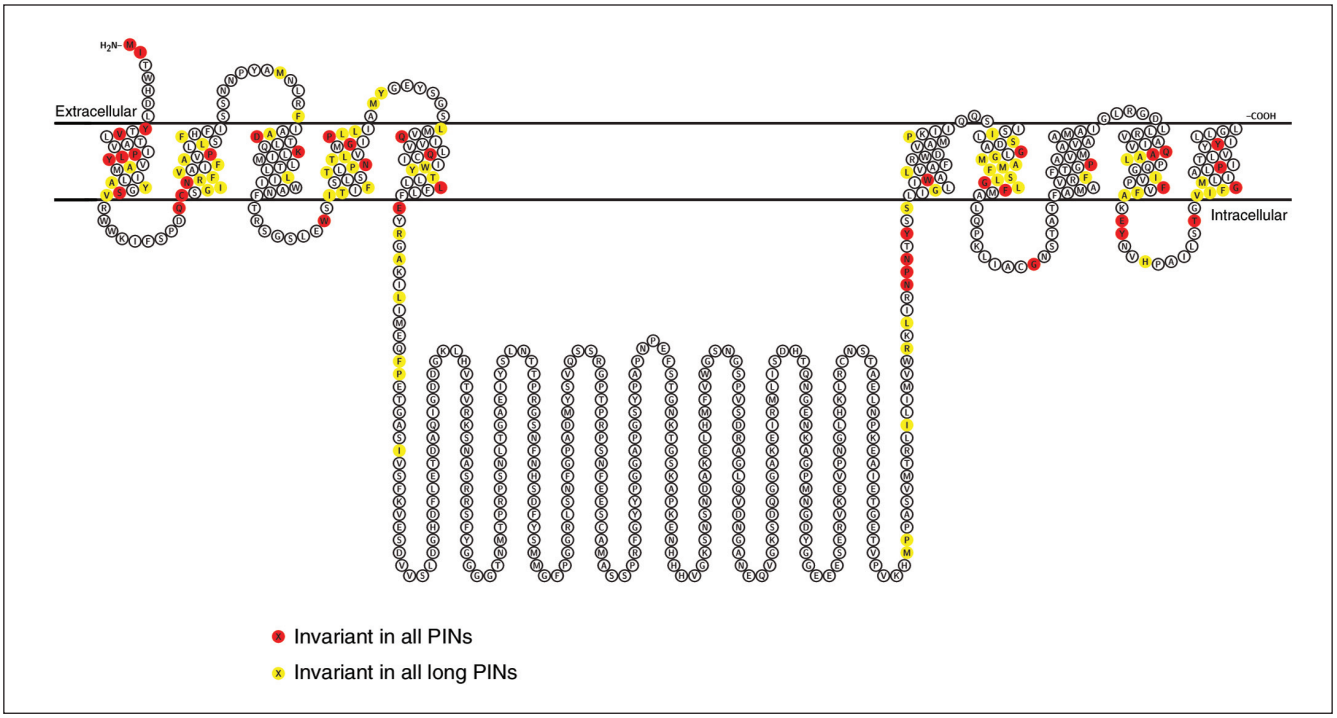

Figure 2

The predicted structure of PIN proteins. The sequence shown is derived from *At*PIN7; the positions marked in yellow are invariant in sequences of all 'long' PINs, the positions marked in red are invariant in sequences of all PINs.

in Figure 3. Groups 5 (which is divided into subgroups 5a and 5b) and 6 form one monophyletic clade (Figure 3). Subgroup 5a contains the archetypal *At*PIN1 and its other dicot-specific orthologs, while subgroups 5b and group 6 contain only monocot-specific sequences. Even though the groups differ markedly in terms of the hydrophilic loop, they may be classified as orthologous on the basis of sequence similarity in the transmembrane regions. Indeed, experimental observations show that *Zm*PIN1a (maize) [17] and *Os*PIN1b (rice) [18] in monocots display expression patterns and have developmental roles that are analogous with the expression and developmental role of *At*PIN1 in dicots. The last group of sequences in seed plants (group 8) is related to *At*PIN6 and contains genes that differ considerably from the other long PIN groups (the canonical PINs). The central hydrophilic loop is markedly reduced and recent data suggest that *At*PIN6 is predominantly localized in the endoplasmic reticulum membrane [13].

With the possible exception of the PIN6-related proteins, the general function of all long PINs from seed plants is to transport auxin out of the cell. The groups differ in the regulation of their expression, localization and activity rather than in the auxin-transport function itself. It has been shown, for example, that *At*PIN1 and *At*PIN2, which are distinct representatives of the long PINs, can functionally replace each other *in planta* when expressed in the same cells and localized at the same side of the cell [16,19].

The second major *PIN* gene subfamily encodes proteins with the central hydrophilic loop virtually absent ('short' PINs) and comprises *At*PIN5 and *At*PIN8. Sequence diversification within the subfamily of short PINs tends to be higher than between the long PINs. From this subfamily, only *At*PIN5 has been characterized so far [13], and reveals a striking difference from the canonical long PINs in its subcellular localization and thus in its physiological function (see below). The short PINs appear to localize to a large extent to the endoplasmic reticulum, and although they presumably act as auxin transporters, they do not directly facilitate auxin transport between cells but mediate intracellular auxin compartmentalization and homeostasis [13].

The precise origin of PIN proteins in the evolutionary history of plants is not known. The basal split of the Viridiplantae - that is, the separation of the Streptophyta (the clade containing land plants (Embryophyta) and some green algae) from the Chlorophyta (representing the majority of green algae) - probably occurred some 725-1,200 million years ago [20] (Figure 1). All green algae with genomes sequenced so far (*Chlamydomonas*, *Ostreococcus*

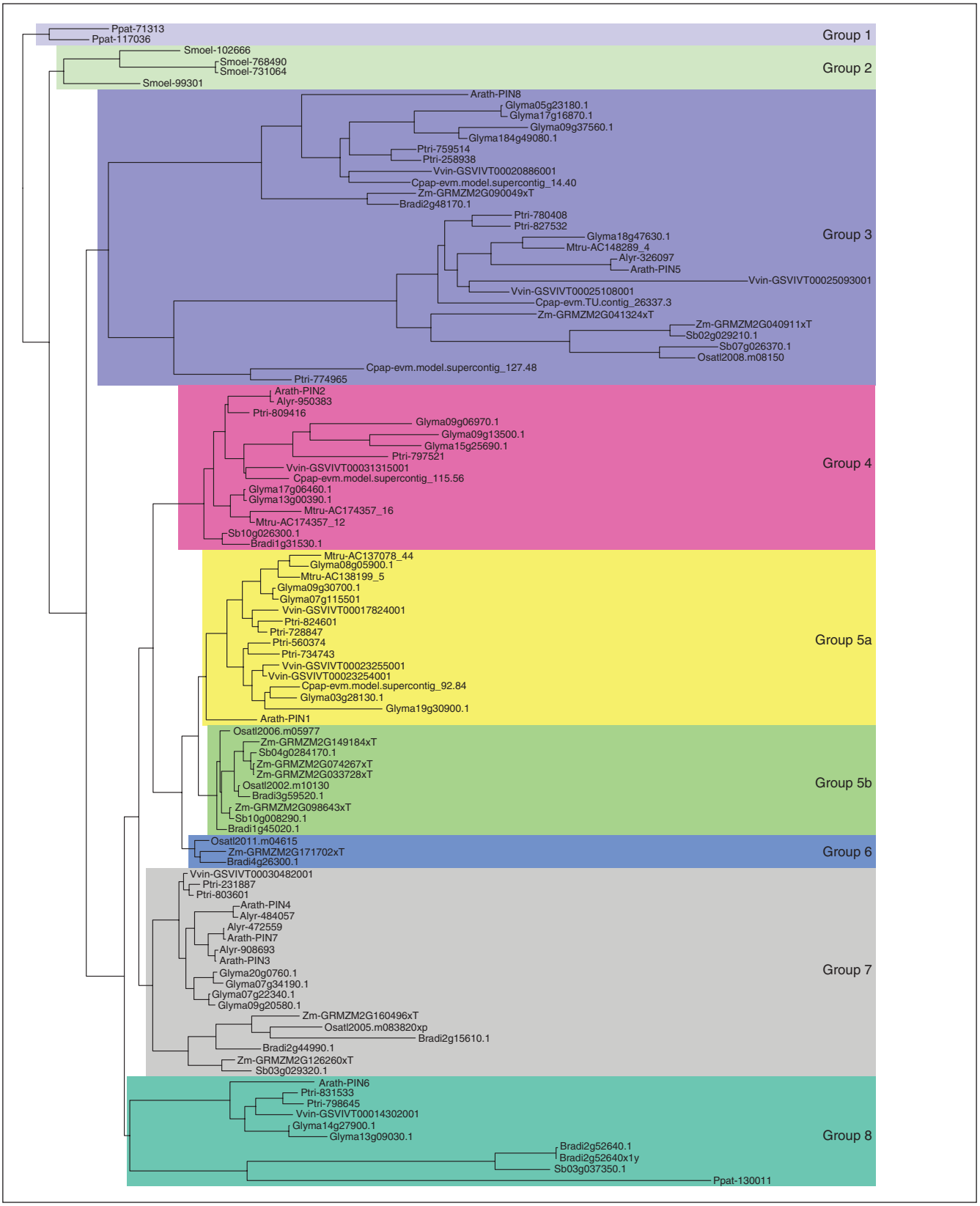

Figure 3

Continued overleaf.

