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# Master and servant: Regulation of auxin transporters by FKBPs and cyclophilins

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Plant development and architecture are greatly influenced by the polar distribution of the essential hormone auxin. The directional influx and efflux of auxin from plant cells depends primarily on AUX1/LAX, PIN, and ABCB/PGP/MDR families of auxin transport proteins. The functional analysis of these proteins has progressed rapidly within the last decade thanks to the establishment of heterologous auxin transport systems. Heterologous co-expression allowed also for the testing of protein–protein interactions involved in the regulation of transporters and identified relationships with members of the FK506-Binding Protein (FKBP) and cyclophilin protein families, which are best known in non-plant systems as cellular receptors for the immunosuppressant drugs, FK506 and cyclosporin A, respectively. Current evidence that such interactions affect membrane trafficking, and potentially the activity of auxin transporters is reviewed. We also propose that FKBPs andcyclophilins might integrate the action of auxin transport inhibitors, such as NPA, on members of the ABCB and PIN family, respectively. Finally, we outline open questions that might be useful for further elucidation of the role of immunophilins as regulators (servants) of auxin transporters (masters).

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## 1. Introduction

Numerous developmental processes in multicellular organisms rely on the establishment of tissue polarities [1]. Spatial developmental information in plants is conveyed in part through the directional distribution of the essential hormone, auxin [2]. Auxin accumulation and its directional distribution among neighboring cells, referred to as polar auxin transport, represent the core of the ability of auxin to elicit differential effects on plant growth and development [1]. In this manner, polar auxin transport is a primary mechanism in the regulation of plant cell physiology and development [1]. Therefore, auxin transport has been a matter of extensive interest and investigation ever since the emergence of the auxin concept more than a century ago [3]. Although seemingly a simple problem, it turned out to be very difficult to rigorously address the physiology and function of auxin transport proteins at the molecular level [4]: auxin transport studies have been found to be complicated by the diffusion component of auxins ([5,6]; see Section 2 and Box 1). Moreover, membrane proteins are generally difficult to analyze functionally because of their low solubility at conditions that preserve their native structure and function. Thus,

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**Box 1: Diffusion versus primary and secondary active auxin transport in plants.**

Much of the complex structure of biological membranes is dedicated to the regulation of solute transport. Passive diffusion of ions and other hydrophilic molecules across the hydrophobic cellular membranes is low. The transport of substrates that cannot diffuse freely across membranes, moreover their directional movement against a concentration gradient, as in the case of polar auxin transport, requires energy. Primary transporters are fueled in most cases by ATP hydrolysis whereas secondary transporters are driven by tapping the electrochemical gradients existing across biological membranes [24]. These are usually $Na^+$ gradients in animal cells provided by the action of $Na^+/K^+$-ATPases or $H^+$ gradients in fungi and plants generated by the action of $H^+$-ATPases on the plasma membrane, respectively. Channels, in contrast, when open let ions diffuse rapidly down electrical and concentration gradients resulting in detectable currents. ABCBs were clearly shown to utilize ATP as a direct energy source and to transport auxin steadily across cellular membranes, suggesting ABCBs act in most cases as primary auxin pumps [4,6,127]. PINs and AUX1/LAXs are thought to act as secondary active auxin exporters dependent on an electrochemical gradient. AUX1/LAXs are similar to bacterial permease-like amino-acid transporters [23] that most likely act as $H^+$/IAA symporters [24]. The energization of PINs is less clear and hypothesized based on the fact that PINs do not possess ATP-binding domains [128]. Interestingly, complex functional interactions among some PINs and ABCBs have been reported [19–21], however, the biological relevance and mechanism of these interactions are far from being understood.

the energy-coupling mechanisms and activity regulation of auxin transporters have remained poorly understood. In contrast, significant progress has been made during the last several years in understanding the membrane targeting of auxin transporters and their effects on plant development [5]. Some advances have been also made toward the elucidation of the functional interactions of auxin transporters with additional regulatory proteins of the immunophilin class. These may affect auxin-transporter trafficking and/or activity [7–10]. The progress in this more recent field is summarized and critically discussed here.

## 2. Auxin transport across biological membranes

According to the chemiosmotic model of auxin transport [11–14], a substantial portion of IAA is protonated in the apoplast and able to enter cells *via* bilayer diffusion, whereas IAA inside the cells is less protonated and its efflux requires active transport. As to our knowledge, whether this is completely so has never been proven experimentally. In this context it is worth mentioning that drug leakage into cells by lipophilic diffusion versus hitchhiking of transporters is currently highly debated in the animal literature [15].

To date, three major families of membrane localized proteins involved in auxin transport have been characterized in *Arabidopsis* and other plant species: PINs, named after the *pin-formed1* (*pin1*) Arabidopsis mutant, ABCBs (ATP-binding cassette, type B) and AUX1/LAXs (AUXIN1/LIKE AUXIN1) [5,16]. Mutations in *PIN1* and combined mutations in other *PIN* genes with PIN1 result in organogenesis defects, indicating that PINs mediate directional auxin flow that regulates organogenesis [17,18]. In contrast, *abcb*1, 19 mutants although significantly dwarfed display only subtle morphological defects [19,20] suggesting that ABCBs function primarily in export of auxin out of meristematic tissues with high auxin concentrations, and in maintenance of long-distance auxin flows required for physiological processes (reviewed in Refs. [4,21]).

AUX/LAX have been discussed to generate auxin sinks as a driving force for auxin transport streams in the stele in the context of lateral root development [22]. The contribution of AUX1 in generating auxin sinks is in agreement with the total loss of gravitropic responses in *aux1* [23].

