PIN proteins perform a rate-limiting function in cellular auxin efflux

One-sentence summary: PIN proteins are directly involved in efflux of phytohormone auxin from cells, and provide the process with specificity, saturability and rate limit.

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

Auxin, a regulatory compound, plays a major role in the spatial and temporal co-ordination of plant development (*1-3*). The directional active cell-to-cell transport controls asymmetric auxin distribution, which underlies multiple patterning and differential growth processes (*4-7*). Genetic approaches in *Arabidopsis thaliana* identified candidate genes coding for regulators of auxin transport, among them permease-like AUX1 (*8*), plant-

specific PIN proteins (*9* and fig. S1) and homologues of human multiple drug resistance/P-glycoproteins PGP1 and PGP19 (*10, 11*). PGP1 have been shown to mediate efflux of auxin from *Arabidopsis* protoplasts and heterologous systems such as yeast and HeLa cells (*12*). Similarly, PIN2 in yeast conferred decreased retention of structural auxin analogues (*13, 14*). Plants defective in PIN function show altered auxin distribution and diverse developmental defects, all of which can be phenocopied by chemical inhibition of auxin efflux (*1*, *4-7*, *9*). All results demonstrate that PINs are essential components of the auxin transport machinery, but the exact mechanism of their action remains unclear.

Studies of the molecular function of PINs have been hampered mainly by the technical inability to quantitatively assess auxin flow across PM in a multicellular system. We therefore established *Arabidopsis* cell suspension culture from the *XVE-PIN1* line, where we placed the *PIN1* sequence under control of the estradiol-inducible promoter (*15*). Treatment with estradiol led to the activation of *PIN1* expression as shown by the co-expressed GFP reporter and RT-PCR of *PIN1* in seedlings (Fig. 1A) and cultured cells (fig. S2). In estradiol-treated *XVE-PIN1* cells, the overexpressed PIN1 was localized at PM (Fig. 1B, C). The synthetic auxin naphthalene-1-acetic acid (NAA) enters cells easily by diffusion, is a poor substrate for active uptake but an excellent substrate for active efflux (*16*). Therefore, change in accumulation of radioactively labelled NAA inside cells provides a measure of the rate of auxin efflux from cells. Untreated *XVE-PIN1* cells as well as non-transformed cells displayed [^{3}H]NAA accumulation kinetics indicative of saturable auxin efflux, and sensitive to a well-established (*1, 9*), non-competitive inhibitor of auxin efflux, 1-naphthylphthalamic acid (NPA) (Fig. 1D). Estradiol did not influence control cells

but led to substantial decrease of [^{3}H]NAA accumulation in *XVE-PIN1* cells (Fig. 1D, E). This demonstrates that PIN1 overexpression leads to the stimulation of efflux of auxin from *Arabidopsis* cultured cells.

Since the use of not enough friable *Arabidopsis* cultured cells for transport assays is limited, we have used tobacco BY-2 cells, a well established model for quantitative studies of cellular auxin transport (*17*). PIN7, the most representative member of the *PIN1,2,3,4,6,7* subfamily (fig. S1), was placed under the control of dexamethasone (DEX)-inducible system (*18*), and stably transformed into BY-2 cells. The resulting line (*GVG-PIN7*) showed upregulation of *PIN7* expression as early as 2 hours after DEX treatment and the upregulated PIN7 protein was detected at the PM (Fig. 2A). Non-transformed cells displayed saturable, NPA-sensitive [^{3}H]NAA efflux, which was unaffected by DEX (Fig. 2B). Induction of expression of PIN7 or its close (PIN4) and the most distant (PIN6) homologues (fig. S1) resulted in a decrease in [^{3}H]NAA accumulation, to roughly half levels (Fig. 2C). The kinetics of NAA efflux after the initial loading of BY-2 cells (Fig. 2D), as well as displacement curves using competitive inhibition by non-labelled NAA (fig. S3A), clearly confirm that PIN7 overexpression stimulates saturable efflux of auxin from cells. The efflux of other auxins, such as synthetic 2,4-dichlorophenoxyacetic acid (2,4-D) or naturally occurring indole-3-acetic acid (IAA), but not its precursor tryptophan, was also stimulated (Fig. 2E, G). The PIN7-dependent efflux of all auxins was NPA-sensitive (Fig. 2G), competitively inhibited by non-labelled NAA, and unaffected by the structurally-related, but biologically inactive weak organic acid, benzoic acid (BeA) (fig. S3B). Furthermore, the increasing levels of induced PIN7, as achieved using different

concentrations of DEX for induction, and monitored by dot blot, clearly correlated with the gradual increase in [^{3}H]NAA efflux (Fig. 2F). In summary, these data imply that different PIN proteins are rate-limiting factors in NPA-sensitive, saturable efflux of auxins from BY-2 cells. This similarity in the molecular function along with the diversity in the regulation of different PINs provides a basis for the complex functional redundancy observed *in planta* (*6, 19, 20*).

