Genome Biology

Protein family review

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

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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

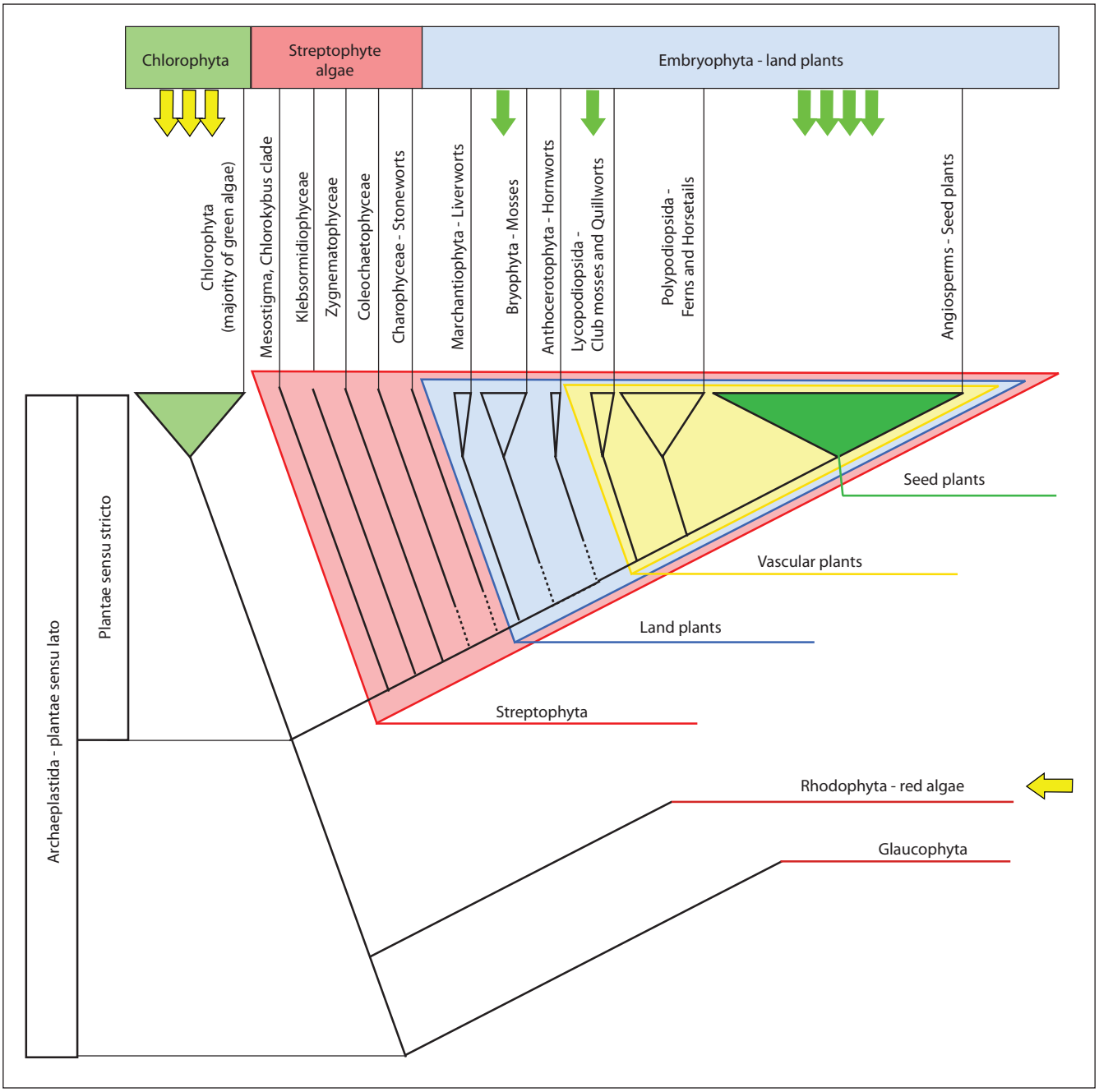

Figure 1

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

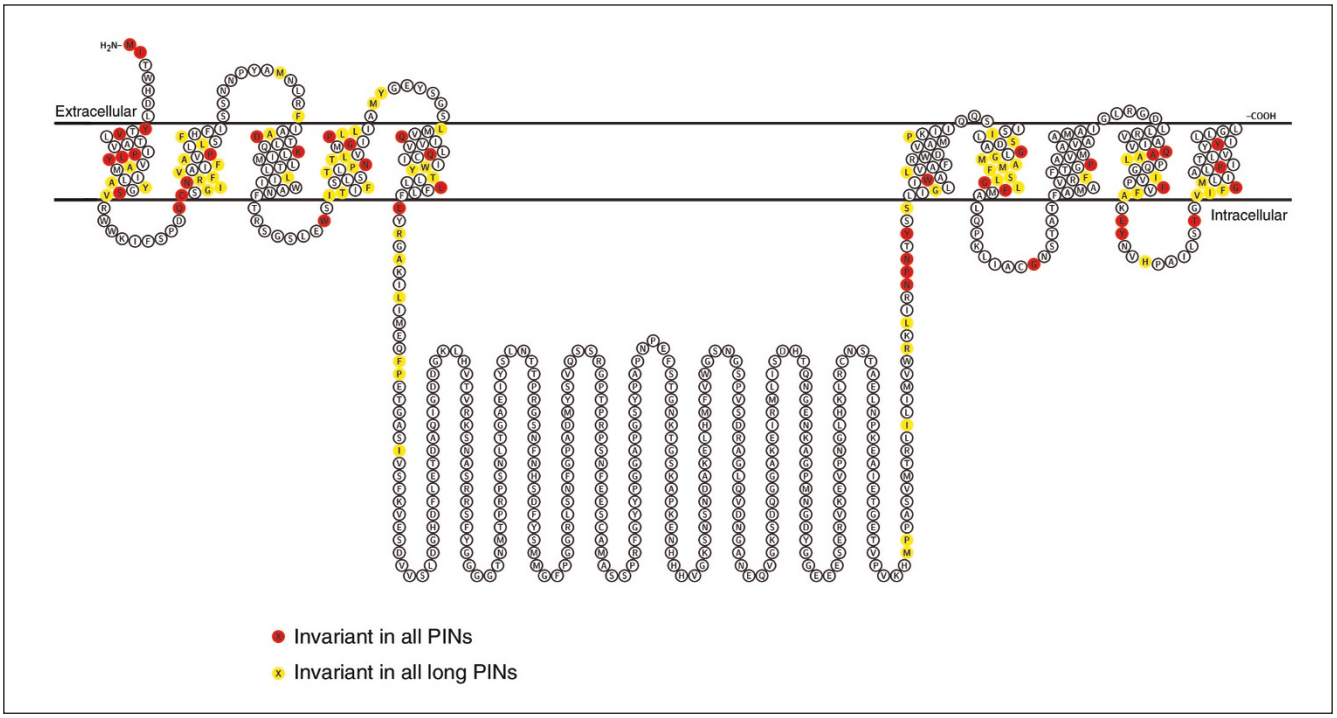

Figure 2

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*