Depending on the direction of transport, the systems are termed influx transporters and efflux transporters. Members of plasma membrane located AUX1/LAX family are grouped as auxin importers [24]. Auxin exporters are primarily represented by plasma membrane localized PINs (so-called long PINs, see below) and members of the ABCB family, which have so far all been found to be plasma membrane-embedded [6,25–27]. However, it should be mentioned that recently ABCB4 and ABCB21 were characterized as facultative importer/exporters whose transport directionality seems to be triggered by intracellular auxin levels [28].

The regulation of plasma membrane presence and polarity of auxin transporters has been studied extensively. Decent progress has been also made in respect to individual transporter regulation by protein degradation, phosphorylation and protein–protein interaction (for reviews, see Refs. [7,29,30]). Long PINs (PIN1–4,7) comprising a large cytoplasmic loop [5] are often polarly localized to a specific face of the plasma membrane in Arabidopsis root cells, in good correlation with the direction of the auxin flow [31]. By contrast, short PINs (PIN5,6,8) are localized in the endomembrane system (most likely the endoplasmic reticulum (ER)) and appear to function in intracellular auxin transport and homeostasis [32,33]. Recently, a second family of ER-localized auxin transporters, called PILS (for PIN-LIKES), was also shown to regulate auxin homeostasis [34], although their role in auxin transport is less clear. AUX1/LAX and ABCB proteins are plasma membrane localized like long PINs, but are most often apolar (reviewed in Ref. [1]).

All classes of plasma membrane-based auxin transporters are brought to their specific membrane domains by vesicle trafficking along actin tracks [35]. However, based on different sensitivities to brefeldin A the cycling of ABCBs and PINs as well as that of individual members of each family, respectively, seems to reveal specific trafficking mobilities as summarized in excellent reviews [35,36].

Recent analyses in heterologous, non-plant auxin-transport systems (such as yeast, HeLa cells and Xenopus oocytes) demonstrated that AUX1/LAX influx, and ABCB and PIN-mediated IAA efflux activity is substrate-specific and rate limiting [6,19,25,26,37–42], providing experimental evidence that these proteins are *bona fide* auxin transporters. Although plant ABCBs belong to the large superfamily of multidrug resistance transporters, they were found to own a high degree of substrate specificity toward only a few auxinic compounds but not to transport closely related substances (such as the anti-auxin 2-NAA or benzoic acid [6]) or classical substrates of mammalian ABCBs (such as rhodamin123, daunomycin and vinblastine [43]). ABCB-mediated auxin transport was dependent on ATP hydrolysis [6,37] as expected for primary active pumps, and sensitive to inhibitors of auxin efflux (such as NPA, and flavonols [6,37,43,44]) along with known inhibitors of mammalian multidrug ABC transporters (such as cyclosporine A and verapamil [6,43]). Recently, expression of ABCB19 in HeLa cells was also reported to result in anion currents that were inhibited by applying an anion channel blocker [45]. Currently it is unclear if this indicates a unique ion channel function for ABCB19, ion channel function additional to pump function, or an artifact of the heterologous expression. Furthermore, it is unknown whether these currents represent $IAA^-$ fluxes. It is worth mentioning though that ion channel function has been described for some mammalian ABC transporters [46]. It has also been reported that co-expression of ABCB and PIN combinations leads to synergistic transport rates and alters substrate specificities and inhibitor sensitivities [20,21]. The individual roles

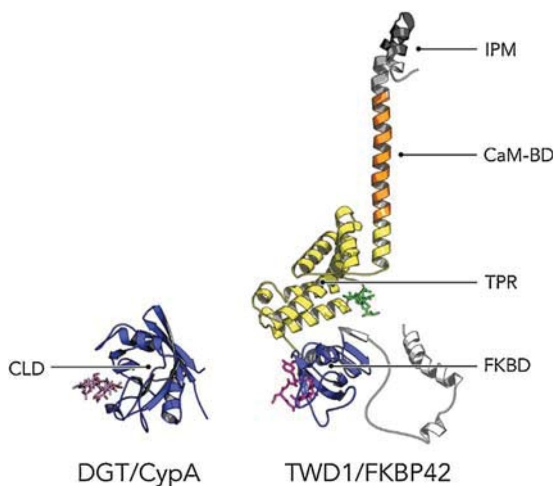


**Fig. 1.** Domain and molecular structures of tomato DGT/CypA and Arabidopsis TWD1/FKBP42. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Cartoon diagrams indicate functional domains in the two different immunophilins and their canonical drug binding sites. Protein structures presented here were generated using the I-TASSER server [129] from full-length peptide sequences without constraints. Threading templates were PDB entries 1e8k, 1dyw, 4i9y, 4e1q, 4jjm, and 2cpl for DGT (estimated TM-score = 0.98 ± 0.05, estimated RMSD = 1.5 ± 1.4 Å), and 2if4, 1kt0, 1qz2 for TWD1 (estimated TM-score = 0.52 ± 0.15, estimated RMSD = 10.3 ± 4.6 Å). Functional domains are labeled: CLD, cyclophilin-like domain; FKBD, FK506-binding domain; TPR, tetratricopeptide repeat domain; CaM-BD, calmodulin-binding domain; IPM, in-plane membrane anchor. Binding sites for immunosuppressant drugs cyclosporin A (PDB 4hy7; pink) and FK506 (PDB 1bkf; magenta) to the CLD and FKBD domains of DGT and TWD1, respectively, are indicated. The location of the MEEVD peptide of HSP90 (PDB 1qz2; green) docking to the TPR domain of TWD1 is given.

of ABCBs and PINs in these interactions, e.g., which protein functions as a transporter and which as a regulator, are far from being understood. Nonetheless, these data point to complexity and cooperation among auxin transport systems at least in the case of heterologously over-expressed proteins.