The evidence from cultured cells shows that PIN proteins are key rate-limiting factors in cellular auxin efflux. This approach, however, cannot distinguish whether PINs play a catalytic role in auxin efflux or act as positive regulators of endogenous plant auxin efflux catalysts. To address this issue, we used a non-plant system - human HeLa cells, which contain neither PIN-related genes nor auxin-related machinery and allow efficient heterologous expression of functional eukaryotic PM proteins (*21*). We transfected HeLa cells with *PIN7* and its more distant homologue *PIN2*. Transfected cells showed strong PIN expression (Fig. 3A), which resulted in a substantial stimulation of net efflux of natural auxin [^{3}H]IAA, compared to empty vector controls (t-test: P<0,001) (Fig. 3B). Efflux of [^{3}H]BeA was also stimulated but to a lesser extent. These data show that PIN proteins are capable of stimulating cellular auxin efflux in the heterologous HeLa cell system, albeit with decreased substrate specificity.

To test the role of PIN proteins in another evolutionary distant non-plant system, we used yeast (*S. cerevisiae*). PIN2 and PIN7 were expressed in yeast and showed localization at PM (Fig. 3A and not shown). Kinetics of relative [^{3}H]IAA retention demonstrated that expression of PIN2 led to a substantial increase in IAA efflux (Fig. 3C). Efflux assays in

conjunction with control experiments, including testing metabolically less active yeast in the stationary phase, or after glucose starvation (Fig. 3D), confirmed an active PIN-dependent export of IAA and - to a lesser extent - of BeA from yeast (Fig. 3C, and fig. S4B). To test the requirements of the subcellular localization for PIN2 action in yeast, we performed a mutagenesis of the PIN2 sequence to isolate mistargeted mutants. One of the mutations, which changed Serine97 to glycine (pin2Gly97), led to the localization of pin2Gly97 in intracellular compartments (Fig. 3A). When tested in the [^{3}H]IAA efflux assay (fig. S4A), pin2Gly97 failed to mediate auxin efflux but rather increased [^{3}H]IAA accumulation inside cells (Fig. 3D). This shows that pin2Gly97 is still functional but fails to mediate auxin efflux, suggesting importance of PIN localization at PM. Overall, the results suggest that in yeast as well, PM-localized PIN proteins mediate, although with decreased specificity, a saturable efflux of auxin.

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

To compare the roles of PINs and PGPs in auxin efflux, we constructed *GVG-PGP19:HA* cell line of BY-2. DEX treatment led to the upregulation of PGP19:HA protein, which was detected at PM (Fig. 4A), and to a decrease in [^{3}H]NAA accumulation, similar

to that observed in *GVG-PIN4,6,7* lines (Fig. 4B, cf. Fig. 2C). BeA did not interfere with [^{3}H]NAA accumulation and [^{3}H]Trp accumulation did not change after DEX treatment (data not shown). However, compared with PIN-mediated auxin efflux, the PGP19-mediated NAA efflux was significantly less sensitive to NPA. While PIN-mediated transport was completely inhibited by NPA, around 20% of PGP19-dependent transport was NPA-insensitive (Fig. 4C).

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

Rate-limiting, saturable, and specific action of PIN proteins in mediating auxin movement across PM out of plant cells largely clarifies a role of PIN proteins in intercellular auxin transport. Furthermore, the polar, subcellular PIN localisation provides a vectorial component to the directional auxin flow (*22*). Therefore, transport function of

PINs together with their asymmetric subcellular localisation defines directional local auxin distribution underlying different developmental processes.

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24. Materials and methods are available as supporting material in Science Online.

Figure legends

Figure 1. PIN1-dependent auxin efflux in *Arabidopsis* cultured cells.

(A) Upregulation of PIN1 expression in *XVE-PIN1 Arabidopsis* seedlings following estradiol (EST, 1 μM, 4 hours) treatment. The expression of coupled GFP reporter, RT-PCR of *PIN1* (PGP19 expression used as a control). (B, C) anti-PIN1 immunostaining (red) at the PM of *XVE-PIN1* cultured cells following EST (1 μM, 24 hours) treatment (C), no signal in the untreated control (B). Nuclear counterstain in blue. Scale bars 10 μm. (D) Auxin accumulation in *Arabidopsis* wild type cells. NPA (10 μM) increased [^{3}H]NAA accumulation inside cells demonstrating inhibition of auxin efflux. EST treatment (1 μM, 24 hours) had no effect on [^{3}H]NAA accumulation. (E) [^{3}H]NAA accumulation kinetics in *XVE-PIN1* cells demonstrating PIN1-dependent stimulation of NAA efflux following PIN1 overexpression. Error bars represent SEs of the mean (n = 4); where invisible, they fall on the symbols.