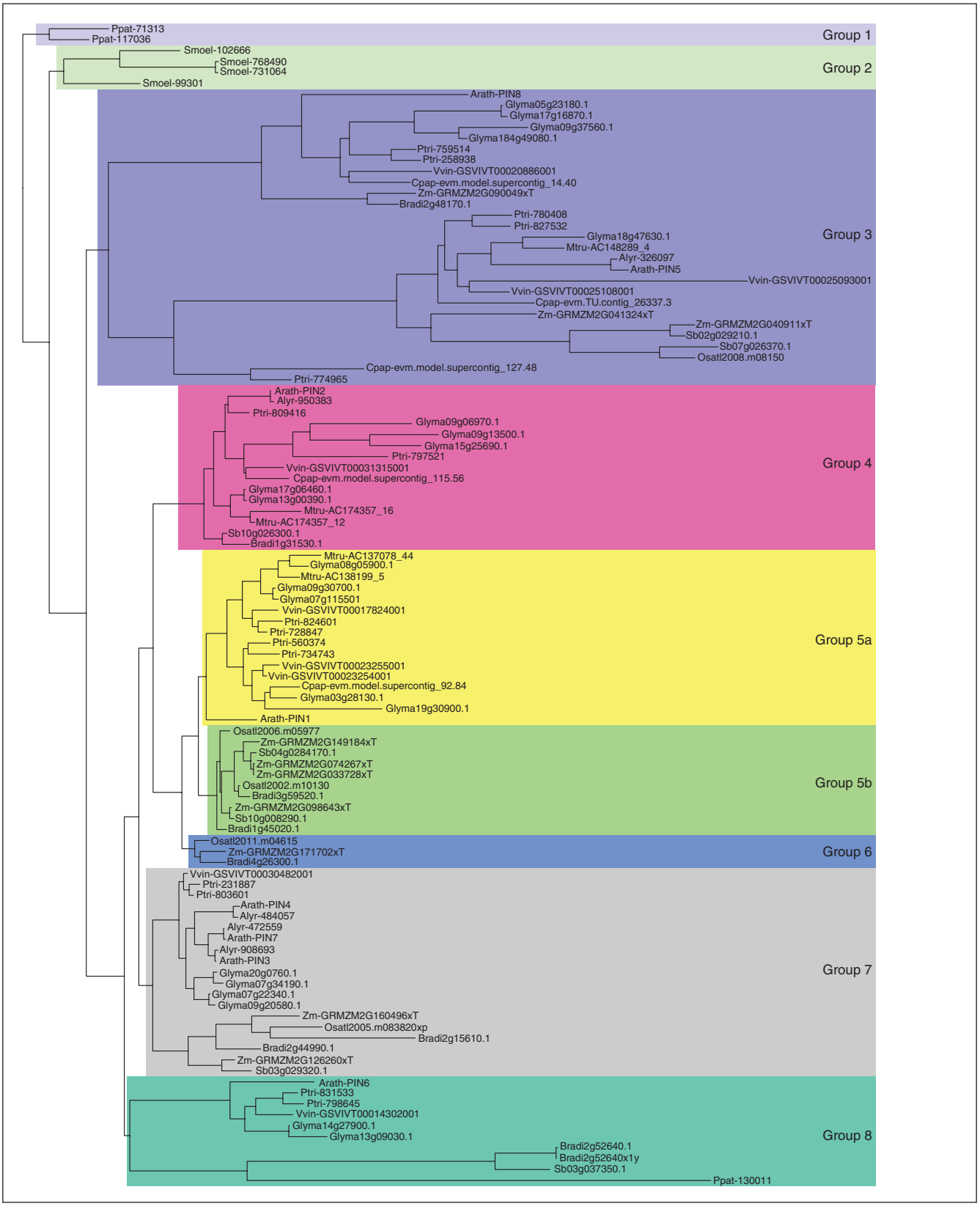

Figure 3

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

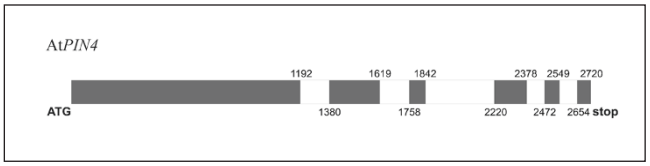

Figure 4

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.

