

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

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

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

## INTRODUCTION

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

The PIN protein family consists of plant-specific integral plasma membrane proteins that have been identified based on mutants defective in organogenesis (*pin-formed1* or *pin1*) (Okada et al., 1991; Gälweiler et al., 1998) and tropism (*pin2/agr1/eir1*) (Luschnig et al., 1998). The *Arabidopsis* genome encodes eight PIN-related sequences, most of which have been already characterized at cellular and developmental levels (reviewed by Vieten et al., 2007; Zažímalová et al., 2007). PIN proteins are expressed in different parts of the plant and are almost universally required for all aspects of auxin-related plant development, including embryogenesis (Friml et al., 2003), organogenesis (Benková et al., 2003; Reinhardt et al., 2003), root meristem patterning and activity (Friml et al., 2002a; Blilou et al., 2005), tissue differentiation and regeneration (Scarpella et al., 2006; Xu et al., 2006; Sauer et al., 2006a), and tropisms

(Luschnig et al., 1998; Friml et al., 2002b). Most phenotypic aberrations in *pin* loss-of-function alleles can be phenocopied by external application of auxin efflux inhibitors, such as 1-naphthylphthalamic acid (NPA) (Tanaka et al., 2006). When expressed in plant and non-plant cultured cells, PIN proteins perform a rate-limiting function in cellular auxin efflux (Petrášek et al., 2006). Importantly, PIN proteins show distinct polar subcellular localization that determines auxin flux direction, as predicted by classical models of directional auxin transport (Wiśniewska et al., 2006). The dynamic regulation of the intracellular movement of PINs, their polar targeting and their protein stability provides a means to regulate directional throughput of auxin flow (Friml et al., 2004; Paciorek et al., 2005; Abas et al., 2006; Michniewicz et al., 2007). Moreover, the PIN-dependent auxin distribution network involves redundancy and auxin-mediated crossregulation of PIN expression and PIN targeting (Sauer et al., 2006a; Blilou et al., 2005; Vieten et al., 2005). A crucial role for PIN-dependent auxin efflux in generation of morphogenetic asymmetric auxin distribution has recently been suggested by mathematical modelling (Grieneisen et al., 2007).

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

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

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

## MATERIALS AND METHODS

### Plant material and DNA constructs

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

### Growth conditions

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

### BY-2 cell lines

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

## Immunotechniques and microscopy

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

### Phenotype analyses

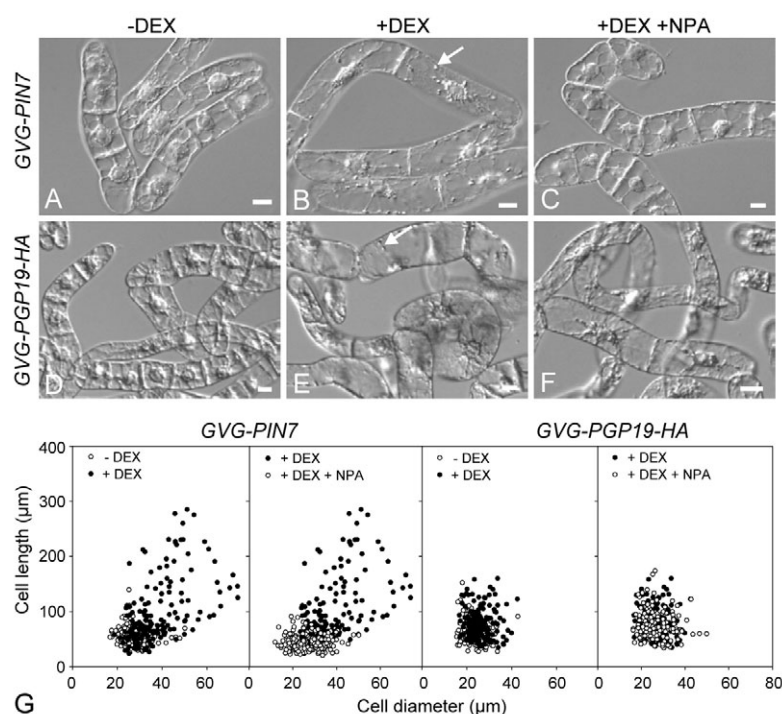
Plates with grown seedlings (5 or 10 days old) were scanned on a flatbed scanner and measured with ImageJ software (<http://rsb.info.nih.gov/ij/>). The vertical growth index (VGI) was calculated as described (Grabov et al., 2005). The hypocotyl twisting index was determined as the relation between hypocotyl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