Figure 3 continued.
Cladogram of PIN proteins. The protein sequences of PINs were obtained from a repository of genomic sequences [62] and were aligned by the package MAFFT (program mafft-linsi, default setting) [63]; the non-homologous parts of the hydrophilic loop were edited out. The cladogram was computed by MrBayes [64] with parameters: lset=invgamma; ngammacat=6; prset aamodelpr=fixed(wag). The computation was run for 5,000,000 generations, sampled every 100 generations and the first 10,000 generations were discarded. The sequences are divided into different groups according to the sequence similarity of the hydrophilic loop. All members of group 5 have a similar sequence in the hydrophilic loop but subgroup 5a has a site for phosphorylation by PINOID kinase whereas subgroup 5b lacks it. Species abbreviations: At, *Arabidopsis thaliana*; Alyr, *Arabidopsis lyrata*; Bradi, *Brachypodium distachyon*; Cpap, *Carica papaya*; Glyma, *Glycine maxima*; Mtru, *Medicago truncatula*; Osat, *Oryza sativa*; Ppat, *Physcomitrella patens*; Ptri, *Populus trichocarpa*; Smoel, *Selaginella moellendorffii*; Sb, *Sorghum bicolor*; Vvin, *Vitis vinifera*; Zm, *Zea mays*.

and *Micromonas*) belong to the clade Chlorophyta and none of these organisms contains a *PIN* gene. On the other hand, sequence data from the most primitive land plants available - the moss *Physcomitrella patens* and the club moss *Selaginella moellendorffii* - have revealed the presence of *PIN* genes of groups 1 and 2, both belonging to the long PIN subfamily. Nonetheless, to assess the evolutionary origin of PIN proteins more precisely, the genomic data from algae more closely related to land plants (that is, from the Streptophyta) and also from the liverworts, land plants even more ancient than the club mosses, is needed. Interestingly, the *P. patens* and *S. oellendorffii* PINs do not cluster with PINs of seed plants or with each other (Figure 3, groups 1 and 2), suggesting separate evolutionary establishment of *PIN* families in each of the lineages. The only exception is *P. patens* *Pp*PIND (accession number XP_001765763), which is in the same group as *At*PIN6. However, its intron sequences suggest the possibility of horizontal transfer of this gene from monocots [13].

The intron/exon organization of *PIN* genes is highly conserved. With a few exceptions, the genes are composed of six exons. The first corresponds to the amino-terminal transmembrane segment and most of the central hydrophilic loop. The second exon spans the rest of the variable region of the loop and also the first part of the carboxy-terminal transmembrane domain. It is followed by four small conserved exons coding for the rest of the second transmembrane segment (Figure 4). Several exceptions to this organization exist only in short PINs, and in long PINs related to At*PIN2* (group 4 PINs), where some orthologs display a split of the first canonical exon into two exons.

Characteristic structural features

The predicted structure of canonical long PIN proteins is similar to the structures of secondary transporters - that is, membrane transport proteins that use the electrochemical gradient across the membrane, rather than ATP hydrolysis, to power transport. The PIN proteins have two hydrophobic domains (each with five transmembrane helices) that are separated by a hydrophilic domain with a presumably cytoplasmic orientation. This predicted structure is based only on bioinformatic analyses of the sequences

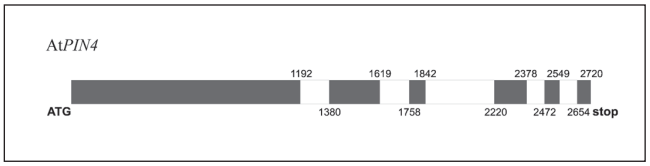

Figure 4

Typical genomic organization of the At*PIN* genes using At*PIN4* as the example. Exons are displayed as black squares and introns as white squares with the positions of exon/intron borders marked.

available and has not been verified experimentally. The hydrophobic domains of PIN proteins are highly conserved in sequence, mainly in the transmembrane helices, which tolerate no insertions or deletions; the loops between the transmembrane helices within the hydrophobic domains exhibit much greater variability both in size and sequence. The hydrophilic domains of PIN proteins from the same group (Figure 3) are very similar in sequence, but there is only limited sequence similarity between hydrophilic domains of PINs from different groups.

There is a substantial difference in the sequence variability of the hydrophobic domains between short and long PINs. The hydrophobic domains of long PINs contain positions that have the same amino acid in all available sequences - that is, they are invariant - but not all of these positions are invariant in the short PINs. However, there are no amino-acid positions that are invariant in short PINs but not in the long PINs (Figure 2). This indicates that the positions that are invariant only in long PINs must be crucial for some important function of long PINs that has not been retained in short PINs.

Two motifs important for intracellular trafficking of PIN proteins can be predicted. One comprises two diacidic motifs presumably important for trafficking of proteins from the endoplasmic reticulum that are located in the amino-terminal part of the hydrophilic domain of all long PINs. The other is a tyrosine-based internalization motif present in all PINs that is important for recruitment of the protein into clathrin-dependent vesicles. The importance of these residues for PIN action, however, remains to be demonstrated.

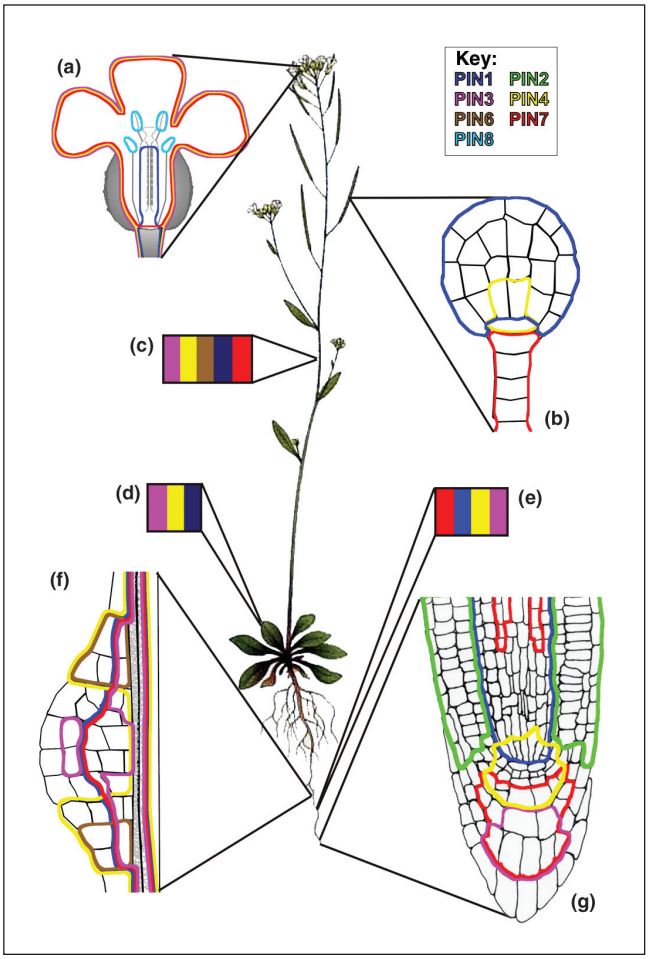

Figure 5

Expression map of *Arabidopsis thaliana PIN* genes compiled from both promoter activity data and protein localization. Each *PIN* gene-expression domain is marked out by a colored line (see key in upper right corner. The organs depicted are **(a)** flower; **(b)** embryo (late globular stage); **(c)** stem; **(d)** rosette leaf; **(e)** mature part of the primary root; **(f)** lateral root primordium (stage 5); **(g)** root tip. The figure is based on the data from [11,12,14,22,23,65,66]. Note that *PIN5* expression is not depicted, as it is expressed weakly throughout the aerial part of the plant with maxima in the hypocotyl, the guard cells of stomata, and cauline leaves [13,65].

Localization and function

Tissue distribution and subcellular localization

Many PIN proteins have specific developmental roles that are largely determined by their highly specific tissue expression (Figure 5), which is in turn based on the diversification of *PIN* gene promoters. Promoters of *Arabidopsis PIN* genes confer specific and partially overlapping expression patterns, reflecting their roles in different developmental processes and their functional redundancy. *At*PIN1 is the major non-redundant member of the family involved in aerial development; it is expressed in apical parts of early embryos, throughout the vascular tissues, in the shoot apical meristem and in developing organs [6,21,22]. The *At*PIN7, on the other hand, shows complementary expression in the basal lineage in the embryo and later can be found in the root tip [14]. *At*PIN2, *At*PIN3 and *At*PIN4 also act in the root tip, mediating the auxin maximum and auxin redistribution for root gravitropism there [7,11,12]. Among the short PINs, *At*PIN5 is relatively weakly and ubiquitously expressed whereas *At*PIN8 shows a very specific expression pattern exclusively in the male gametophyte - the pollen.