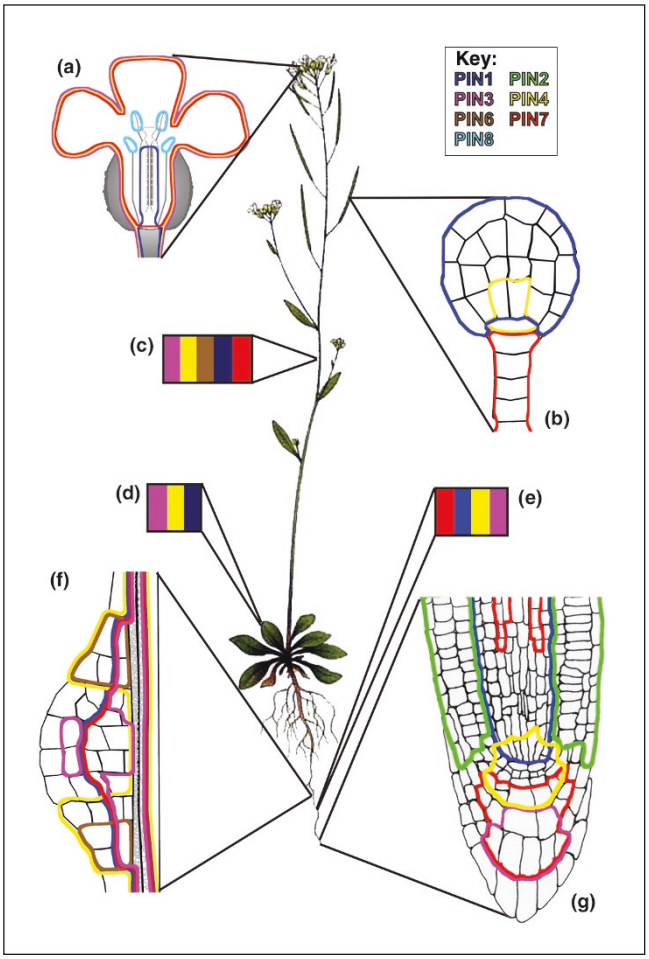

Figure 5

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.

