# Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression

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## Summary

Plant development displays an exceptional plasticity and adaptability that involves the dynamic, asymmetric distribution of the phytohormone auxin. Polar auxin flow, which requires polarly localized transport facilitators of the PIN family, largely contributes to the establishment and maintenance of the auxin gradients. Functionally overlapping action of PIN proteins mediates multiple developmental processes, including embryo formation, organ development and tropisms. Here we show that PIN proteins exhibit synergistic interactions, which involve cross-regulation of PIN gene expression in *pin* mutants or plants with inhibited auxin transport. Auxin itself positively feeds back on PIN gene expression in a tissue-specific manner through an AUX/IAA-dependent signalling pathway. This regulatory switch is indicative of a mechanism by which the loss of a specific PIN protein is compensated for by auxin-dependent ectopic expression of its homologues. The compensatory properties of the PIN-dependent transport network might enable the stabilization of auxin gradients and potentially contribute to the robustness of plant adaptive development.

Key words: *Arabidopsis*, Auxin transport, PINs, Functional redundancy

## Introduction

Plants cannot respond to the environment by a motile behavioural response but have evolved a highly flexible and adaptive developmental programme instead. In postembryonic development, meristems, which contain stem cell populations, provide new cells for growth at both ends of the main body axis. New structures, such as flowers, leaves and lateral shoot and root branches form de novo and connect to existing body structures by newly differentiated vascular strands. Both shoots and roots modify their growth direction in response to external signals such as light and gravity (Jürgens, 2003). Differential distribution of the plant hormone auxin within tissues and organs underlies adaptation processes, including the regulation of root meristem activity (Sabatini et al., 1999), organogenesis (Benková et al., 2003) and vascular tissue differentiation (Mattson et al., 2003), as well as tropic growth (Friml et al., 2002b). These dynamic auxin gradients result from an active, directional (polar) auxin transport between cells, which requires differentially expressed auxin transport facilitators of the PIN family. The direction of the auxin flow is believed to be determined by the asymmetric cellular localization of PIN proteins (Friml, 2003). Rigorous proof for the function of PIN proteins as auxin transporters is still lacking, but numerous circumstantial evidences demonstrate that multiple PIN proteins play a central role in auxin transport (Friml and Palme, 2002). Despite the proposed uniform function of PIN proteins in auxin transport, genetic analysis implicates different PINs in various, seemingly unrelated, developmental processes (Friml, 2003). In *Arabidopsis*, PIN1 mediates organogenesis and vascular tissue differentiation (Gälweiler et al., 1998; Benková et al., 2003; Reinhardt et al., 2003), PIN2 root gravitropic growth (Müller et al., 1998), PIN3 shoot differential growth (Friml et al., 2002b), PIN4 root meristem activity (Friml et al., 2002a), and PIN7 early embryo development (Friml et al., 2003b). Strong, embryo lethal phenotypes of *pin1,3,4,7* quadruple mutants, which contrast with much weaker and often not fully penetrant defects in most of the single *pin* mutants, suggest a functional redundancy within the PIN gene family (Friml et al., 2003b). Moreover, recent analysis of various combinations of *pin* mutants revealed ectopic expression of PIN proteins in some mutant combinations (Blilou et al., 2005), but the underlying mechanism and biological importance of this effect is unclear. The PIN-dependent auxin distribution system displays an extensive plasticity at the subcellular level. It represents an entry point for both environmental (such as gravity) as well as developmental signals, which can modulate the polarity of PIN localization and hence the direction of auxin flow (Friml et al., 2002b; Benková et al., 2003; Friml, 2003). It remains unclear how the multiple environmental and developmental

signals are integrated and can result in a stabilized modulations of the PIN-dependent auxin distribution network, which are required to initiate and perpetuate a particular adaptation response.

Here we identify and describe synergistic interactions within the auxin transport network, which correlate with specific ectopic expression and proper polar targeting of PIN proteins in certain cells. This phenomenon involves feedback between auxin distribution and PIN gene expression as well as PIN protein stability. The identified complex regulations provide a mechanistic basis for compensatory properties of a functionally redundant auxin distribution network.