PIN promoter activity can be flexibly regulated, which accounts for a compensatory type of functional redundancy. Several *pin* knockout mutants in *Arabidopsis* show ectopic activity of other PIN proteins compensating for the lost PIN activity [23]. This phenomenon seems to account for the high degree of functional redundancy among *PIN* genes, masking most of the phenotypic manifestations expected to result from single, and some double, *PIN* gene inactivations [14,23,24].

In the case of the PIN proteins, subcellular localization is more important than for other transporters. Localization differs fundamentally for canonical long PINs and short PINs (Figure 6). Long PINs are targeted to the plasma membrane and often show asymmetrical, polarized localization to particular faces of the cell, which determines the direction of intercellular auxin flow and thus contributes to auxin distribution within tissues [16] (Figure 7). In contrast to this, the short PINs (typically *At*PIN5 and *At*PIN8) have been shown to be localized predominantly to the endoplasmic reticulum, where they mediate auxin flow between the cytoplasm and endoplasmic reticulum lumen to regulate subcellular auxin homeostasis (Figure 6).

Factors regulating the function of PIN proteins

The PIN proteins mediate asymmetric auxin distribution within tissues, and various endogenous and exogenous signals modulate auxin distribution and thus plant development by acting on PIN proteins. PIN protein activity can be regulated at many levels, including regulation of transcription, protein degradation, subcellular trafficking (endocytic recycling and polarized targeting) and transport activity [3,4,25]. For many of the *Arabidopsis PIN* genes, regulation by other hormonal pathways has been demonstrated. Auxin itself upregulates the transcription of many long PINs. In contrast, the 'short' At*PIN5* is downregulated by auxin [13]. Other phytohormones and plant growth regulators also influence the activity of the PIN promoters to various degrees. The effects are organ- or even cell-type-specific and strongly depend on the particular part of the plant examined and growth regulator used (brassinosteroids [26-28], cytokinins [29-31], gibberellins [32], ethylene [33], flavonoids [34,35]). PIN abundance is also regulated at the level of protein stability.

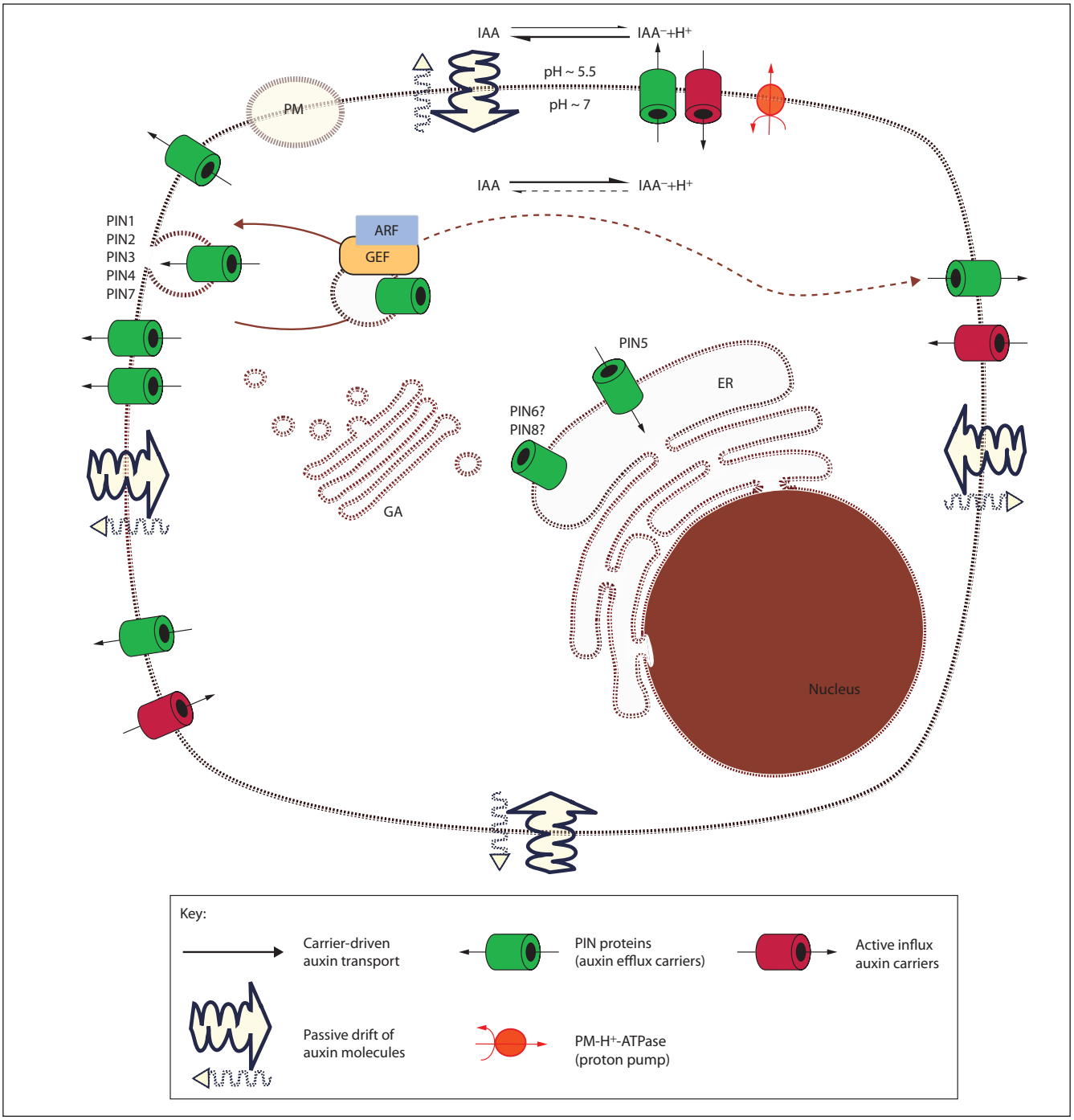

Figure 6

Schematic diagram of an idealized plant cell and the role of specific PIN proteins in auxin management at the cellular level. The low pH in the apoplast (the region outside the cell membrane comprising the plant cell wall) is maintained by the activity of the plasma membrane H^+-ATPase. In the acidic environment of the apoplast, a relatively high proportion of auxin molecules stay protonated (un-ionized; indole-acetic acid (IAA)) and these can enter the cell directly via passive diffusion. In its ionized (dissociated) form ($IAA^- + H^+$), auxin cannot cross membranes by passive diffusion; it needs to be actively transported by carriers. Ionized auxin molecules can enter cells via active transport by auxin-influx carriers. In the relatively higher pH of the cytoplasm, auxin molecules undergo almost complete dissociation. The asymmetric positioning of the auxin-efflux carriers from the 'long' PIN subfamily at the plasma membrane then determines the direction of auxin efflux from the cell. Localization of *At*PIN5 (from the 'short' PIN subfamily) at the membranes of the endoplasmic reticulum leads to compartmentalization of auxin into the lumen of the endoplasmic reticulum, where it undergoes metabolic conversion. PM, plasma membrane; ER, endoplasmic reticulum; GA, Golgi apparatus.