## RESULTS

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

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

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



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

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

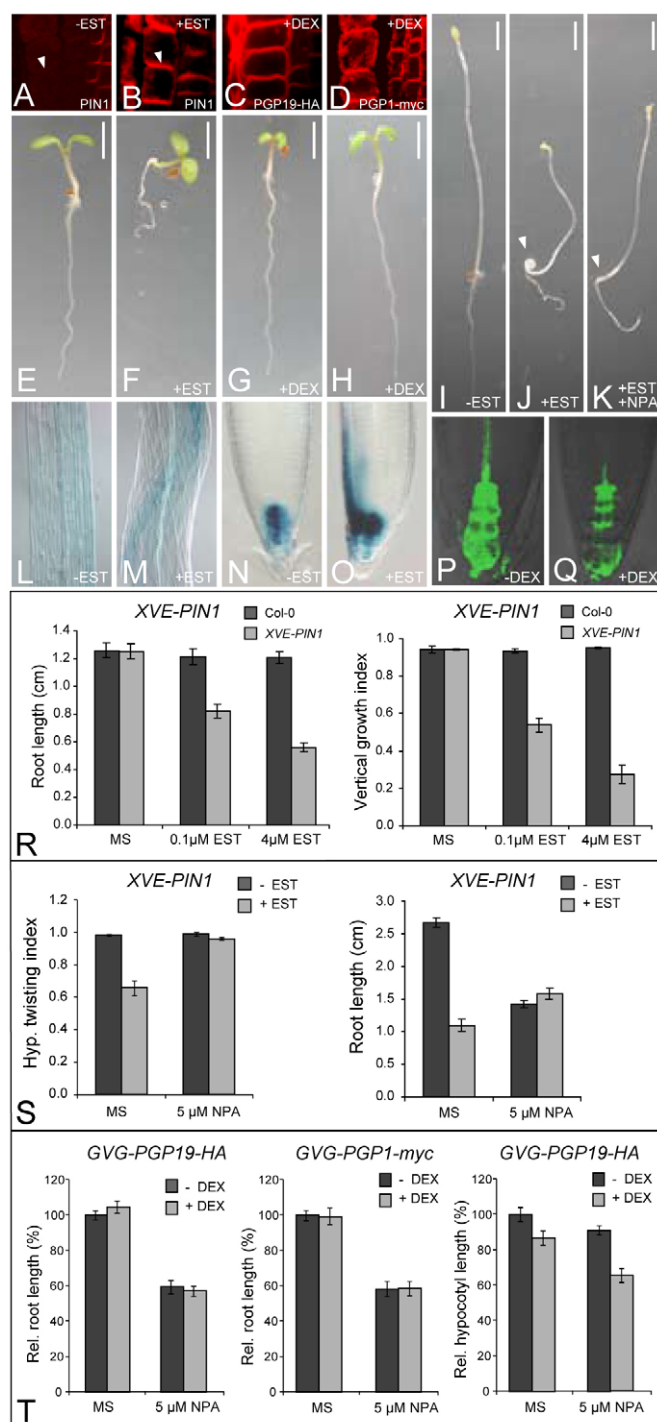
To study what effects overexpression of PINs and PGPs may have at the multicellular level, we analysed transgenic lines overexpressing PIN1, PGP1 or PGP19. After induction of PIN1 expression in the estradiol-inducible *XVE-PIN1* line (Petráček et al., 2006) (Fig. 2A,B), seedlings lost the gravitropic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

shown) and in other PIN1-overexpressing lines, such as DEX-inducible *GVG-PIN1* and constitutive *35S::PIN1* (data not shown). This confirmed that the phenotypic changes and changes in patterns of DR5 activity observed are due to the overexpression of PIN1 protein.