## Materials and methods

### Used materials

The *PIN1,2,3,4,7::GUS*, *pin4-3*, *pin3-2*, *pin3-3*, *pin7-1*, *pin7-2*, *pin1,7*, *pin1,3,4,7*, *PIN1::PIN1:GFP* (Benková et al., 2003), *pin1,2*, *pin1,7*, *PIN7::PIN7:GFP* (Blilou et al., 2005), *PIN7:GUS* (Friml et al., 2003b), *PIN2::PIN2:GFP* (Xu et al., 2005), *HS::axr3-1* (Knox at al., 2003), *pin1* (Okada et al., 1991), *slr-1* (Fukaki et al., 2002), *agr1* (Chen et al., 1998) and *eir1-1* (Luschnig et al., 1998) have been described previously. *PIN4::PIN4:GFP* was generated by insertion of mGFP into the PIN4 coding sequence (nucleotides 1032 to 1035 from ATG). The *PIN2::PIN2:HA* construct was generated by fusion of the *PIN2* promoter (1302 bp) and the *PIN2* (AF086906) cDNA with the nine-amino-acid HA epitope tag at the C-terminus in the kanamycin version of the pS001 plasmid (Reiss et al., 1996).

### Growth conditions and drug treatments

*Arabidopsis* seedlings were grown in a 16 hours light/8 hours dark cycle at 18-25°C on 0.5 × MS with sucrose. Short-time exogenous drug application was performed by incubation of 4-5-day-old seedlings in liquid 0.5 × MS with or without 1% sucrose supplemented with indole-3-acetic acid (IAA); 2,4-Dichlorophenoxyacetic acid (2,4-D); N-1-naphthylphthalamic acid (NPA) or 1-naphthalene acetic acid (NAA) for 24 hours. Long-time treatment was done by growing seedlings for 4 days on 0.5 × MS with 1% sucrose and NAA. The sirtinol treatment was done by growing the seedlings for 5 days on 0.5 × MS with 1% sucrose plus 20 μmol/l Sirtinol.

### Quantitative RT-PCR and Northern blot analyses

RNA was extracted using the RNeasy kit (QIAGEN) from root samples. Poly(dT) cDNA was prepared out of 1 μg total RNA using Superscript III Reverse Transcriptase (Invitrogen, Belgium) as recommended by Invitrogen. Quantifications were performed on a Bio-Rad Icycler apparatus with the qPCR Core Kit for SYBR green I (Eurogentec) upon recommendations of the manufacturer. PCR was carried out in 96-well optical reaction plates heated for 10 minutes to 95°C to activate hot start Taq DNA polymerase, followed by 40 cycles of denaturation for 60 seconds at 95°C and annealing-extension for 60 seconds at 58°C. Target quantifications were performed with specific primer pairs designed using Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA). Expression levels were normalized to *ACTIN2* expression levels. All RT-PCR experiments were at least performed in triplicates and the presented values represent means. The statistical significance was evaluated by *t*-test. Northern analysis of *PIN2* expression was performed with Col-O seedlings (6 DAG) grown in liquid 0.5 × MS under continuous illumination. Prior to the experiment, seedlings were transferred into the dark for 16 hours. NAA (10 μmol/l) was added and samples were harvested at indicated time points. Total RNA (10 μg) was loaded per lane. As a loading control, *UBQ5* was used. For the quantification of PIN2::GUS activity, the GUS activity was determined as described (Sieberer et al., 2000). *PIN2::GUS* seedlings (6 DAG) were pre-adapted in the dark for 16 hours, treated with 10 μmol/l NAA and subsequently processed at indicated time points. Protein concentrations were normalized with Bradford reagent (Biorad).

### Expression profiling experiments

Growth conditions were as described (Himanen et al., 2004). For the timecourse experiments, plants were grown on 10 μmol/l NPA for 72 hours before they were transferred to 10 μmol/l NAA containing medium. For the RNA preparation only the differentiated segments were used. The root apical meristems were cut off and the shoots were removed by cutting below the root/shoot junction. RNA was isolated using RNeasy kit according to the manufacturer's instructions. A more detailed description of the microarray, including the data evaluation, is given elsewhere (Vanneste et al., 2005).

