Cell. Mol. Life Sci. 64 (2007) 1621 – 1637
1420-682X/07/131621-17
DOI 10.1007/s00018-007-6566-4

Cellular and Molecular Life Sciences

Review

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

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Received 28 December 2006; received after revision 16 February 2007; accepted 26 March 2007
Online First 27 April 2007

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].

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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].

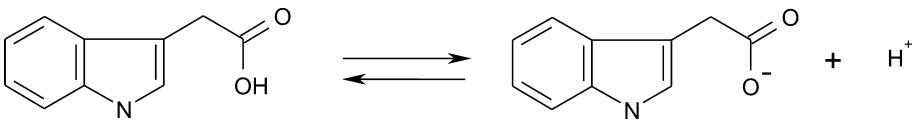

Figure 1. Reversible dissociation of the molecule of native auxin – indole-3-acetic acid (IAA).

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

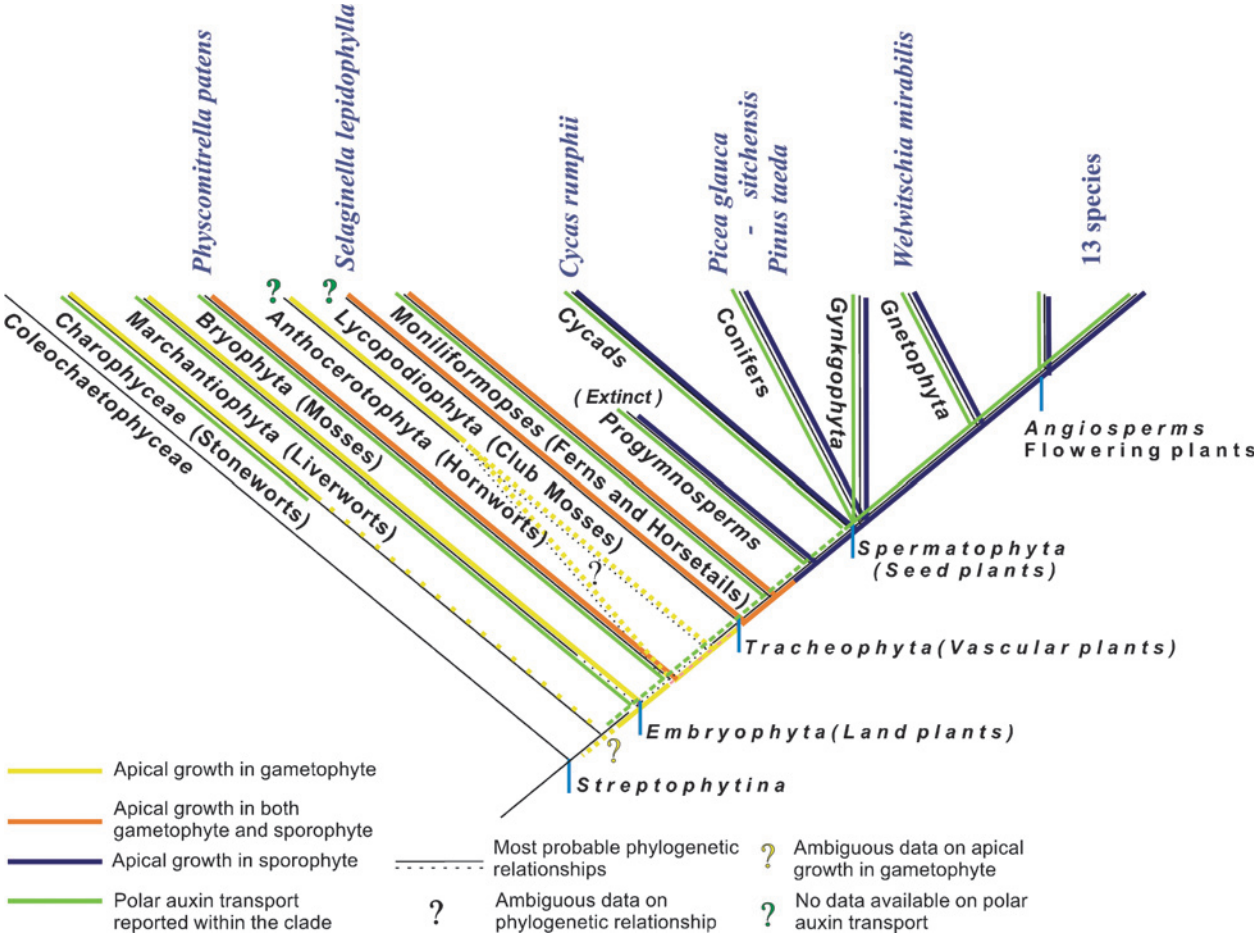

Figure 2. Series of branching events in the plant evolution process with respect to the predictability of *PIN*-like gene distribution. The layout of the groups' relationships and the depicted evolution of apical growth in the diagram were adapted according to Friedman et al. [45] and Friedman and Carmichael [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