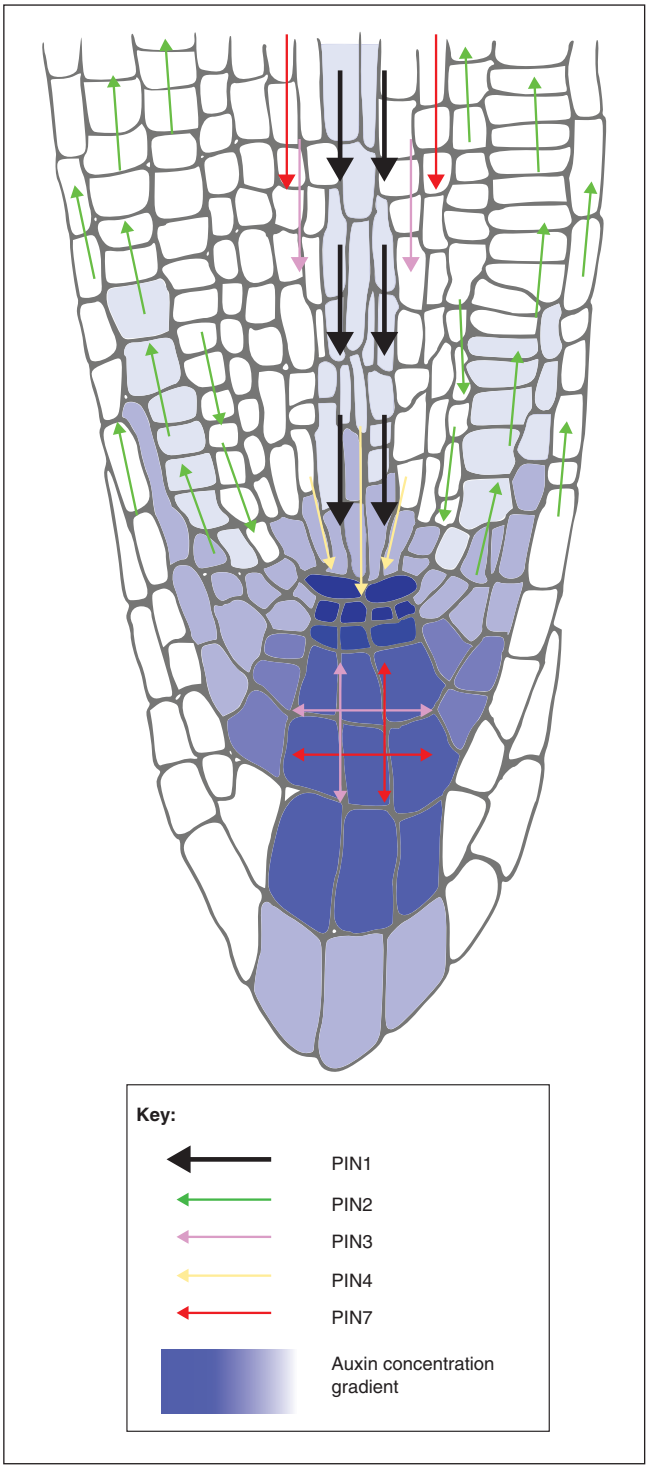

Figure 7

Auxin distribution and PIN-dependent auxin-transport routes in the *Arabidopsis thaliana* root tip. Auxin distribution (depicted as a blue gradient) has been inferred from DR5 activity and indole-acetic acid (IAA) immunolocalization. The localization of auxin transporters is based on immunolocalization studies and on *in vivo* observations of proteins tagged with green fluorescent protein. Arrows indicate auxin flow mediated by a particular PIN transporter.

Several PIN proteins, mainly *At*PIN2, exhibit pronounced auxin-regulated turnover based on PIN trafficking to the vacuole and their degradation there [36-38].

Constitutive intracellular recycling of PIN proteins is an important regulatory mechanism in PIN action [39]. It consists of clathrin-dependent endocytosis of plasma membrane PINs [40] and their recycling to the membrane mediated by guanine-exchange factor of ADP-ribosylation factor (ARF-GEF)-dependent exocytosis [41] (Figure 6). Auxin itself has been shown to inhibit PIN internalization and increase the numbers and activity of PIN proteins at the plasma membrane [42]. Rearrangements of PIN locations, which change the direction of auxin efflux, have been observed in many developmental processes, such as embryogenesis [14], organogenesis [22,43,44], vascular tissue development [21] and gravitropism [11]. These are related to a transcytosis mechanism involving constitutive cycling of PIN between the plasma membrane and endosomal compartments [45]. PIN recruitment to the different trafficking pathways is related to its phosphorylation status [46]. Several sites in the central hydrophilic domain can be phosphorylated by serine/threonine protein kinases [19]. The sequences around the phosphorylated amino acid are conserved within each (sub)group of PINs (Table 1). The protein kinases PINOID and D6PK phosphorylate PIN proteins specifically, with different functional consequences. Phosphorylation by PINOID kinase regulates the localization of the protein [47] and it is counterbalanced by the protein phosphatase 2A [46]. D6PK is presumed to regulate PIN activity [48]. The transport activity of PINs can also be regulated by synthetic compounds, auxin-transport inhibitors, and flavonoid endogenous regulators; however, the mechanism of action of these compounds is not yet fully understood [49-51].

Mechanism

In general, PIN proteins function as auxin transporters - at the plasma membrane for intercellular transport (long PINs) [15] or at the endoplasmic reticulum membrane for intracellular regulation of auxin homeostasis (short PINs) [13]. The directionality of auxin flow, which is due to the polarized location of long PINs, is the key element in the formation of the auxin gradients and auxin maxima that underlie many developmental processes in land plants [25]. These include the establishment of embryonic apical-basal polarity [14], root patterning [12,24], organogenesis and organ positioning [22,43,44]. Polarized auxin transport controlled by long PINs is also involved in responses of plants to environmental stimuli such as gravity - in the case of gravitropisms [8,11]. The loss-of-function phenotypes in long PINs demonstrate their crucial role in these developmental processes (Figure 8).

The only genetically characterized member of the short PIN subfamily is *At*PIN5. Its auxin-transport function

Table 1

Identified phosphorylation sites in PIN proteins

Sequence	Protein and group	Reference	Notes
LQSSRNPTPPRG**SS**FNH*	AtPIN1; group 5	[56]	
TPRP**S**N	AtPIN1; group 5	[56]	
YPAPNPXF**S**P	AtPIN1; subgroup5a	[46,56]	Phosphorylated by PINOID kinase
AAGKD**TT**PVA*	AtPIN6; group 8	[56]	

The phosphorylated amino acid is in bold type. All members of the designated group share the sequence. *It is not known which of the two neighboring amino acids is phosphorylated.

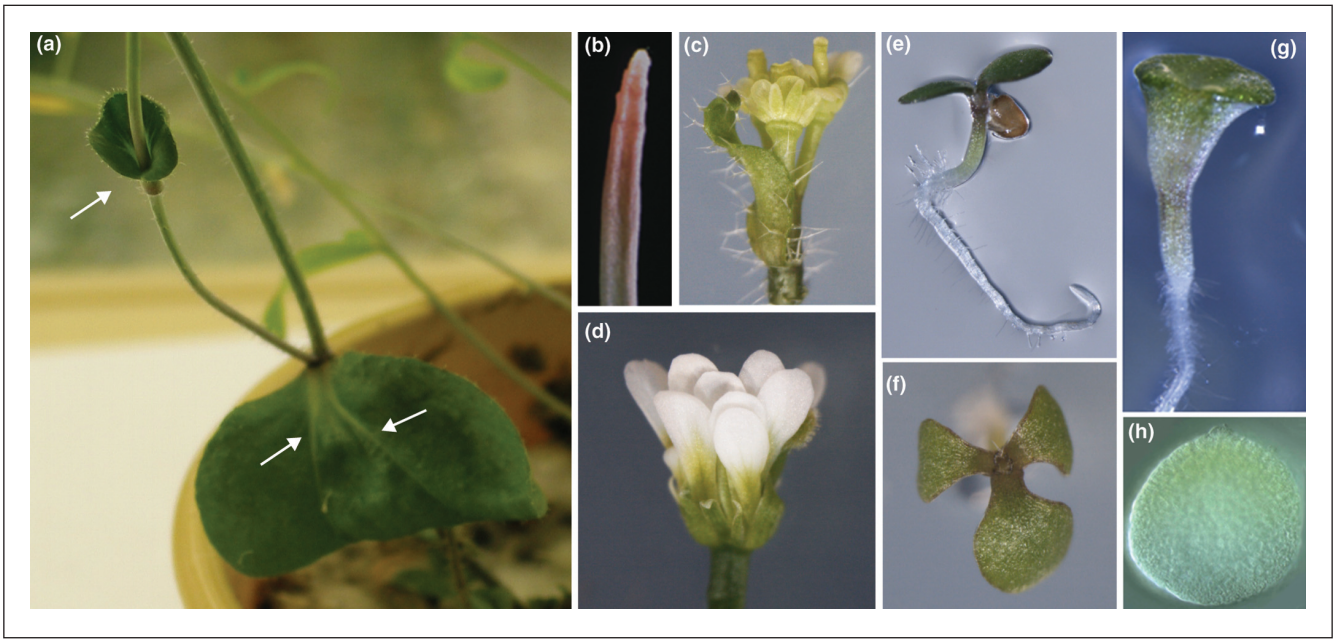

Figure 8

Examples of *pin* loss-of-function phenotypes. **(a-d,f)** *pin1* mutants can have (a) fused leaves, (b) pin-like inflorescence, (c,d) defective flowers and (f) three cotyledons in the seedling. **(e)** *pin2* mutant showing agravitropic root growth. **(g)** Fused, cup-shaped cotyledons of triple-mutant *pin1,3,4* seedling. **(h)** No apical-basal patterning in a triple-mutant *pin1,3,4,7* embryo.

(shown in yeast cells) together with its subcellular localization at the endoplasmic reticulum membrane implies the transport of auxin molecules from the cytosol into the lumen of endoplasmic reticulum. As a result of this translocation, auxin molecules are exposed to metabolic enzymes localized in the endoplasmic reticulum, leading to metabolic changes that decrease the availability of free active auxin molecules in the cytosol. In this way, *At*PIN5 contributes to control of intracellular auxin homeostasis [13].

In contrast to the wealth of data on the developmental roles of PIN proteins, there is only limited knowledge on their structure, their structure-function relationships and the mechanism of transport. Earlier physiological experiments [52] established that auxin efflux requires a membrane H^+ gradient. Moreover, no ATP-binding motifs suggesting ATP-dependent transport have been recognized in PIN protein sequences. These findings, together with PIN topology in the membrane, suggest that the PIN proteins are gradient-driven secondary transporters. In particular physiological situations, they can act cooperatively with the ATP-dependent auxin transporters of the ABCB (ATP-binding cassette B) family [53,54].