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

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





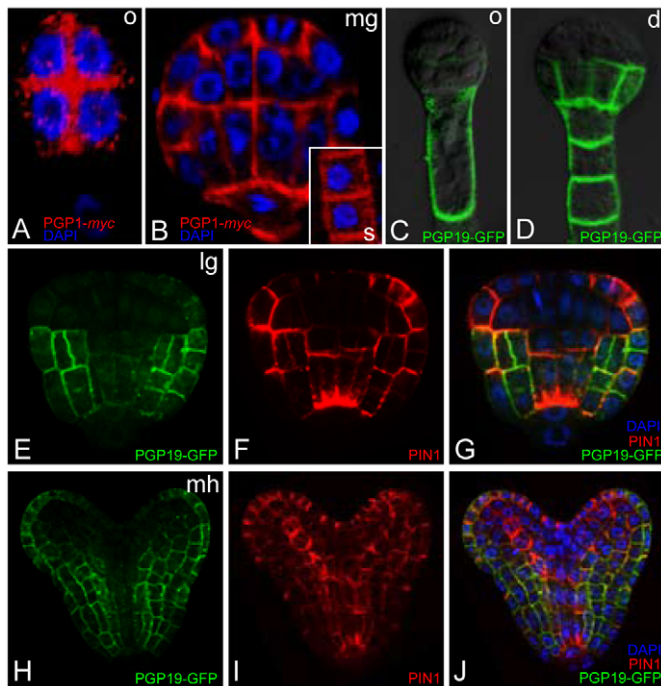
### PGP proteins are expressed and synergistically interact with PIN1 protein during embryogenesis

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

First, we studied the involvement of PIN- and PGP-dependent transport during *Arabidopsis* embryogenesis, which is known to be regulated by PIN-dependent asymmetric auxin distribution (Friml

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

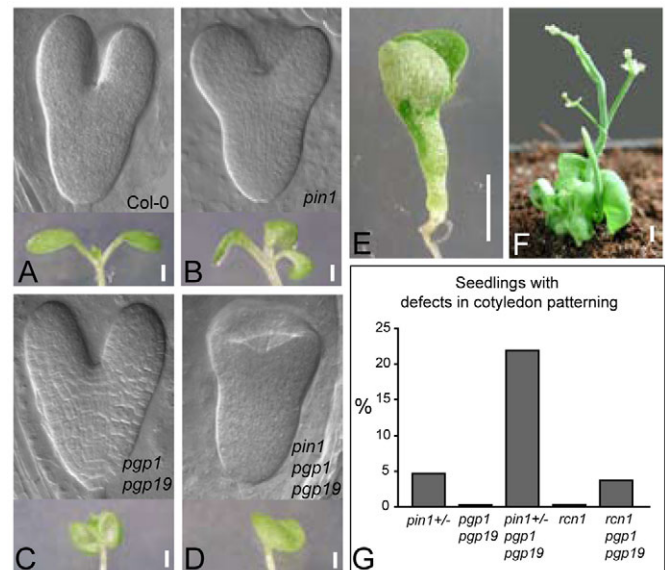
et al., 2003; Weijers et al., 2005). Various PIN proteins are expressed at different stages of embryogenesis in distinct cells, and *PIN* gene mutants (single and double), as well as mutants with defective PIN localization, are defective in embryo patterning (Steinmann et al., 1999; Friml et al., 2003;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