Detailed structural studies of auxin transport proteins are currently largely lacking. A structural modeling approach using mouse ABCB1 as template has identified IAA-specific substrate binding domains in AtABCB1 [47]; however, these data await their experimental verification. Further structural analyses are much needed, particularly for PIN proteins, which represent a plant-specific class of transporters with very low level of similarity to well characterized transporters from non-plant systems. Such studies should have highest priority because they would widely enhance our knowledge on individual auxin transport mechanisms.

## 3. Plant immunophilins are implicated in regulation of development

Immunophilins belong to two evolutionary non-related groups of proteins originally discovered and classified based on their ability to bind two different classes of immunosuppressant drugs: FK506-binding proteins (FKBPs) bind macrolides, FK506 (tacrolimus) and rapamycin (sirolimus), *via* their FK506-binding domain (FKBD) (see Fig. 1). Cyclophilins (also called rotamases and referred to either as Cyps, or ROCs for rotamase Cyps) are named after their high affinity for the cyclic peptide, cyclosporin A [48–50], which binds to the core cyclophilin-like domain (see Fig. 1). A family of chimeric immunophilins that contain both Cyp and FKBP domains was reported in *Toxoplasma gondii* and designated as FCBPs for FK506 and cyclosporin A binding proteins [51] but FCBPs do not appear broadly conserved across different species.

In mammalian immune cells, FK506–FKBP and cyclosporin A-cyclophilin complexes interact with the calcium-dependent serine/threonine protein phosphatase calcineurin, interrupting the phosphorylation signaling pathway and eventually leading to immunosuppression [48]. Rapamycin-bound FKBPs interact with the protein kinase TOR (target of rapamycin) causing cell cycle arrest as well as inducing immunosuppression [52].

In addition to their ability to interact with immunosuppressants, immunophilins also possess peptidylprolyl *cis–trans* isomerase activity (PPIase; also referred to as rotamase) that catalyzes the rotation of *cis*-peptidylprolyl bonds [53]. The PPIase activity is inhibited by binding of the corresponding immunosuppressant, but is not directly involved in the immunosuppression mechanisms [53]. In addition, some immunophilins have been demonstrated to possess chaperone or co-chaperone activity that is independent of their PPIase activity and unaffected by immunosuppressant drugs [48,49,54,55]. These are usually larger immunophilins that possess additional structural domains. For example, mammalian Cyp40 is a co-chaperone present in steroid receptor-HSP90 complexes where it participates in steroid receptor assembly. Studies with deletion mutations have mapped the co-chaperone function of Cyp40 between its N-terminal PPIase domain and its C-terminal HSP90-binding tetratricopeptide repeat domain [56].

Surprisingly, the principal human cyclophilin, HsCypA, which consists only of the PPIase core domain, has also been reported to exhibit chaperone-like function [57]. *In vivo*, the PPIase and chaperone activities of immunophilins might be involved together or separately in assisting folding of newly synthesized proteins, protein trafficking, activity, assembly of protein oligomers, and protein stability. Yet, non-plant immunophilins have been rarely associated with specific processes of development. One example is a *Drosophila* cyclophilin named ninaA, which is required for the synthesis of rhodopsin in R1-6 photoreceptors [58].

FKBPs and cyclophilins are represented by particularly large families in plants [48,59]. The Arabidopsis genome encodes for 23 FKBP genes compared to *Saccharomyces cerevisiae* (4), *Caenorhabditis elegans* (8) and *Homo sapiens* (15). *Arabidopsis* has seven multi-domain FKBPs and 16 single-domain FKBPs [55]. The *Arabidopsis* cyclophilin family is also very large (29 members) compared to *S. cerevisiae* (8) and *H. sapiens* (16) (reviewed in Ref. [60]). An even larger plant cyclophilin family has been recently reported in soybean (*Glycine max*; 62 members; [61]). Currently the reason for the vast expansions of the FKPB and cyclophilin families in plants is unclear, but these expansions might reflect a shift toward roles in development, stress response and redox regulation of chloroplast function (as reviewed in Ref. [55]). In the following text and Table 1, we summarize properties of plant immunophilins and their function in development.

Among plant FKBPs, the FKBP42 TWISTED DWARF1 (TWD1)/ULTRACURVATA (UCU2) has been extensively studied. The *twd1/ucu2/fkbp42* mutants is marked by a characteristic twisting of the root epidermal cell files and displays a defect in root gravitropism, an auxin-transport regulated phenotype [8,9,62,63] (Table 1). Over-expression of a mutated version of TWD1 lacking its membrane anchor also results in a hypermorphic (“giant”) growth phenotype caused by enhanced cell elongation, which is probably due to altered apoplastic auxin concentrations caused by enhanced auxin transport [64] (see Section 4). All attempts to demonstrate PPIase activity in TWD1 have failed [62,65], probably because only 3 out of the 11 key residues inside the hydrophobic pocket of its FK506-binding domain involved in PPIase function are conserved [55] (see Fig. 1). The unusual FK506-binding domain in TWD1 was instead found to participate in protein interaction with ABCB-type auxin transporters and is apparently involved in the functional regulation of these transporters [44] (see Section 4). TWD1 also possesses a tetratricopeptide repeat domain (common

**Table 1**
Examples of plant immunophilins with functions in plant development. As a note, nomenclature of cyclophilins is not clear in the literature, and proteins are grouped here for simplicity based on molecular size. ER, endoplasmic reticulum; PM, plasma membrane; nd, not determined.