Frontiers

Out of the eight PIN proteins in *Arabidopsis*, the canonical long PINs are already well characterized and their developmental roles in generating intercellular auxin distribution patterns have been demonstrated [55]. On the other hand, the existence of auxin transport into the endoplasmic

reticulum and its role in regulating auxin homeostasis is a novel and unexpected finding and there is still lot of work needed to elucidate the details and physiological importance of this activity. From the evolutionary point of view, it would be interesting to know which function of PINs is the older: the plasma-membrane-based intercellular auxin transport by long PINs or the endoplasmic-reticulum-based control of intracellular auxin homeostasis by short PINs? The most ancient PIN proteins currently known, from mosses, are localized to the endoplasmic reticulum, which suggests that intracellular function is evolutionarily ancestral, but this remains to be experimentally verified. The other obvious open questions relate to experimental information on PIN protein structure and membrane topology. This, as well as more detailed biochemical characterization of PIN-driven auxin transport is still largely lacking.

Acknowledgements

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7 DISCUSSION

Polar auxin transport (PAT) can be considered as a key process for plant growth, development and behavior, generally - for survival of each (higher) plant. Carrier-mediated auxin efflux represents crucial step within the PAT, limiting both intensity of auxin flow and its direction. In fact, cellular auxin efflux controls delivery of auxin molecules on the site of their action (signal transduction), and there, auxins play their role in the regulation of cell fate destination and plant development as discussed in the Chapter 4 in detail.

Nevertheless, identity of individual molecular players in the auxin transport machinery has not been clear. As described above in the Chapter 4.5, the sought-after auxin efflux carriers were critical component to fill the chemiosmotic hypothesis in. However, even after the discovery of promising and polarly localized candidates (some of the PINs, Geldner et al. 2001), the chemiosmotic model machinery has not been fully understood. ABCB-type auxin transporters are PM transport proteins with well-known motif in their sequences to power the transport activity (i.e. cleavage of ATP) and besides, they were reported to cooperate in yeast with other proteins involved in transmembrane transport, and helping to determine substrate specificity for ABCB (Geisler and Murphy 2006). Because of this, it was reasonable to think, that co-operation of ABCB transporters (with apolar distribution and known energy source) and polarly localized PIN transporters (with unknown energy source) would be a conceivable alternative (Blakeslee et al. 2007; Bandyopadhyay et al. 2007). Presumed transport complex might be fully functional only in the case, where both proteins are present, and as long as the PM-PINs are localized mostly in a polar manner, this mutualism would result in polarized stream of auxin, exactly according to the chemiosmotic model. That is why it is of crucial importance to understand the molecular function of PINs as well as the nature of their co-operation with ABCBs.

7.1 PM-targeted PINs play a key role in polar auxin transport

Convincing evidence for auxin efflux catalyst function of PIN proteins has been missing. Because it is difficult to investigate biochemical aspects of auxin transport on the level of tissue or whole plant, we have utilized beneficial character provided by the suspension-cultured tobacco BY-2 cells. They represent model system with relatively homogeneous cell population suspended in liquid medium, and together with the relative ease they can be transformed with, they are advantageous for quantitative transport studies at the level of a single cell and its compartments (Petrášek and Zažímalová 2006). Actually, already the important study conducted by Rubery and Sheldrake (1974), bringing the chemiosmotic polar diffusion model in, and later studies by Delbarre (1996; 1998) describing different substrate specificities of uptake and efflux carriers for different auxins, were based on data measured on plant cell suspensions. Nevertheless, the transformation of BY-2 cell line by genes coding for putative efflux carriers, preferably under inducible promoters, permits to create homogenous expression systems to monitor and juxtapose actions of individual overexpressed PM transport-protein candidates. This approach allowed us to propose the direct involvement of tested transport proteins for auxin transport across PM.

As shown in the Chapter 6.1, to test the proposed role of PIN proteins in the regulation of auxin transport, BY-2 cells were transformed with PIN4, PIN6 and PIN7 genes from *Arabidopsis thaliana* under control of inducible promoter, along with ABCB19 for comparison. Accumulation of radiolabeled auxins in cells reflects total sum of actions of all auxin influx and efflux processes. By this method the efflux rate can be characterized in the case of constant auxin influx, i.e. if such an auxin is used which enters cells almost entirely by passive diffusion but it is a good substrate for efflux carrier(s). NAA is such candidate (Delbarre et al. 1996). All investigated members of *At*PIN gene family displayed convincing accumulation kinetics of radioactively labeled NAA (Fig. 2C in Petrášek et al. 2006), showing marked decrease upon induction of PIN overexpression. Regarding the difference between PIN-dependent action and that one of the ABCB19, the auxin accumulation decreases after induction were comparable in extent; nevertheless, significant difference was in sensitivity towards the well-established inhibitor of auxin efflux, NPA. Whereas PIN-overexpression stimulated auxin efflux was reverted, about 20% ABCB19-dependent transport remained unaffected by NPA.

Moreover, with partial exception of PIN6, strong inducible overexpression of the tested PINs – and also that of ABCB19 - resulted in remarkable changes in cell phenotypes, such as cessation of cell division, marked elongation and accumulation of starch granules. This is especially true for cell lines overexpressing PIN7 and PIN4, belonging to the same subgroup within 'long' PINs (see Křeček and Skůpa et al. 2009). Similar changes in cell morphology have been reported previously to be the typical response to auxin depletion (e.g. Sakai et al. 2004), correlating the phenotypes of PIN overexpressing cells to those with limited IAA availability. However, PIN6 showed markedly less pronounced PIN-dependent increase in cell length (Fig. S1H in Mravec et al. 2008). Importantly, like in the case of auxin accumulation, the 'long' PIN-dependent cell phenotype changes could be rescued by NPA. Again, the ABCB19-line displayed much less sensitivity towards NPA. So, it has been obvious that further work was needed to clarify, whether *in planta* PM-PINs and ABCBs are working entirely independently or in a coordinated fashion. The results in the paper by Mravec at al. (2008) strengthened the notion, that the two transport systems (PM-PIN-dependent and ABCB-dependent) work independently on each other, at least in the sense of potential protein-protein interaction(s) *in situ*. The paper offered the model for interaction of ABCBs/PGPs and PINs compensational mechanism in roots: Whereas ABCBs limit the effective cellular auxin concentration available for the PM-PIN-mediated transport, PINs act exclusively on transversal PM and drive the auxin flow through the channel. Only the combination of ABCB/PGP activity-limiting auxin availability with PIN-driven directional auxin transport defines how much auxin flows through the auxin channels.

With reference to the study on 'short' PINs (Mravec et al. 2009) and regarding the subcellular topology of PIN6 observed there (see also the Chapter 4.5.4), it is interesting to note, that upon overexpression PIN6 proteins had the impact on BY-2 cell morphology different from that of PIN4 and PIN7. The average additional increase in cell length related to PIN4- and PIN7-overexpressing cells was about 130% and 90%, respectively, while only 22% was observed for PIN6. In A*rabidopsis* PIN4 together with PIN7 and PIN3 are close paralogs with highly similar sequences, most probably originating from some recent duplication event (see the Chapter 7.2). They are put together into subgroup 7 of the classification system suggested in Křeček and Skůpa et al. (2009; enclosed in the Chapter 6.3 of this thesis). Single *pin* mutants, such *as pin4,3 or 7* exhibit no obvious phenotypes (Friml et al. 2002a; Vieten et al. 2005), and it is

probably related to their tendency to substitute each other through ectopic expression if a single one is missing (functional redundancy, Vieten et al. 2005). Nevertheless, in spite of the weak mutant phenotypes, they are all developmentally very relevant (reviewed in Petrášek and Friml 2009), with PIN4 and PIN7 having important role in embryogenesis and root patterning (Friml et al. 2002a; Friml et al. 2003; Weijers et al. 2005 Plant Cell; Blilou et al. 2005) and PIN3 having crucial role in gravitropic and phototropic responses (Benková et al. 2003; Friml et al. 2002b) by means of transcytotic deposition of the PIN3 protein to different PM domain upon gravi/photo-stimulation (Kleine-Vehn et al. 2010). From the point of view of the developmental relevance it is hardly surprising, that PIN4 and 7 (and presumably PIN3) are functional PM-targeted auxin transporters.

PIN6, on the other hand, is one of the least studied PIN proteins so far, and its developmental role was not elucidated yet. Benková et al. (2003) reported the tissue expression pattern of PIN6, with prominent expression in margins of newly emerging lateral roots. So, based on its tissue-specific location, PIN6 seems to play role in lateral root emergence. From Petrášek et al. (2006) we know, that PIN6 can mediate auxin efflux in tobacco cells; however, in Mravec et al. (2009) it was shown that, along the "short PINs", PIN6 is localized in the membranes of ER. Interestingly, Pernisová et al. (2009) reported that PIN6 is the only PIN, which is upregulated by high concentrations of cytokinin (kinetin), whereas most PINs are either downregulated or relatively stable after cytokinin treatment. In the light of the above mentioned findings, together with structurally limited similarity of PIN6 with any of the PIN family subgroups, we can only speculate, what developmental role PIN6 plays. It may well differ from the function of both the "long" and "short" PINs.