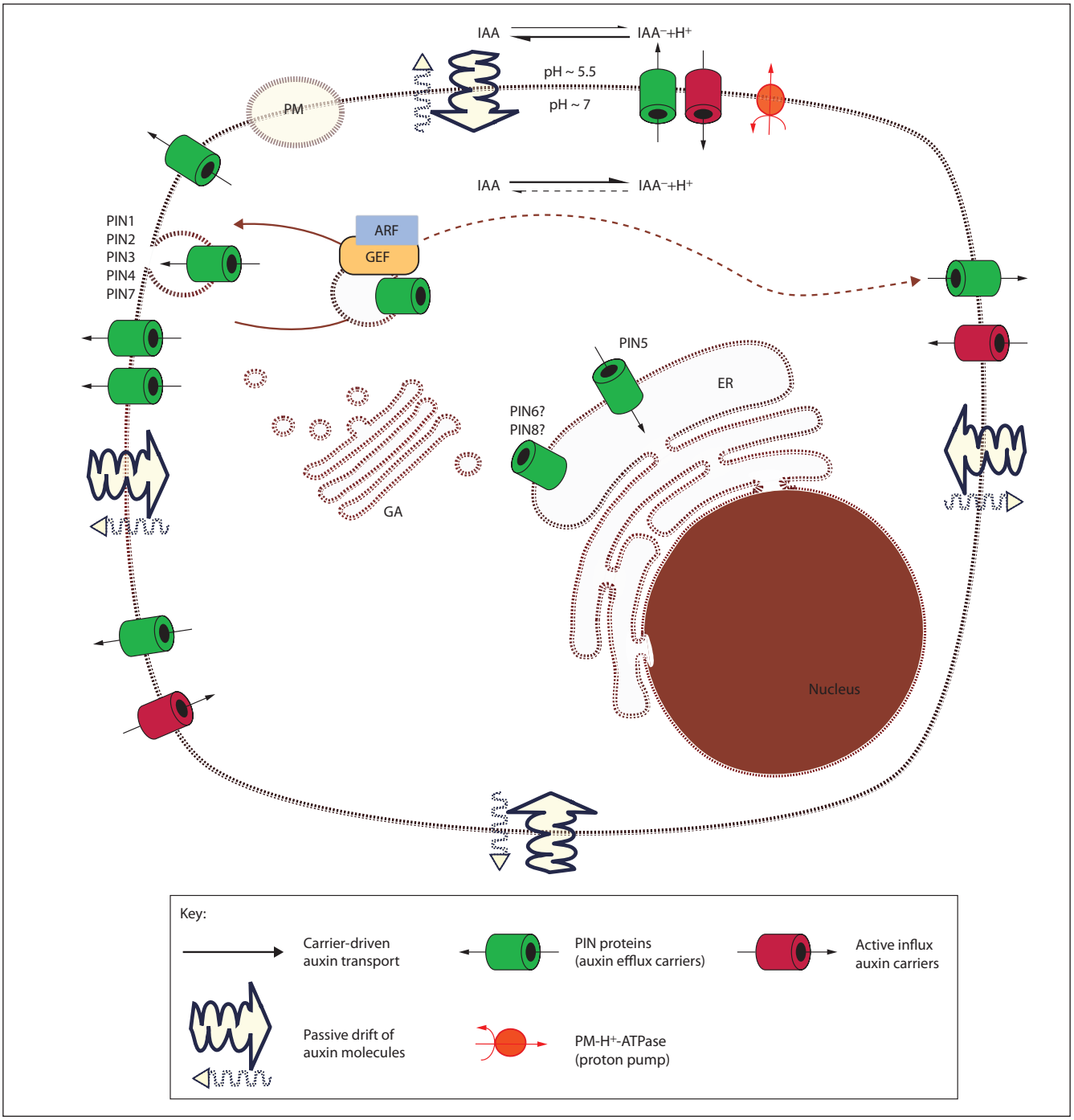

Figure 6

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.