| FKBP/cyclophilin | Plant species | Location | PPIase activity | Function and protein interactions in plant development |
|---|---|---|---|---|
| FKBP12 | *V. faba* [72]<br>*A. thaliana* [72,73]<br>*Z. mais* [72]<br>*O. sativa* [131]<br>*P. wilsonii* [74] | Cytoplasm | nd | No obvious phenotype in *A. thaliana* [132]<br>Interacts with AtFIP37, which has a role in embryo development [73]<br>Interacts with PwHAP5, role in pollen tube development [74] |
| FKBP42/TWD1/UCU2 | *A. thaliana* [62,63,65]<br>*O. sativa* [131]<br>*Z. mais* [133] | Cytoplasm-facing ER [8,9], PM [8,62,99] and tonoplast locations [65,66] | No [62,65] | Dwarf phenotype caused by reduced cell elongation, reduced helical orientation of epidermal layers in root and shoot, disoriented growth [62,63]<br>Regulation and PM-to-ER trafficking of ABCB-type auxin transporters [8,9]<br>Interaction with ABCB transporters on PM (*via* FKB-domain) [8,62], ABCC transporters on tonoplast (*via* TPR domain) [66], with HSP90 (TPR domain) [62,65] and calmodulin (calmodulin-binding domain) [66]<br>Over-expression of TWD1 lacking its membrane anchor results in hypermorphic growth [64] |
| FKBP72/PAS1 | *A. thaliana* [69]<br>*O. sativa* [131]<br>*Z. mais* [133] | Unclear (C-terminal membrane anchor), nuclear [68,134] | Low, inhibited by FK506 and rapamycin [134] | Up-regulated cell division, leaf fusions, short hypocotyls, sterile [69]<br>Chaperon during translocation of NAC-like transcription factor (AtFAN) into nucleus [68]<br>Regulation of very long fatty acid elongation [70] |
| – | | | | |
| DGT/CypA/ROC1/CYP1/CYP2/ | *P. patens* [10]<br>*A. thaliana* [77]<br>*S. lycopersicum* [78]<br>*O. sativa* [91,92,135]<br>*R. communis* [79] | Nuclear and cytoplasmic [10,90], phloem sieve elements [79] | Significant [77,78], inhibited by CsA [78] | Regulates growth [90], gene expression [84,90,92], patterned cell division [88], phloem function [79], ROS balance in root apical meristem [87] |
| Cyp20-2 | *A. thaliana* [93], *T. aestivum* [94] | Predicted chloroplast [49] | Stronger in *A. thaliana* than in *T. aestivum* [93] | Hormonal responses [93,94], flowering time [93,94], protein conformational change [93] |
| Cyp40/SQT | *A. thaliana* [95–97] | Predicted cytosol [49] | Unknown | Protein complex assembly [97,136], vegetative phase change [95], meristem function [96], microRNA function [97] |
| Cyp71 | *A. thaliana* [137] | Nucleus [137] | Unknown | Meristem function, leaf morphology and vein pattern, root elongation [137] |

in many large immunophilins) that was shown to interact with vacuolar ABC transporters of the C subclass and HSP90 [65,66], as well as a calmodulin-binding domain [66] (see Fig. 1). Furthermore, TWD1 contains a unique C-terminal in-plane membrane anchor domain [64,67]. The functional importance of HSP90 and calmodulin binding to TWD1 is not known but analogy to human FKBP38 and other multi-domain FKBPs suggests that both HSP90 and calmodulin might act as modulators of TWD1. Interaction with the classical chaperone, HSP90, also suggest that TWD1 might function as co-chaperone (reviewed in Refs. [48–50,54,59]).

Another frequently studied Arabidopsis FKBP protein, PASTICCINO (AtFKBP72/PAS1), is involved in the control of cell proliferation and differentiation and was suggested to chaperone the nuclear translocation of a NAC transcription factor [68,69]. Further, it seems to participate in the biosynthesis of very long fatty acids. Loss of PAS1 results in irregular cell division due to interrupted synthesis of very long fatty acids, leading to a '*pasticcino*' (cupcake) mutant phenotype [70]. Both, the interaction with the very long fatty acid elongase complex and that with the NAC transcription factor, respectively, is mediated by the C-terminal region of PAS1 that contains a calmodulin-binding domain [53]. The C-terminal domain of PAS1 also contains a membrane anchor domain similar to that in TWD1 [64,67,71] that seems to be important for the cellular distribution of PAS1 and its exclusion from the nucleus except during cell division [71]. Another Arabidopsis FKBP, FKBP12, interacts with AtFIP37, a DNA-binding protein involved in mRNA splicing, cell cycle regulation and embryo development [72,73] although the developmental importance of this interaction is unclear. In the conifer *Picea wilsonii*, FKBP12 has a role in pollen tube development through interaction with PcHAP5, a subunit of a histone-associated transcription factor [74].

Interaction with immunosuppressants is generally not conserved in plant FKBPs. Higher plants (including Arabidopsis) were found to be insensitive to both FK506 and rapamycin apparently because the primary receptor, plant FKBP12, has lost its capacity to bind these drugs and thus to inhibit calcineurin and TOR signaling [72,75]. This is supported by the finding that expression of yeast FKBP12 in Arabidopsis provides rapamycin sensitivity to a similar level as found for yeast and animals [75]. PPIase activity is also not well conserved in plant FKBPs (Table 1). Instead, some plant FKBPs appear to operate as regulators of specific protein–protein interactions [27,48,49,54,55,76].