7.2 PIN protein family evolution and *PIN* gene family diversity – complex relationships, paralogs and orthologs

Together with ABCBs/PGPs contributing to load auxin into the auxin stream pathway (Mravec et al. 2008), various PIN proteins evolved to generate the network of auxin transport components, underlying flexible developmental program necessary for the survival of a sessile organism.

As the evolution pushes forward, genomes frequently duplicate, creating duplications of the previous gene sequences. Presuming that neither copy of the duplicates had lost its expression, one can conclude that the selection pressure on the genes was somewhat alleviated. Duplicated genes in eukaryotic genomes are frequent and they are thought to be at least in the beginning mutually redundant in function. Anyway, generally, gene duplication is thought to be one of the major driving forces of evolutionary innovation, and the organismal complexity is thought to be tightly coupled to the evolution of new gene functions arising subsequently to gene duplication (Force et al. 1999).

Actually, not only *PINs*, but most of the genes in *Arabidopsis thaliana* are members of gene families. How do the members of gene families arise, and how are gene copies maintained in the family after its inception? Several studies have concluded that the genome of *A. thaliana*, or more precisely that one of its ancestors, has undergone at least three whole genome duplications during approximately the last 120 million years (Ermolaeva et al. 2003; Proost et al. 2011). Some gene families may evolve primarily through tandem duplication and high rates of gene birth and death in clusters (Cannon et al. 2004).

Actually, although being a superb model system for plant genetics, the genome of *A. thaliana* might be rather exceptional, with its many genome duplications, huge amount of gene losses, and recent genome shrinkage (Proost et al. 2011). Analysis of the *A. thaliana* genome, and comparison with other plant genomes that have been sequenced later, unveiled a very complex evolutionary history of the genome (Proost et al. 2011) and that of its dicot ancestors (see also Fig. 4).

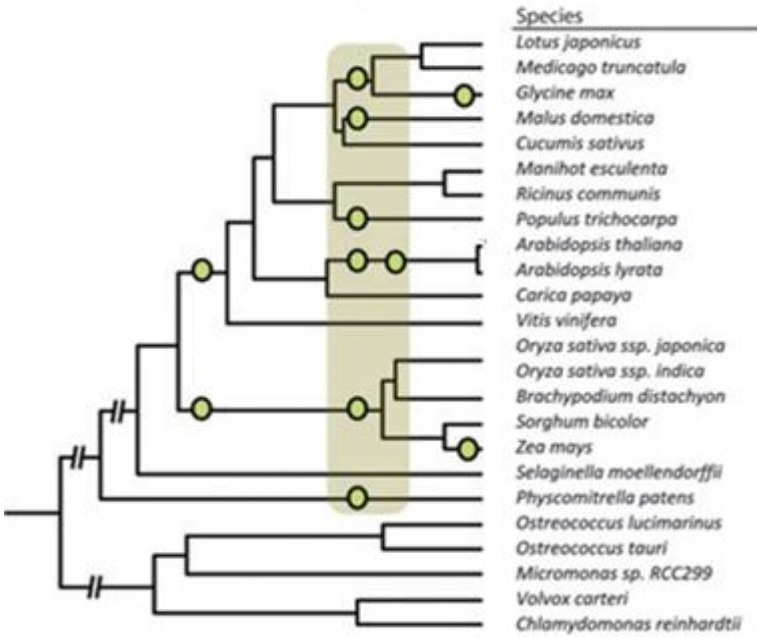

Figure 4. Schematic and highly pruned phylogenetic tree of green algae and land plants for which the genome sequence has been determined. Dots on the tree denote whole genome duplications. The scheme highlights very different duplication histories of different plant models. Size and composition of protein families is based on complete evolutionary history of the genome. Accordingly, the composition of PIN family can be very variable between different species. Image modified from Proost et al. (2011).

Duplicated genes are thought to be initially redundant in function. As mentioned above, presuming that neither copy of the duplicates had lost expression implies that the evolution pressure on the two sequences is alleviated. Here, subfunctionalization (see below) represents a model that explains how redundant genes may evolve after the event of the gene duplication (Force et al. 1999; Flagel and Wendel 2009). Originally both, later just one of the extra copies is usually under relaxed selection, due to the fact that degenerative mutation that normally would render the gene nonfunctional and would handicap the plant in competition, is not harmful to the organism as long as it occurs to just one of the copies.

According to the current consensus, with some reservation, two likely scenarios may happen after the gene duplication (Flagel and Wendel 2009): One scenario leading to the so-called gene neofunctionalization, when one of the former copies remains fully functional and the other one acquires new unexpected role after mutation, and the second scenario of gene subfunctionalization, proposed by Allan Force (Force et al. 1999) and now usually considered to be more likely. The

subfunctionalization model suggests that mutation in one of the copies is more likely to nullify just one of the several of possible sub-functions of the ancestral gene in the targeted copy. By such a mutation, gene will be not rendered dysfunctional in entireness and remains to contribute to overall plant fitness by the retained part of its functionality. With different mutations occurring to the other copy in some future events, the two copies may become disconnected in terms of evolutionary constraints, each of the copies will gradually retain part of the whole original developmental role of ancestral gene and they will start to evolve independently.

PIN family within *Arabidopsis* is represented by 8 paralogs (see the Chapter 4.5.4). Paralogous sequences provide useful insight into the way how genomes evolve. As the family evolves and incorporates bigger diversity of functional copies, possible functional plasticity increase due to the potential given to the evolution pressure; thus, one protein is sub-functionalized into two, with each separately regulated. This might be true especially for auxin transporters, as they are essential determinants of plant body developmental program.

While various PIN proteins contribute to create flexible network of auxin transport within plant body, most of 'long' PINs seem to retain the original transport function with only small, if any changes. However, they do still vary in their developmental roles due to different tissue-specific or stimuli-regulated expressions, differential regulations of their transport activity or protein internalization and subcellular targeting. As mentioned above, genes are thought to be initially redundant in function after duplication. Redundancy in function of similar proteins and their developmental role may conversely indicate short history of gene evolution for gene/proteins concerned. There is known and prominent developmental redundancy between some of the members of the PIN family in *Arabidopsis* (Vieten et al. 2005). So, if we have a look upon the developmental/functional redundancy between *At*PIN3, PIN4 and PIN7, we might get some insights. PINs 3,4,7 are arguably very similar in many aspects. First, they all are structurally and sequentially closely related, their intron structure is almost identical (Křeček and Skůpa et al. 2009). In contrast to PIN1 and PIN2 proteins (and PIN1 and PIN2 orthologs) their targeting to PM tends to be sometimes apolar (Blakeslee et al. 2007). They all play a significant developmental role in organogenesis, and root tip patterning (discussed in the Chapter 7.1), they are the key players in the root tip auxin redistribution and as such, they are the key players of the tropic responses in the root. Also their tissue-specific expression is rather close, and even in

those positions on which they do not express natively they can be upregulated in case of the mutation of other member of this subgroup (Vieten et al. 2005). Moreover, various PIN proteins ectopically expressed in various *pin* mutants always adopt the correct polar localization (Vieten et al. 2005).

This all is consistent with the scenario, that *PIN3,4 and7* genes all recently originated from a single copy and they still retain a huge part of original similarity not only of their structurally conserved function, but - most interestingly - they share to some degree the way of regulation of their subcellular and tissue-specific expression.

However, in spite of all the similarities described above, *PIN3,4 and 7* genes show also first divergences and they start to develop original traits showing subfunctionalization in the process. Diversification of PIN gene promoters may be attributed to the type of first diversity which typically arrives first between sister sequences. The *PIN* promoter regions are becoming much more variable than gene/protein sequences themselves and as a result, PIN protein transcriptional regulation tends to diversify. This reasoning leads to the question: what was the original functional, developmental and transcriptional state of the ancestral gene for *PIN3,4* and *7*? As we reported in the paper by Křeček and Skůpa et al. (2009; enclosed in the Chapter 6.3 of this thesis), in the classification system dividing PIN sequences upon similarity in hydrophilic loop, these PINs are included together in one of subgroups within "long" PINs - together with orthologs from other species. Not surprisingly, while *Arabidopsis* has three representatives in this subgroup 7 (i.e. PIN 3,4 and 7 – see Křeček and Skůpa et al. 2009) most of other species have just one ortholog there. Based on the similarity in the hydrophilic loop and phylogenetic closeness (defined in terms of position in gene cladograms), those orthologous sequences might be expected to act in a developmental place of these three *At*PINs in other species. It may be worth knowing how the analogous but solitary PINs work in other species and how does that determine/undermine plasticity of the plant body development and/or its response to environment.

According to PIN classification suggested in Křeček and Skůpa et al. (2009) 'long' PINs can be divided into 4 subgroups in Arabidopsis and one more is found in monocots: The above described subgroup 7 represents PIN3-like proteins, PIN1 belongs into subgroup 5, and there are also subgroups 4 containing PIN2-like sequences and the least similar to the other three is subgroup 8 encompassing PIN6-like sequences.