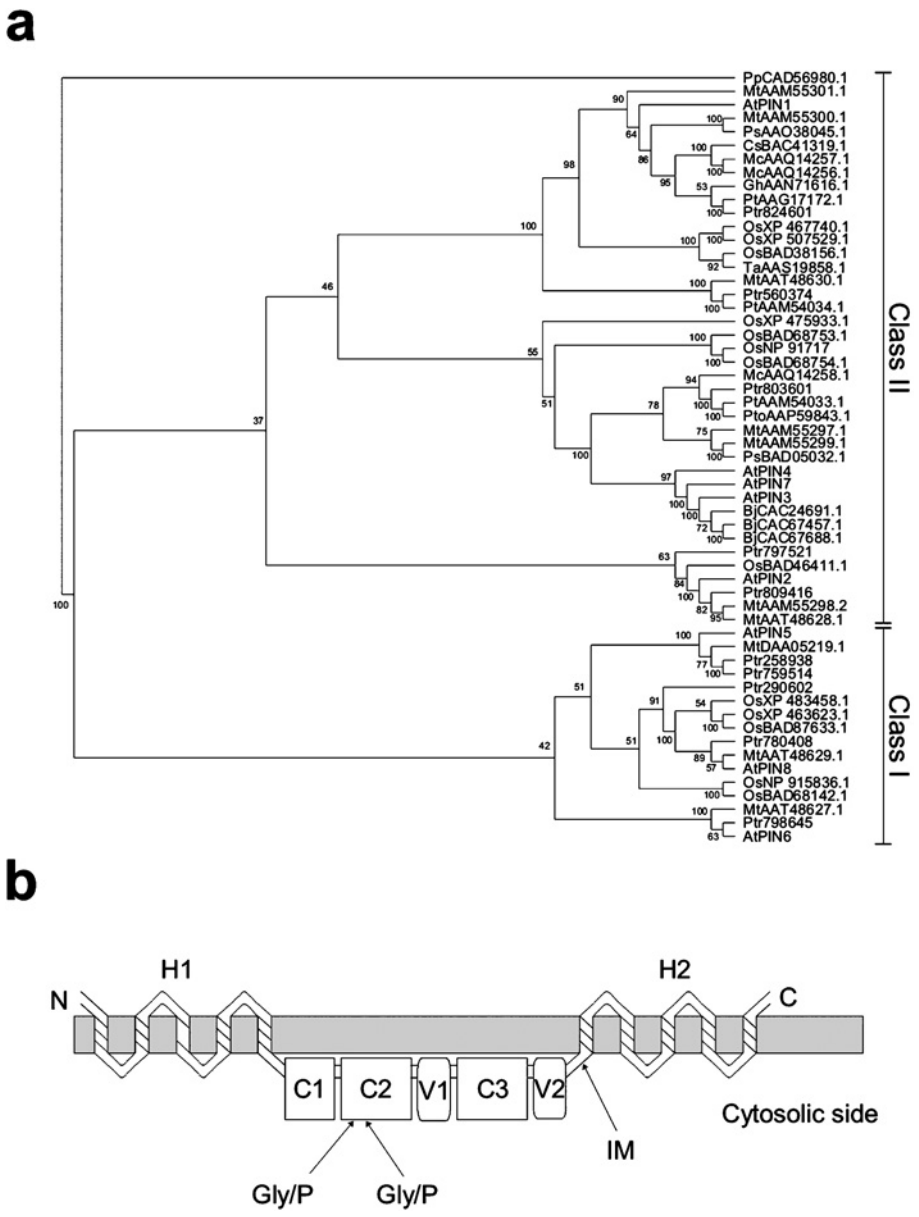

Figure 3. The cladogram and structure of PIN proteins. **(***a***)** The cladogram of PIN proteins: the names of the proteins begin with the first letters of the scientific name: At, *Arabidopsis thaliana*; Bj, *Brassica juncea*; Cs, *Cucumis sativus*; Gh, *Gossypium hirsutum*; Mt, *Medicago truncatula*; 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