In contrast to the loss of sensitivity to immunosuppressants in plant FKBPs, plant cyclophilins display conserved sensitivity. For example, cyclosporine A was found to inhibit the PPIase activity of Arabidopsis chloroplasts stromal extracts [77], and Arabidopsis ROC1/CypA and tomato DIAGEOTROPICA (DGT/CypA) were sensitive to cyclosporine A in *in vitro* studies [78]. PPIase activity also seems conserved in plant cyclophilins, and strong activity has been experimentally demonstrated in all tested proteins, e.g.,

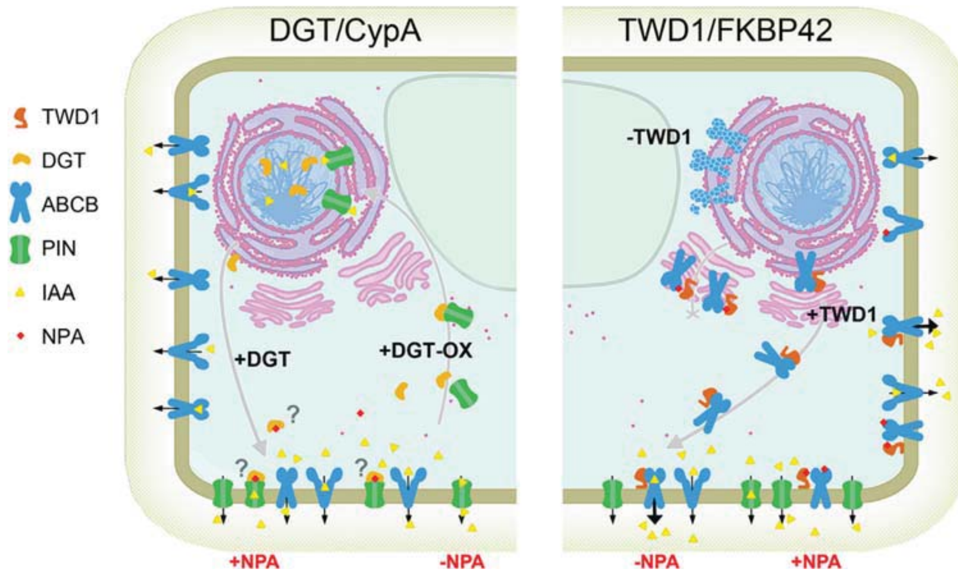


**Fig. 2.** Working model of tomato DGT/CypA and Arabidopsis TWD1/FKBP42 functions in regulation of auxin transporters.
Interactions with DGT/CypA may be regulating PIN membrane exchanges and activity (left panel) [10,124]. This hypothesis is based on observed inhibition of PIN auxin efflux activity upon PIN co-expression in a yeast transport system. When expressed together with PIN1 or ABCB1 in *N. benthamiana* leaves, DGT-dependent relocation of plasma-membrane localized PIN1 to the nuclear periphery was observed, suggesting that DGT regulates PIN trafficking, whereas both PIN1 and ABCB1 increased DGT's presence on the plasma membrane. Auxin transport in the *dgt* root tip was completely NPA-insensitive [10] suggesting that DGT is involved in NPA inhibition of PIN-driven auxin efflux. Because both DGT and NPA appear to be negative regulators of PIN auxin transport, NPA is more likely to promote DGT interaction of PINs rather than to disrupt it (see +NPA). However, it is unknown whether DGT engages in direct physical interactions with PINs (or ABCBs) and whether DGT binds NPA.
In analogy, TWD1/FKBP42 was shown to have a regulatory role on ABCB transporters (right panel). TWD1 is located in endomembrane systems, such as the ER and the tonoplast but seems to exhibit its activity predominantly at the plasma membrane. A chaperone-like function for TWD1 during ER to plasma membrane trafficking of ABCBs was suggested based on the finding that ABCB1,4,19 are retained on the ER and degraded in the *twd1* mutant [8,9].
ABCB-type auxin transporters and TWD1 have been both identified as targets of NPA [37,41,62,111,112]. Micromolar concentrations of NPA were also shown to disrupt the ABCB1–TWD1 interaction (see +NPA) supporting the hypothesis that NPA binds at the ABCB–TWD1 interface [44,130].

**Box 2: Immunophilins in regulation of plant development and auxin transport.**
Immunophilins can function by either binding of small ligands (such as immunosuppressive drugs and other compounds that generate protein-binding surfaces), by peptidyl–prolyl isomerisation of their targets, or as chaperones. From bacteria to humans, these versatile proteins regulate protein structure, activity and stability. In higher plants, the FKBP and cyclophilin families are dramatically over-repesented, and display a functional shift toward specific developmental functions. Whereas FKBPs of plants show reductions in their affinity for immunosuppressive drugs as well as decreases in their PPIase activity, plant cyclophilins display conserved affinity for immunosuppressive drugs and presence of PPlase activity. Recent studies identified plant immunophilins as regulators of polar auxin transport, and suggested that immunophilins function in either auxin-transporter cellular trafficking or activity, or both [8–10,43,44,62]. Whereas Arabidopsis TWD1/FKBP42 shows preference for binding and functional interaction with ABCB type of auxin transporters [8], tomato cyclophilin DGT/CypA displays high functional preference for PIN auxin transporters [10]. Furthermore, whereas TWD1 is a positive regulator of its ABCB auxin transporter targets [43,62], DGT appears to function as a negative regulator of PINs [10]. Auxin transport in both, Arabidopsis *twd1* and tomato *dgt* mutants, is insensitive to NPA [10,44] suggesting that immunophilins also mediate auxin-transporter sensitivity to auxin transport inhibitors.