Contrariwise to PIN3-like sequences, PIN1 developmental function might be shared among several PIN1 orthologs in other species. While there is only one representative for this subgroup in Arabidopsis, for many analyzed monocots and dicots, two or multiple PIN1 orthologs were found. Especially in all analyzed monocots so far, more than one PIN1 ortholog was confirmed. Experimental observations show that *Os*PIN1b (rice; Xu et al. 2005) and *Zm*PIN1a (maize; Carraro et al. 2006) in monocots display expression patterns and have developmental roles that are analogous with the expression and developmental role of AtPIN1 in dicots. According to functional characterization in maize (Carraro et al. 2006; Gallavotti et al. 2008; Skirpan et al. 2009) *Zm*PIN1a and *Zm*PIN1b play fundamental role in meristem function and inflorescence organogenesis in a fashion reminiscent that of *At*PIN1; nevertheless, even though in maize development the two maize PIN1s share the role analogous to that of *At*PIN1, they have distinct expression patterns in maize tissues. In relation to body architecture it may be of importance to assess how do duplicated orthologs of PIN genes/proteins contribute to modularity of auxin redistribution and finally, to observed differences in the morphologies of various species of flowering plants.

On the larger scale and according to the phylogenetic study conducted in Křeček and Skůpa et al. (2009), the subdivision of the family on the short PINs and the 'long' PINs approximately to phylogenetic trajectory. This is providing us an interesting opportunity to ponder whether or not the short PINs represent ancestral state of the protein function and to assess the impact of the diversification of PINs deeper in the past.

7.3 Short PINs and subcellular auxin compartmentalization

*At*PIN5 is the representative of subgroup of PINs with protein sequence strikingly different from canonical "long" PINs, and where the otherwise-variable, hydrophilic loop connecting two hydrophobic domains is almost completely missing (Fig. 2). For a long time, PINs from this subgroup were not characterized. To test their function, several approaches were conducted in the paper by Mravec et al. (2009) - as presented in the Chapter 6.2.

Based on inference of Matsuoka et al. (1993) we could presume that hydrophilic loop in transmembrane transporters probably has rather regulatory than transport

function. Studies focused on the regulatory motifs within PIN protein sequences showed that there is a number of such elements residing in the central loop, particularly sites for phosphorylation (summarized in Křeček and Skůpa et al. 2009). Those are necessary for context-dependent regulation of PIN protein targeting into different PM domains (Friml et al. 2004; Michniewicz et al 2007). Nevertheless, it was not clear whether this presumption is complete and whether or not the central hydrophilic loop is necessary for the transport function of PIN transporters as well. In this respect, the functional characterization of *At*PIN5 aid both our understanding of the regulatory function of hydrophilic loop and transport function of the "long" (canonical) PINs. Moreover, it revealed a new mode of auxin management and a fresh insight into the evolution of diversity of PIN sequences. The observation that auxin is transported across the ER membrane was unexpected, no previous models of auxin transport or auxin action had proposed regulation at this level (Friml and Jones 2010).

As we have shown in Mravec et al. (2009) (presented in the Chapter 6.2), the indirect immunofluorescence visualization showed the ER-like pattern of PIN5 localization in both Arabidopsis plants and tobacco BY-2 cells. In contrast, other well-characterized PIN proteins localize preferentially on the transversal plasma membranes, determining the directionality to the flow of auxin through the file of cells. Importantly, BY-2 cells showed dramatic changes in their IAA metabolic profile after induction of *At*PIN5 expression: free IAA was quickly metabolized, IAA-sugar conjugates decreased, and the capacity to conjugate IAA to amino acids, such as Asp or Glu, increased. These relatively rapid and major changes in the metabolic fate of IAA in response to manipulations with the PIN5 activity at the ER membrane suggest that a substantial part of the auxin metabolic pathways is compartmentalized to the ER (there is also discussion about this topic in the Chapters 4.4.3, 4.5.4. and 6.2).

Unfortunately, subcellular localization of components involved in IAA metabolic changes has been often overlooked, and so the localization of most of IAA-metabolizing enzymes is still unknown. Nevertheless, so far known auxin metabolic enzymes and regulatory proteins were reported to reside in the ER (Bartel and Fink 1995; Schuller et al. 2006; Woodward and Bartel 2005). Moreover, predicted ER localization of the IAA-amino acid hydrolases (Davies et al. 1999; Campanella et al. 2003; Rampey et al. 2004) directly implies the important role of ER in auxin management. Anyway, there is still at least one major question to be answered: what

is the energy source for the cross-endomembrane transport of auxin and how it is maintained (Peer et al. 2011).

It is plausible that the ER is more than just a “trash bin” for free auxin. Auxin in the ER may also participate more directly in signaling. It is well known that the majority of the candidate auxin receptor for ‘non-genomic’ auxin signaling, ABP1, in the cell is retained at the ER. This was demonstrated by both localization studies of ABP1 and the presence of the ER-retention signal KDEL at the C-terminus of the ABP1 sequence (Tian et al. 1995), although it seems that the KDEL retention signal evolved just recently in flowering plants (Tromas et al. 2010). It had been speculated that ABP1 can bind auxin only poorly in the ER because of the unfavorable pH; however, this notion was relevant to the situation, when transport system capable to mass large quantities of auxin in the ER was not considered (Friml and Jones. 2010). Due to PIN5, auxin could reach high concentrations in the ER, thus providing conditions sufficient for auxin binding to ABP1 in this compartment. With respect to the fact, that compartmentalization of auxin signal may contribute to diversity in auxin signaling, the finding by Yin et al. (2009) that another auxin receptor (GLP4) resides in GA is noteworthy.

7.4 Evolutionary aspects in auxin action and auxin action in evolution

The proper function of PAT is crucial for many, often very diverse plant developmental processes and/or situations, and it delimits basic processes such as embryogenesis, polarity maintenance and growth responses to environment. PAT in higher plants is the process with very complex regulation. When studying PAT, plant models representing individual stages in course of plant phylogeny may help to assess early development of the auxin management in plant history and in turn by matching the respective plant body plans with the degree of auxin regulation achieved, we might succeed in assorting different pathways according to their necessity for plant adaptation and survival. In general it would give us better insight into the basis of the whole process (discussed in Zažímalová et al. 2007 – enclosed in the Chapter 6.3)

Understanding the auxin biology in flowering plants with their many redundant pathways working to the same goal from different functional perspectives, is particularly difficult for separation into independent components and for extraction of

the necessary core ones, for the purpose of further characterization. Many of those auxin-related processes are elusive and they avoided to be solved for that reason (for example, heavy redundancy in auxin biosynthetic pathways prevents researchers to isolate useful mutant(s) with decreased production of auxin and makes it difficult to progress forward in deciphering individual steps of auxin biosynthesis).

Literature on the auxin transport has been very limited in terms of the non-flowering plants, even of ferns, and also review-papers tend to avoid this area and they just carefully present limited conclusions to set of species and models to which the findings apply. As a result, there was not a review with an opinion whether auxin transport is question of only seed plants, vascular plants, all land plants or whether it applies also to some antecedent(s) of today‘s plants.

Therefore, we tried to collect potential clues about presence of auxin transport in indirect fashion. We have assembled known sequences coding for the homologues of auxin efflux carriers of PIN family and resulting data were related to available information on the distribution of PAT, apical and polarized growth and other PAT-related characteristics during plant evolution. We have also suggested some outlines for the next possible steps in data-mining strategies related to studies of co-evolution of PINs together with various forms of auxin transport, and some possible implications of PAT for land plant evolution (Zažímalová at al. 2007). Interestingly, polar auxin transport was reported only in the sporophytes all land plants including mosses, notwithstanding moss sporophyte – the seta - being merely an anatomical extension of the moss gametophyte from morphological point of view. However, PAT is missing so far in all reports on gametophyte development.

Local auxin biosynthesis ‘*de novo*’ contributes significantly to the generation and maintenance of local auxin gradients (reviewed in Zhao 2008; Chandler 2009) and represents key regulatory factor for developmental processes such as female gametophyte development in *Arabidopsis* (Pagnussat et al. 2009). Actually, this is somewhat akin to the moss for which gametophyte represents dominant generation. It seems that the body plan in gametophyte of mosses may not be relying on (polar) auxin transport (Fujita et al. 2008); nonetheless auxin homeostasis is tightly regulated in these plants. The role of auxin conjugation/deconjugation in auxin homeostasis was a subject of various evolutionary studies (Sztein et al. 1999; Cooke et al. 2002; Poli et al. 2003; Ludwig-Müller 2011; De Smet et al. 2011) showing, that while there is a significant shift in the amount of conjugated molecules with evolution, the auxin

conjugation as such is an ancient way to regulate auxin physiological activity and to control pool of active (free) auxin in the cell. The capacity to deactivate IAA through conjugation increases in time and with increased complexity of the plant body; in evolutionary more advanced species it becomes more dominant and its regulatory role changes and evolves in time (Cooke et al. 2002; Cooke et al. 2003; Sztein 1999). Interestingly, PIN*A* from *Physcomitrella patens* localizes to ER in BY-2 cells (Mravec et al. 2009); this insinuate that Physcomitrella PINs might be functionally related to the ER-localized 'short' PINs and it may play a key role in auxin metabolic changes as PIN5 does in higher plants.