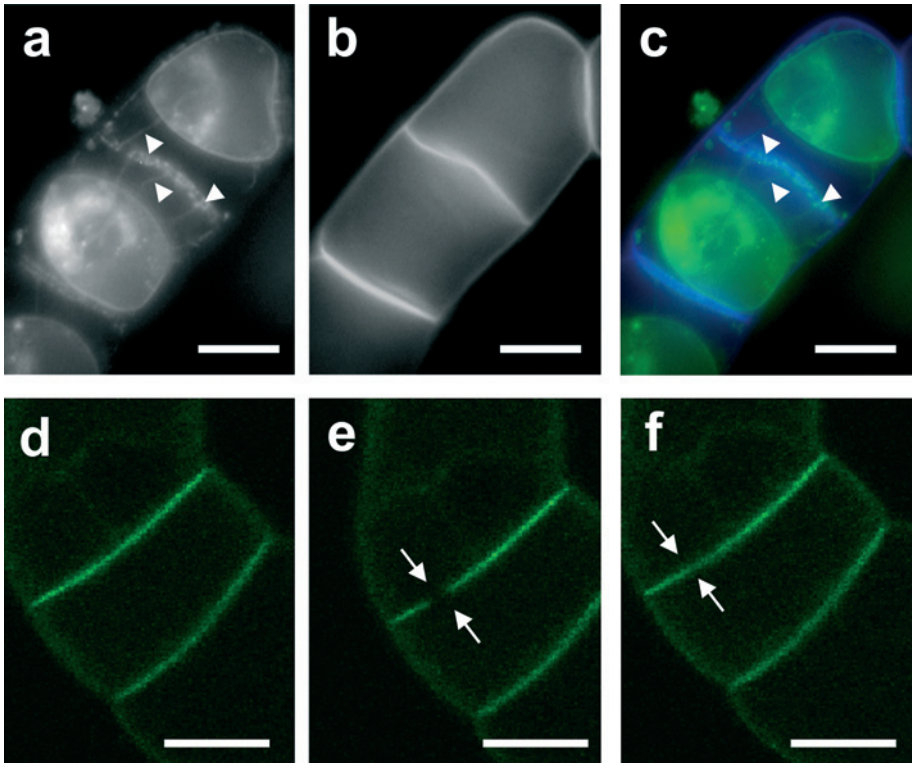

Figure 4. *In vivo* localization (*a–c*) and dynamics (*d–f*) of *Arabidopsis thaliana* PIN1-GFP proteins in stably transformed 2-day-old tobacco BY-2 cells. Cytoplasmic localization of PIN1-GFP after mild plasmolysis with 0.45 M mannitol (5 min) with GFP signal in Hechtian strands and plasma membrane-cell 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

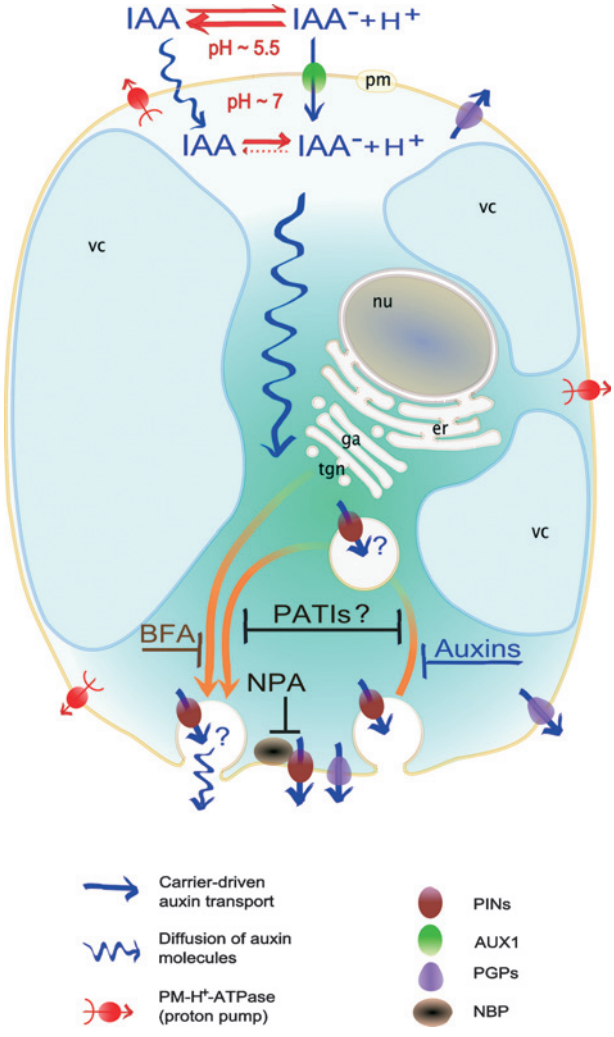

Figure 5. Scheme for the role of PINs in the polar auxin transport machinery in plant cells. Various transporters (auxin influx carrier, i.e. permease AUX1; auxin efflux carriers of the PIN and PGP type) are depicted together with PIN constitutive cycling. Plasma membrane H^+-ATPase, involved in maintenance 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.

Acknowledgements. The authors thank J. Neustupa for valuable suggestions and M. Jiřina for critical reading of the manuscript. The authors are supported by grants awarded by the Grant Agency of the Academy of Sciences of the Czech Republic, project no. A6038303 and KJB600380604, and by the Ministry of Education of the Czech Republic, project LC06034.

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