Arabidopsis ROC1, tomato DGT [78], *Ricinus communis* and rice CYCLOPHILIN1 [79]. Interestingly, similar to plant FKBPs, plant cyclophlins have been strongly linked to regulation of development (Box 2). Mutations in tomato *DGT* result in auxin-related phenotypes such as lack of lateral roots, deregulation of gene expression, ROS imbalance, and defects in polar auxin transport [80–88] (Table 1). DGT is both nuclear and cytoplasmic localized [10] (Table 1, Fig. 2), and can move from shoot to root in grafted plants partially restoring the lack of lateral roots in *dgt* mutant rootstocks [89]. A DGT-like small cyclophilin from *Physcomitrella patens* is also important for auxin-regulated growth [90], whereas a related cyclophilin from rice (LATERAL ROOTLESS2, LRT2/OsCYP2) plays a crucial role in auxin-regulated lateral root formation [91] and has been shown to directly regulate the stability of the auxin-responsive transcriptional repressor protein, OsIAA11, *via* peptidylprolyl isomerization [92]. The Arabidopsis cyclophilin CYCLOPHILIN20-2 regulates flowering time by modulating the conformation of BRASSINAZOLE-RESISTANT1 in Ref. [93].

Interestingly, whereas Arabidopsis AtCyp20-2 over-expressing lines flower earlier, the *gaid* wheat mutant that over-accumulates TaCyp20-2 results in a dwarf phenotype and late flowering [94] suggesting the function of some cyclophilin orthologs might vary across plant species. The *squint* mutation in the Arabidopsis *SQN*/*Cyp*40 cyclophilin gene results in a delay of vegetative shoot maturation [95] and a delay of flower meristem termination when combined with other mutations [96]. Similar to TWD1/FKBP42 [65], Arabidopsis *SQN*/*Cyp*40 forms a complex with HSP90, facilitating HPS90-mediated protein complex assembly [97].

## 4. Functional interactions of immunophilins with auxin transporter proteins regulate auxin transport

The regulatory role of TWD1/FKBP42 on ABCB transporter activity and ABCB presence on the plasma membrane has been extensively documented [8,9,43,44,62]. ABCB1/PGP1 was identified in a yeast two-hybrid screen using TWD1 as bait [62], although a role of ABCB1 in hypocotyl elongation had been described earlier [27]. In heterologous auxin-transport systems, TWD1 strongly modulates the transport activity of ABCB1 [8,43,44,62] and ABCB19

[43] (see Fig. 2). In agreement, Arabidopsis *abcb1 abcb19* and *twd1* mutants have widely overlapping (although not entirely identical) dwarfed phenotypes [8,9,62], with a peculiar, non-handed helical rotation of the epidermal cell files, designated as "twisting". Functional FKBP–ABCB interaction seems to be conserved throughout evolution as judged from the fact that the activity of murine ABCB3/MDR3 transporter expressed in *S. cerevisiae* requires the presence of yeast FKBP12 [98]. Yeast FKBP12 also seems to compete with TWD1 for regulation of Arabidopsis ABCB1 activity in a *S. cerevisiae* auxin-transport system [43], which is further supported by the finding that FKBP12 partially complements the *twd1* mutant phenotype when expressed in Arabidopsis [99].

The exact mechanism through which TWD1 regulates ABCB auxin transporters is far from being understood. A chaperone-like function for TWD1 during ER to plasma membrane trafficking of ABCBs has been proposed based on the finding that ABCB1,4,19 are retained on the ER and degraded in the *twd1* mutant [8,9] (see Fig. 2). This hypothesis agrees with the fact that the human TWD1/FBP42 ortholog, FKBP38, promotes ER to plasma membrane delivery of the cystic fibrosis transmembrane conductance regulator, CFTR/ABCC7 [100], a chloride channel whose malfunction is responsible for the genetic disorder mucoviscidosis. The interaction with FKPB38 is thought to result in isomerization of prolyl bonds of CFTR, allowing for the establishment of functional CFTR protein at the plasma membrane [100]. However, it is not clear whether FKBP42/TWD1 functions in the same way in ABCB transporter regulation because, as already mentioned, PPIase activity has not been confirmed in TWD1. A second functional difference between TWD1 and FKBP38 lies in the fact that TWD1 interacts with ABCB1 *via* its N-terminal FKBD domain [62] whereas FKBP38 interaction with CFTR involves not only its FKPB/PPIase domain but also its tetratricopeptide repeat domain [100]. In TWD1, the tetratricopeptide repeat domain is involved in interaction with the vacuolar ABCC1/ABCC2 protein [66] suggesting that TWD1's tetratricopeptide repeat domain is ABCC-specific.

No significant effect of TWD1 on PIN1–4-mediated auxin transport activity was detected in heterologous auxin-transport systems (Laurent and Geisler, unpublished). However, BRET data gained after transient co-expression in tobacco leaves suggests a weak physical interaction between TWD1 and PIN1 [8]. Functional interaction between the proteins is supported by the fact that the *pin-formed* phenotype of *pin1* and the twisting phenotype of *twd1* are partially rescued in a *pin1 twd1* double mutant (Friml and Geisler, unpublished). Because the PIN1 and PIN2 localizations were shown to be unchanged in *twd1* [43] it is possible that TWD1 does not alter PIN trafficking but rather PIN transport activities; this possibility needs further experimental verification.