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

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

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

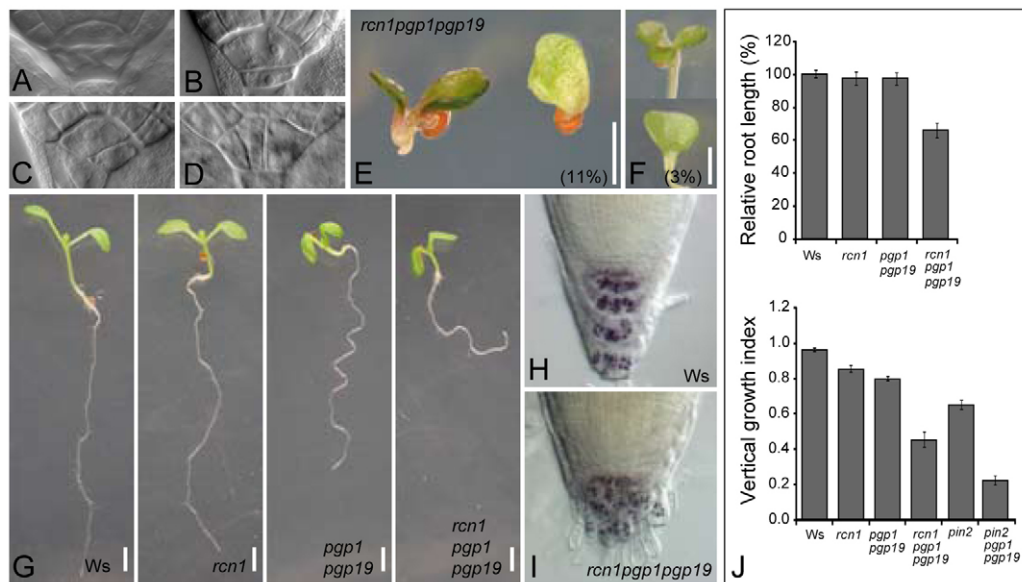


**Fig. 4. Genetic interaction of PGPs with PIN1 during embryonic leaf formation.** (A-D) Synergistic interaction of *pgp1pgp19* and *pin1* during cotyledon formation. Typical defects in cotyledon formation during embryogenesis and their postgermination appearance are shown: wild type (A), *pin1* (B),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

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

### PGP genetically interacts with RCN1 in embryogenesis and root development

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



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

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

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

### Diverse functions of PGP1 and PGP19 in lateral root organogenesis

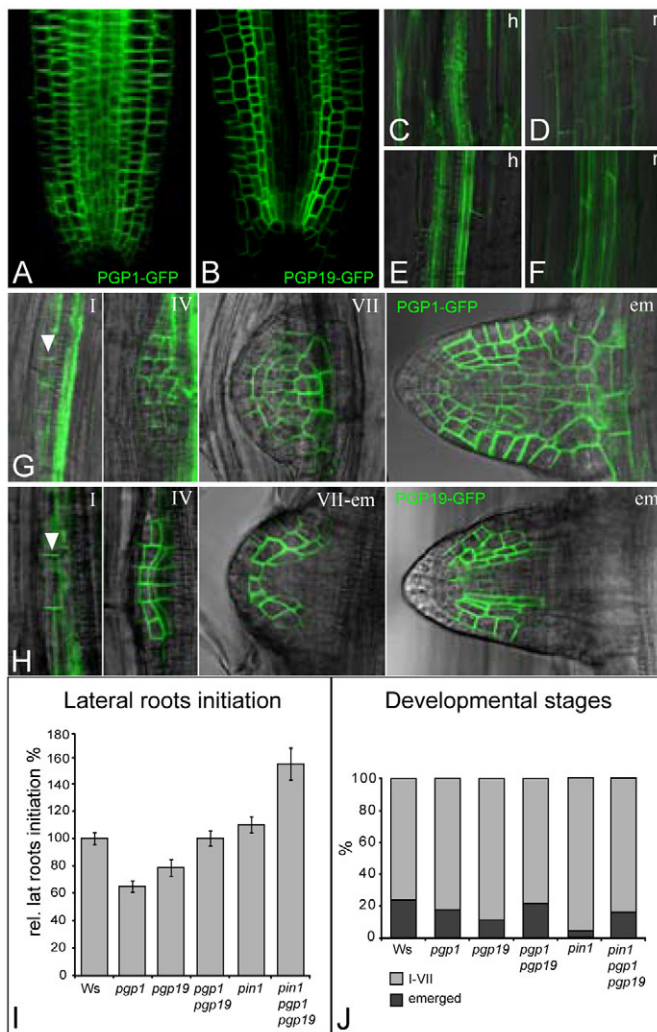
Next, we studied the role of PGP-dependent auxin efflux and its possible interaction with PIN-dependent mechanisms in lateral root initiation and emergence, other processes that involve PIN-dependent auxin transport. Pharmacological or genetic modulation of local transport-dependent auxin distribution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

We determined expression and localization patterns of PGP1 and PGP19 during early post-embryonic development by using *pPGP1::PGP1-GFP* and *pPGP19::PGP19-GFP* constructs. *pPGP1::PGP1-GFP* and *pPGP19::PGP19-GFP* complemented most aspects of the corresponding *pgp1* and *pgp19* mutant phenotypes, such as hypocotyl elongation defect (see Fig. S3A–E in the supplementary material). Similarly to expression during embryogenesis, PGP1-GFP did not exhibit tissue-specific