Figure 2. PIN-dependent auxin efflux in BY-2 tobacco cultured cells.

(A) Inducible PIN7 expression in *GVG-PIN7* tobacco cells. PIN7 immunostaining (green) at the PM after DEX (24 h; 1 μM) treatment but not in the untreated control, RT-PCR of *PIN7* within 24 hours of DEX (1 μM) treatment. Nuclear counterstain in blue. Scale bars 40 μm. (B) Auxin accumulation in BY-2 control cells. NPA (10 μM) increased [^{3}H]NAA accumulation inside cells demonstrating inhibition of auxin efflux. DEX treatment (1 μM, 24 hours) had no effect on [^{3}H]NAA accumulation. (C) [^{3}H]NAA accumulation kinetics in

GVG-PIN4, 6, 7 cells demonstrating PIN4, 6, 7-dependent stimulation of NAA efflux. Non-induced control shown only for PIN7; those for PIN4 and PIN6 were within the range +/- 8 % of the values for PIN7. Data expressed as % of non-induced control at 30 min after application of labeled [^{3}H]NAA. (D) Induced *GVG-PIN7* cells showed decreased retention of [^{3}H]NAA compared to non-induced control. (E) Accumulation kinetics in induced *GVG-PIN7* cells revealed PIN7-dependent stimulation of [^{3}H]IAA and [^{3}H]2,4-D efflux. (F) Treatments with increasing concentrations of DEX led to gradually higher levels of PIN7 in *GVG-PIN7* cells as determined by dot blot (upper part), and to concomitant decrease of [^{3}H]NAA accumulation. (G) NPA inhibition of both endogenous and PIN7-dependent efflux of [^{3}H]NAA, [^{3}H]IAA and [^{3}H]2,4-D. PIN7 overexpression or NPA treatment did not affect accumulation of related compound, [^{3}H]Trp. Non-induced cells (open bars), induced cells (grey bars). For all experiments, error bars represent SEs (n=4); where invisible, they fall on the symbols.

Figure 3. PIN-dependent auxin efflux in mammalian and yeast cells.
(A) PIN2:HA and PIN7:HA expression in HeLa and yeast: anti-HA immunostaining detected PIN2:HA and PIN7:HA at the PM of transfected but not control (empty vector) HeLa cells (upper panel). Anti-PIN2 immunostaining detected PIN2 at the PM and pin2Gly97 in intracellular compartments, compared to empty vector controls (lower panel). (B) Transfected HeLa cells display PIN2- and PIN7-dependent net efflux of [^{3}H]IAA and to smaller extent also of [^{3}H]BeA. (C) The kinetics of [^{3}H]IAA efflux: PIN2 stimulated saturable [^{3}H]IAA efflux in yeast JK93da strain. (D) [^{3}H]IAA retention measured 10 min

after loading: PIN2 and PIN7 mediated [^{3}H]IAA efflux, pin2Gly97 failed to mediate efflux but increased [^{3}H]IAA retention. Yeast in stationary phase or without glucose showed much less [^{3}H]IAA efflux.

Figure 4. Requirement of PGP function for PIN role in auxin efflux.
(A) Inducible PGP19 expression in *GVG-PGP19:HA* tobacco cells. PGP19:HA immunostaining (red) at PM after DEX treatment (24 h, 1 uM) but not in the untreated control; anti-HA immunoblot after 24 hours of DEX (1 μM) treatment. Nuclear counterstain in blue. Scale bars 40 μm. (B) [^{3}H]NAA accumulation decreased upon PGP19 expression revealing function in auxin efflux in BY-2 cells (C) Different sensitivities of PIN7- and PGP19-dependent [^{3}H]NAA efflux to NPA treatment (10μM, 20 min) in *GVG-PIN7* and *GVG-PGP19:HA* cells[24]. (D) Root gravitropism in *XVE-PIN1* seedlings: PIN1 overexpression (4 hours, 4 μM EST) led to gravitopic defects in *pgp1/pgp19* mutants in contrast to gravitropic growth of EST-treated non transformed wild type and *pgp1/pgp19* seedlings. Root gravitropism scored 12 hours after gravity stimulation (n>40).