In contrast to Arabidopsis TWD1, which shows a strong functional interaction with ABCBs and only potential weak interaction with PINs, tomato DGT was shown to exhibit strong functional effect on PINs and insignificant effect on ABCBs [10]. This model is based on the finding that DGT modulates the auxin efflux activities of Arabidopsis PIN1 and PIN2 when the proteins are co-expressed in a heterologous *S. cerevisiae* auxin-transport system (see Fig. 2, Ref. [10]). Co-expression with PIN1 in *Nicotiana benthamiana* leaves results in DGT-dependent relocation of plasma membrane-localized PIN1 to the cellular interior whereas co-expression with ABCB1 does not affect the ABCB1 localization [10]. However, both PIN1 and ABCB1 increase DGT's presence on the plasma membrane suggesting certain interaction of DGT with both types of transporters [10]. PIN2 localization on the plasma membrane is also modified in root epidermis cells of the *dgt* loss-of-function mutant, consistent with a function for DGT in regulation of PIN sorting [10]. Notably, while TWD1 appears to increase the functionality of ABCBs and their presence on the plasma membrane (Fig. 2), [9,64], DGT seems to decrease the functionality of PINs and their presence of on the plasma membrane [10]. Thus, it appears that FKBPs and cyclophilins, belonging to different subgroups of the immunophilin protein superfamily, serve as regulators ("servants") of functionally distinct, primary or secondary energized, transporters of auxin ("masters") (Box 2), respectively. It would be exciting to further explore the differences and similarities between functional TWD1 and DGT exchanges with auxin transporters, as this would certainly uncover novel aspects of auxin transport regulation.

## 5. Imunophilins might mediate auxin transporter sensitivity to NPA

Auxin transport inhibitors, such as the synthetic non-competitive auxin efflux inhibitor NPA (1-*N*-naphthylphtalamic acid) and plant endogenous flavonoids (initially identified by their ability to replace NPA in plasma membrane binding [12]) inhibit auxin efflux through some poorly understood mechanisms. At low micromolar concentrations, auxin transport inhibitors are thought to impair directly the activity of auxin transporters [37,41,101], whereas at higher concentrations (>50 μM) auxin transport inhibitors seem to interfere with the bundling status of the actin cytoskeleton, thus affecting the presence of auxin exporters on the plasma membrane in this manner [7,102–105]. These two modes of actions for NPA on auxin transport, which could be even interconnected [7], might be represented by two distinct NPA binding affinities, as reported for zucchini plasma membrane and speculated to correspond to the auxin transporter itself and an additional NPA-binding regulatory subunit [106]. While the number of NPA binding sites in the plant cell is currently highly debated [107], there is general agreement that NPA binding is peripheral to the plasma membrane and associated with the actin cytoskeleton [108–110].

The protein targets of NPA most closely associated with NPA inhibition of auxin transport have not been elucidated completely. ABCBs-type auxin transporters and TWD1 are valid candidates because both have been demonstrated to bind NPA. However, while the connection of NPA binding to ABCBs and inhibition of auxin transport appears straightforward, the importance of NPA binding to TWD1 is more difficult to validate [37,41,62,111,112]. Root gravitropic response, an auxin-transport regulated process, is NPA-insensitive in the *twd1* root [44] supporting the idea that TWD1 and NPA act in a common pathway. Micromolar NPA was shown to block the auxin-transport activity of ABCB1 [6] and to partially restore the root epidermal twisting of the *abcb1-100 abcb19-3* mutant (speculated to result in part from a defect in auxin transport) suggesting that NPA interferes with auxin transport by directly impairing ABCB functionality [8,9]. Because root gravitropic response [44] and epidermal twisting of the *twd1* mutant are also partially rescued by NPA [8,9], NPA binds to the putative FKBD domain of TWD1 involved in interaction with ABCBs [44], and micromolar concentrations of NPA disrupt the ABCB1–TWD1 interaction [44], it is possible that NPA inhibits auxin transport in part by interfering with TWD1 regulation of ABCBs.

Interestingly, flavonols were even more effective in disrupting the ABCB1–TWD1 interaction, with quercetin being the most successful [44]. This is of interest because flavonols, such as quercetin and kaemferol, have been shown to inhibit plant [6,26,43] and even mammalian ABCB activities [113], and are considered to be plant endogenous auxin transport modulators or inhibitors [101,114,115].

A model for inhibition of PINs by NPA has been also suggested, which was initially based on the finding that Arabidopsis wild type plants grown on NPA (or its functional analog, 2-[4-(diethylamino)-2-hydroxybenzoyl benzoic acid, called BUM [37]) form pin-formed inflorescences phenocopying *pin*1 mutations [37,116,117]. One

could speculate, that independent interactions of NPA with both ABCBs and PINs could in fact account for the two NPA-binding affinities found in zucchini plasma membrane [106]. However, conclusive data are lacking demonstrating NPA binding or direct inhibition of PIN transport proteins in auxin-transport systems [37,39,41]. Therefore, it seems likely that the NPA action on PIN-dependent auxin transport requires additional regulators that are still to be identified [39,103].

ABCBs appear valid candidates as mediators of NPA effect on PIN-driven auxin transport. For example, co-expression of ABCB and PIN combinations in HeLa cells not only increases the auxin substrate specificity of PINs but also confers NPA sensitivity [19], indicating that the NPA sensitivity of PIN-mediated transport relays on PIN interactions with ABCBs. NPA sensitivity of PINs could be also mediated by NPA binding to FKBP42/TWD1, provided that PINs, ABCBs and FKBP42/TWD1 participate in common auxin-transport complexes (see Section 2), or that TWD1 interacts with PINs independently of ABCBs (discussed in Section 4).