expression pattern and was detected in all cells of hypocotyls and roots (Fig. 6A,C,D), except root-tip columella cells. The expression of PGP19-GFP was also found in hypocotyls and main roots, but, in contrast to PGP1-GFP, exhibited a more tissue-specific pattern, with strongest expression in endodermal and pericycle tissues (Fig. 6B,E,F), in agreement with published data on PGP19-HA (Blakeslee et al., 2007) and MDR1(PGP19)-GFP (Wu et al., 2007). In addition, PGP19-GFP expression was detected also in root tip epidermal cells, which is not supported by the published pattern of MDR1(PGP19)-GFP (Wu et al., 2007). Both proteins are also expressed during all stages of lateral root development and persisted after lateral root emergence as shown previously (Geisler et al., 2005; Wu et al., 2007). PGP1-GFP expression was observed from the first stage of lateral root primordium organogenesis on (Fig. 6G). During developmental stage I, PGP1-GFP was localized to anticlinal membranes of short initials, and, later, when lateral root primordia are formed, apolar membrane localization was detected in all cells of primordia. Expression and membrane localization of PGP19-GFP at developmental stage I fully overlapped with PGP1, but at later developmental stages PGP19-GFP expression was more restricted to endodermis and pericycle. In emerged lateral roots, PGP19-GFP was detected also in cortical and epidermal cells, similar to its expression in primary root tips (Fig. 6H).

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

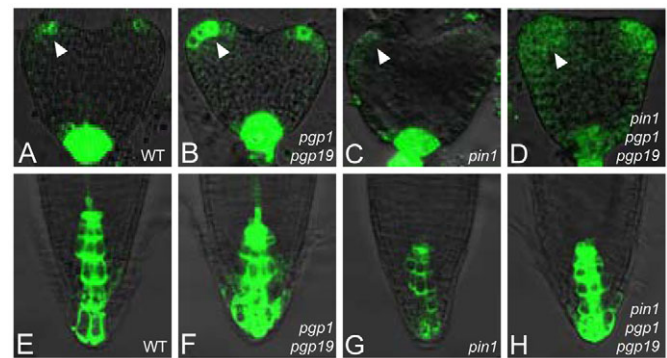




**Fig. 6. Post-embryonic expression and the role of PGP1/PGP19 in lateral root development.** (A,B) Expression and localization of PGP1-GFP and PGP19-GFP in root tips of 5-day-old seedlings. PGP1-GFP is expressed in all cells, except the columella (A); PGP19-GFP expression is more restricted to endodermal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

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

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



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

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

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

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

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

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

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

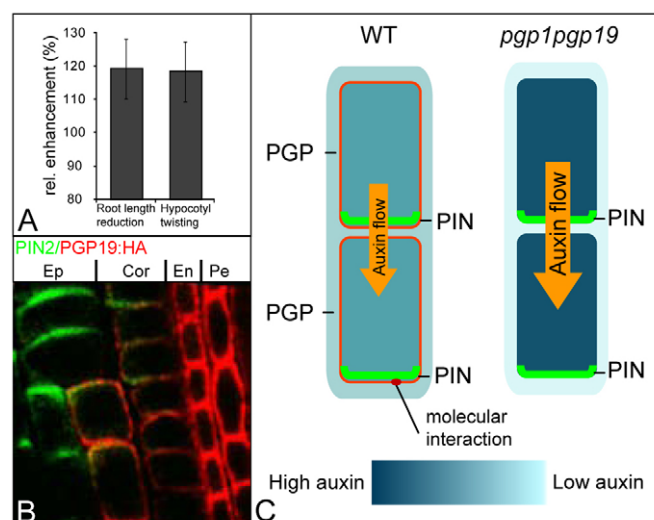
## DISCUSSION

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

### PGPs and PINs define two distinct auxin efflux systems

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

However, the identity of the molecular mechanism that underlies the effect of auxin efflux inhibitors such as NPA still has to be clarified. Previous reports have clearly shown that NPA binds PGP



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

proteins (Noh et al., 2001; Murphy et al., 2002; Rojas-Pierce et al., 2007), that PGP-mediated auxin efflux activity is inhibited by NPA in heterologous systems (Petrášek et al., 2006; Blakeslee et al., 2007) and that NPA inhibits PGP19 action in phototropism (Nagashima et al., 2008). It is possible that NPA and other auxin efflux inhibitors have multiple binding and regulatory sites with different affinities. Moreover, auxin efflux inhibitors might have multiple effects, including modification of actin-based subcellular dynamics (Dhonukshe et al., 2008) or of the PGP-PIN interaction (Blakeslee et al., 2007).

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

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



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

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

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

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

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

Based on previous and novel findings, we propose a model for the functional interaction between PGP- and PIN-dependent auxin transport mechanisms in embryos and root meristems (Fig. 8C). We

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

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

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#### Supplementary material

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

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