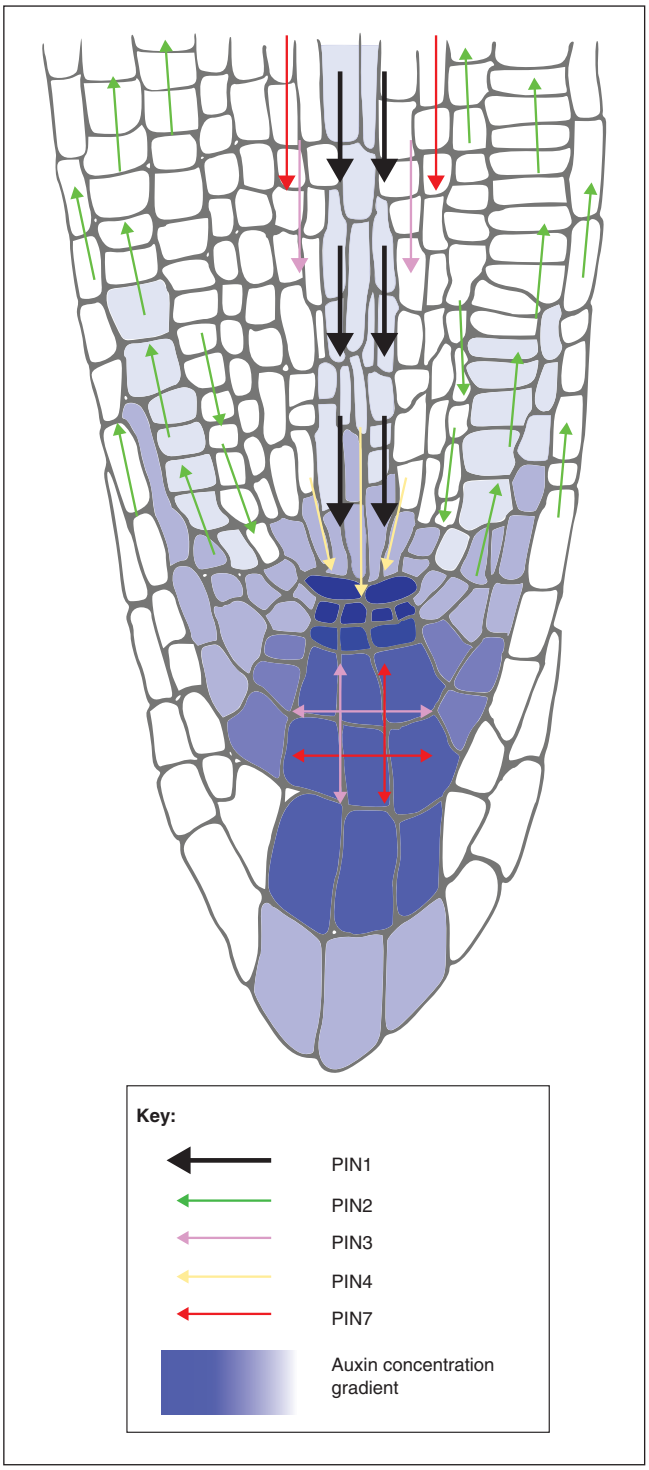

Figure 7

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.

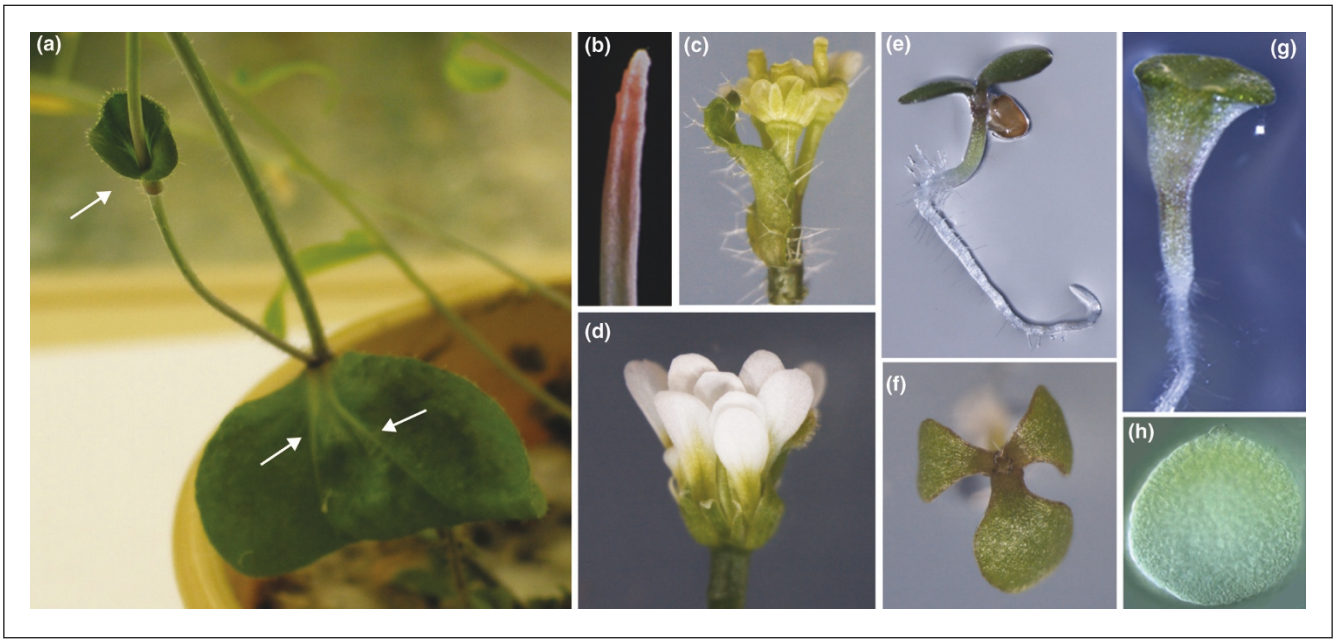

Figure 8

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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