At higher concentrations, NPA could also affect the functionality of auxin transporters by interfering with the actin cytoskeleton and this way with transporter cycling at the plasma membrane. Higher NPA concentrations are thought to block protein trafficking [105,118,119] and could affect auxin transports by interfering with their cycling at the plasma membrane. NPA indeed interferes with PIN1 trafficking after disruption by the lactone antibiotic, brefeldin A [105], as well as in the *tir3* mutant lacking the scaffolding protein, BIG [119]. It was also shown in an earlier study that the number of NPA-binding sites and polar auxin transport are reduced in the *tir3*/*big* mutant [109], collectively supporting a connection between NPA binding to the cytoskeleton, PIN trafficking, and auxin transport. On the other hand, evidence suggests that the interference of auxin-transport inhibitors with the actin status might be indirect. Notably, synthetic auxin-transport inhibitors, TIBA and PBA (and to a lesser degree NPA), were shown to alter actin bundling and vesicle trafficking in plant and non-plant cells, such as yeast and mammalian cell lines [104]. The same study contributed *in vitro* experiments indicating that auxin transport inhibitors do not seem to act on actin filament bundling directly but to require a so far uncharacterized binding protein integrating their remodeling action on the actin skeleton [104] (reviewed in Zhu and Geisler [7]). Auxin binding protein 1 (ABP1) and TWD1 are valid candidates for this integrating function as both proteins bind auxin transport inhibitors as well as control auxin transporter locations and thus auxin transport [7,8,44,120]. However, an effect for TWD1 on actin filament organization and dynamics has not yet been shown. In contrast, a recent report questioned the role of ABP1 in auxin signaling, auxin-mediated actin organization and plant development [121] although this study does obviously not affect previous biochemical proof of NPA binding to ABP1 [122].

The question of whether cyclophilins could also be part of the mechanism that mediates the NPA inhibition of auxin transport has not been thoroughly investigated. Although the root of the tomato *dgt* mutant is extremely resistant to NPA in terms of NPA inhibition of root growth, the binding of NPA to *dgt* root microsomal membranes, NPA-sensitive auxin uptake by *dgt* root segments and NPA uptake into *dgt* root segments were found to be normal [123]. By contrast, in a study that utilized a highly sensitive auxin-specific microelectrode to measure auxin transport, auxin transport at the *dgt* root tip was halved compared to the wild type and was completely NPA-insensitive [10]. Further evidence is required, particularly whether DGT could confer NPA sensitivity to PIN-driven auxin efflux, and whether DGT binds NPA, in order to determine whether DGT mediates NPA inhibition of auxin transport.

## 6. Outlook

The analysis of auxin transport processes has made substantial progress in the last 20 years but has left us with a flurry of urgent questions. Currently, the functional significance of having both primary and secondary transporters for auxin is unclear, and we do need further mechanistic detail on their individual energization, interaction, and regulation. In particular, PIN and AUX1/LAX proteins share little similarity with transporters from non-plant systems, which prevents their structural modeling based on transporters for which the crystal structures have been solved. For none of the three major transporter subclasses structural data are available, and only for ABCBs structural models have been obtained [38,47]. Moreover, the mechanisms conferring remarkable specificity for auxin in all three types are at present unknown.

Recently, the role of immunophilins in regulation of auxin transport has been highlighted [124]. However, open questions remain, as to whether immunophilins regulate auxin transporter refolding after their trafficking through the endomembrane system in order to ensure correct assembly of functional protein on the plasma membrane, or rather regulate transporter activity at the plasma membrane in response to cellular demand. The mechanisms by which immunophilins affect auxin transporters function are other important questions. Does TWD1 have PPIase activity, or does TWD1 rather act as a protein chaperone on ABCB function? Are both activities present in TWD1, and if so, are these activities connected? Although DGT possesses PPIase activity, it is unknown whether the PPIase activity is involved in regulation of PIN transporter function. Furthermore, it is unclear whether DGT and PINs engage in direct physical interactions in analogy to TWD1 and ABCBs and where these interactions take place (see Fig. 2). Answering these urgent questions will allow us to judge whether transporter refolding is another mechanistic toolset for the regulation of transporter activity and thus plant function and development.

The interactions of high-molecular weight immunophilins with other proteins, such a calmodulin or heat-shock proteins, point to connections with other regulatory pathways. However, it is not clear whether documented calmodulin or HPS90 interactions with FKBPs (such as TWD1) or cyclophilins (such as SQN/Cyp40) are involved in regulation of auxin transport or rather represent unrelated functional relationships. While an involvement of calcium in the regulation of auxin transport has been documented [125], the role of heat–shock proteins is entirely unclear.

As pointed out in Section 5, imunophilins of the cyclophilin and FKBP subclasses might mediate auxin transporter sensitivity to auxin transport inhibitors, which has been documented in the case of NPA and TWD1 [44]. However, our concepts of individual NPA/flavonoids binding affinities as well as mechanistic insights of their action have been obsolete. In this context, it would be interesting to determine whether effects of NPA on actin bundling observed at higher NPA concentrations are indeed coordinated by FKBPs, such as TWD1. The function of flavonols in auxin transport and their effect on imunophilin activities would be also worth dissecting. For example, in analogy to NPA flavonols were shown to bind to and to inhibit ABCBs [6,44,126] and to disrupt the TWD1–ABCB1 interaction but not to bind to TWD1 [44], revealing functional differences that need to be explored. Whether DGT is required for NPA inhibition of PIN driven auxin transport also needs investigation, as this is a question related to unknown aspects of PIN transporter regulation. Addressing these questions should provide a greater insight into the mechanistic interplay between auxin transporters and immunophilins and regulatory drugs in regulating plant development.

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