The role of PIN proteins in mediating auxin homeostasis through auxin metabolism in moss gametophyte remains unknown and asks for investigation. It may well correlate with evolution of PIN transporters as reported in *PIN* gene phylogenies. Therefore, the relevant question is whether short PINs are evolutionary older as suggested in Mravec et al. (2009), or not. Another important question is related to origin of plant-specific PIN proteins in general, namely to the point of their "inception" in time, with relevant attributable change(s) in morphology of ancestral algae, namely with increased complexity of their multicellular bodies. It seems to be the case with the discovery of short PIN-like sequence in Streptophyta algae - *Spirogira pratensis* by De Smet et al. (2011), as predicted in Zažímalová et al. (2007). Answer to those questions would provide very useful feedback for understanding the contributions of various processes regulating both auxin homeostasis and perception in terms of plant body plan and development, as well as relevant increase of plant fitness under variable environmental constraints.

8A CONCLUSION AND PROSPECTS

Conclusions:

The main general objective of this thesis was to advance our understanding of the role of the PIN-FORMED (PIN) protein family in management of auxin transport and - in general – in control of cellular auxin homeostasis. Special attention was paid to deeper understanding of diverse roles of different members of the PIN family to the life and development of the plants, also from evolutionary point of view.

The main findings are:

A. There is a diversity within PIN protein family, and with respect to conservation of the internal hydrophilic loop the proteins form two subfamilies (‘long‘ and ‘short‘ PINs), with different developmental roles;
B. PIN4 and to some extent also PIN6 function as the direct auxin transporters and they play a rate-limiting role in the cellular auxin efflux. This function is typical for ‘long’ PINs;
C. ‘Short‘ PINs have unexpected subcellular localization, and they reside in the endomembrane system;
D. The overexpression of *At*PIN5 in tobacco cells results in dramatic change in the auxin metabolic profile, suggesting the key role of endoplasmic reticulum in control of cellular auxin homeostasis;
E. ‘Short‘ PIN proteins seems to be evolutionary older then the ‘long’ PINs;
F. Regulation of auxin conjugation/deconjugation is an ancient way of influencing cellular auxin homeostasis, and evolution of auxin conjugation correlates with increasing level of plant body complexity during plant evolution.

Prospects

With respect to recent findings related to auxin metabolism and to ways of controlling auxin homeostasis, research focus should be oriented to specify subcellular localizations of different known parts of the auxin-metabolizing enzymatic machinery, and the role of intracellular auxin redistribution in regulation of auxin homeostasis

should be clarified. Moreover, two remaining members of the PIN family yet await characterization. Attention should be paid to species of flowering plants other than Arabidopsis, as characterization of orthologous PIN sequences may bring unexpected results. Last but not least, sequence data-mining should be focused on finding the ancestral *PIN* sequences in evolutionary old organisms including multicellular algae, and they should be correlated with key developmental events in those organisms and their life strategies. This will help to understand original and consequently also the present role of PINs in plant development.

8B ZÁVĚRY A PERSPEKTIVY

Závěry:

Hlavním cílem této disertační práce bylo přispět k pochopení úlohy proteinové rodiny PIN (PIN-FORMED) v managementu transportu auxinu a - obecně - v udržování homeostáze auxinu na úrovni buňky. Zvláštní pozornost byla věnována hlubšímu pochopení rozdílných funkcí jednotlivých členů této proteinové rodiny v růstu a vývoji rostliny, též z evolučního hlediska.

Hlavní získané poznatky jsou:

A. Proteinová rodina PINů je s ohledem na sekvenční strukturu centrální hydrofilní smyčky značně různorodá, lze ji však rozdělit na dvě hlavní podrodiny, označované jako ‘krátké‘ a ‘dlouhé‘ PINy, které se významně liší svou úlohou v růstu a vývoji rostlin;
B. PIN4 a do určité míry i PIN6 jsou funkční přenašeče auxinu, které limitují export auxinu z buňky. Tato funkce je typická pro ‘dlouhé’ PINy; .
C. ‘Krátké‘ PINy jsou lokalizovány v rámci buňky nečekaně na endomembránovém systému;
D. Nadexprese *At*PIN5 v buněčných liniích tabáku vede k výrazné změně v metabolickém profilu auxinu. Tento výsledek poukazuje na klíčovou roli endoplasmatického retikula v regulaci buněčné homeostáze auxinu;
E. “Krátké” PINy mohou představovat původní formu PINů, evolučně starší než ‘dlouhé‘ PINy;
F. Regulace (de)konjugace auxinu je evolučně starší formou řízení homeostáze auxinu v buňce, a evoluce konjugace auxinu koreluje se zvyšující se komplexitou rostlinného těla při evoluci rostlin.

Perspektivy

S ohledem na současné poznatky týkající se regulace metabolismu auxinu v buňce a způsobů kontroly auxinové homeostáze, budoucí výzkum by měl být orientován na určení vnitrobuněčné lokalizace komponent podílejících se na enzymatické aktivaci/deaktivaci auxinových molekul a na objasnění úlohy vnitrobuněčné

redistribuce auxinu v kontrole homeostáze auxinu v buňce. Další výzkum si zaslouží také dva dosud necharakterizované proteiny z podrodiny „krátkých“ PINů a obecně pak diverzita v rodině PINů - zvláště pak tato diverzita v evolučním kontextu, protože srovnávací studie mezi vzájemně ortologními PINy mohou přispět k porozumění rozdílné stavby těl porovnávaných druhů.

Pro porozumění původní – a v extenzi i současné roli PINů v auxinovém transportu by bylo užitečné objevit nejstarší vývojovou větev rostlin, která již obsahuje PINovou sekvenci a pracuje s auxinem víceméně obdobným způsobem jako vyšší rostliny. Porozumění úloze PINů a auxinu u nejstaršího společného předka současných rostlin a příbuzných mnohobuněčných řas by přispělo k porozumění komplexní a složité síti regulačních pochodů, kterými je řízena distribuce auxinu u vyšších rostlin.

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10 CURRICULUM VITAE

Name: **Petr Skůpa**

Address/employer:

Institute of Experimental Botany ASCR, Rozvojová 263, 165 02 Prague 6, Czech Republic.

Tel.: +420-225106425, E-mail: skupa@ueb.cas.cz

Education:

1996-2003 Mendel University of Agriculture and Forestry in Brno, the Faculty of Horticulture, Brno, Czech Republic

2003 Degree in Agriculture (Ing., equivalent of Mgr.)

2003-2010 Postgraduate student at Faculty of Science, Charles University in Prague, Department of Plant Experimental Biology, Dissertation thesis at the Academy of Sciences of the Czech Republic, Institute of Experimental Botany; Laboratory of Hormonal Regulations of Plants. Title: PIN family of auxin transporters: Functional and evolutionary aspects.

Research activity in the fields: Particular role of different PIN proteins in the phenomenon of polar auxin transport; Evolutionary aspects of the auxin management in plants; Mechanism of polar auxin transport, dynamics of auxin efflux carriers and auxin metabolism.

Other experience:

Conferences:

- ACPD – 2. International symposium "Auxins and Cytokinins in Plant Development", Prague; 7th – 12th July 2005
- 3rd International meeting on Plant Neurobiology, Štrbské Pleso, Slovakia; 14th – 18th May 2007

- Symposium Plant cell elongation: integration of hormonal and environmental signals, Ghent, Belgium; 20. 12. 2007
- Conference: Society of Experimental Biology Main Meeting, Marseille, France; 6^{th} - 10^{th} July 2008
- ACPD – 3. International symposium "Auxins and Cytokinins in Plant Development", Prague, Czech Republic, July 10^{th} – 14^{th} 2009

Grants:

- Technology Agency of the Czech Republic, project no.: TA01011802 "Auxin herbicides: Development of herbicides with modified effectiveness or changed species selectivity (Auxinové herbicidy: vývoj herbicidů se změněnou účinností nebo pozměněnou druhovou selektivitou)", 2011-2014, Petr Skůpa – principal investigator.

List of publications:

Papers in impacted journals:

- Petrášek J, Mravec J, Bouchard R, Blakeslee JJ, Abas M, Seifertová D, Wiśniewska J, Tadele Z, Kubeš M, Čovanová M, Dhonukshe P, **Skůpa P**, Benková E, Perry L, Křeček P, Lee OR, Fink GR, Geisler M, Murphy AS, Luschnig C, Zažímalová E, Friml J. PIN proteins perform a rate-limiting function in cellular auxin efflux. Science 2006;312(5775):914-8**.** (IF 2010: 31.364)
- Mravec J, Kubeš M, Bielach A, Gaykova V, Petrášek J, **Skůpa P**, Chand S, Benková E, Zažímalová E, Friml J. Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. Development 2008;135(20):3345-54 (IF 2010: 6.898)
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(Several posters were presented at conferences, as listed above)