### In-situ expression and localization analysis

Histochemical staining for GUS activity and immunolocalization were performed as described (Friml et al., 2003a). For PIN2::GUS, stainings were performed with 10-fold lower concentration of the X-GLUC substrate. The following antibodies and dilutions were used: anti-PIN1 (Benková et al., 2003) (1:500), anti-PIN2 (Paciorek et al., 2005) (1:400) and anti-PIN4 (Friml et al., 2002a) (1:200), anti-HA (mouse) (Babco, 1:1000); and FITC (1:200) and CY3-conjugated anti-rabbit (1:500) or anti-mouse (1:500) secondary antibodies (Dianova). For GFP visualization, samples were fixed for 1 hour with 4% paraformaldehyde, mounted in 5% glycerol and inspected. Microscopy was done on a Zeiss Axiophot equipped with an Axiocam HR CCD camera. For confocal laser scanning microscopy, a Leica TCS SP2 was used. Images were processed in Adobe Photoshop and assembled in Adobe Illustrator.

### Phenotype analysis

For embryo phenotype analysis, for each condition and stage, at least 40 embryos were analysed as described (Friml et al., 2003b). Root phenotypes were examined in 4-day-old seedlings. Root length was measured from hypocotyl junction to root apex, and root meristem size from the position in which epidermis cells rapidly elongate to quiescent centre as described (Blilou et al., 2005). Microscopy inspection of roots and embryos was done on a Zeiss Axiophot equipped with an Axiocam HR CCD camera using differential interference contrast optics.

## Results

### PIN functional redundancy in embryo development involves cross-regulation of PIN gene expression

Previous analysis of embryo (Friml et al., 2003b), lateral root (Benková et al., 2003) and primary root (Blilou et al., 2005) development in multiple *pin* mutants demonstrated functionally redundant action of PIN genes. To gain more insights into the mechanism of PIN functional redundancy, we examined root and embryo development in various multiple *pin* mutants in conjunction with PIN localization patterns. In the early embryo, following zygote division, only two PIN members have been detected. PIN1 resides in the apical cell lineage without pronounced polarity and PIN7 at the apical side of suspensor cells. *PIN4* and *PIN3* expression is detected only at the globular and heart stage, respectively, in the root pole region (Friml et al., 2003b) (Fig. 2A). *pin7* mutants were shown to have defects at early embryo stages; however, they recover to a large extent by redundant activity of the remaining embryonically expressed PINs (Friml et al., 2003b). *pin1,7*

Development

Development

early embryo phenotypes did not differ visibly from the *pin7* single mutants (Fig. 1B). Remarkably, a more detailed analysis of defects in multiple *pin* embryos revealed that *pin1,3,4,7* embryos are more severely affected than *pin1,7* or *pin7* embryos also at early, preglobular stages. When compared with *pin1,7* embryos, *pin1,3,4,7* display novel phenotypes with compressed embryos consisting of densely packed, non-elongated cells (Fig. 1A-C) and the frequency of early embryo defects increased (*Col-0*: 5.7%, 10/177; *pin1,7*: 16.1%, 35/218; *pin1,3,4,7*: 28.8%, 61/212). These findings on a redundant role for PIN1,3,4,7 in early embryogenesis could not easily be reconciled with the lack of *PIN3* and *PIN4* expression at this stage and suggested functional cross-regulation between distinct members of the PIN gene family. Indeed, when we examined expression and localization patterns of PIN proteins in *pin7* embryos, *PIN4* was found ectopically expressed in the *PIN7* expression domain as early as the preglobular stage, when *PIN4* is normally not expressed (Fig. 1D-G). Remarkably, ectopically expressed PIN4 protein exhibited the same polar localization as the PIN7 protein that had been replaced. Such a cross-regulation of *PIN4* expression in the *pin7* mutant potentially explains the observed synergistic interactions in early embryo development.

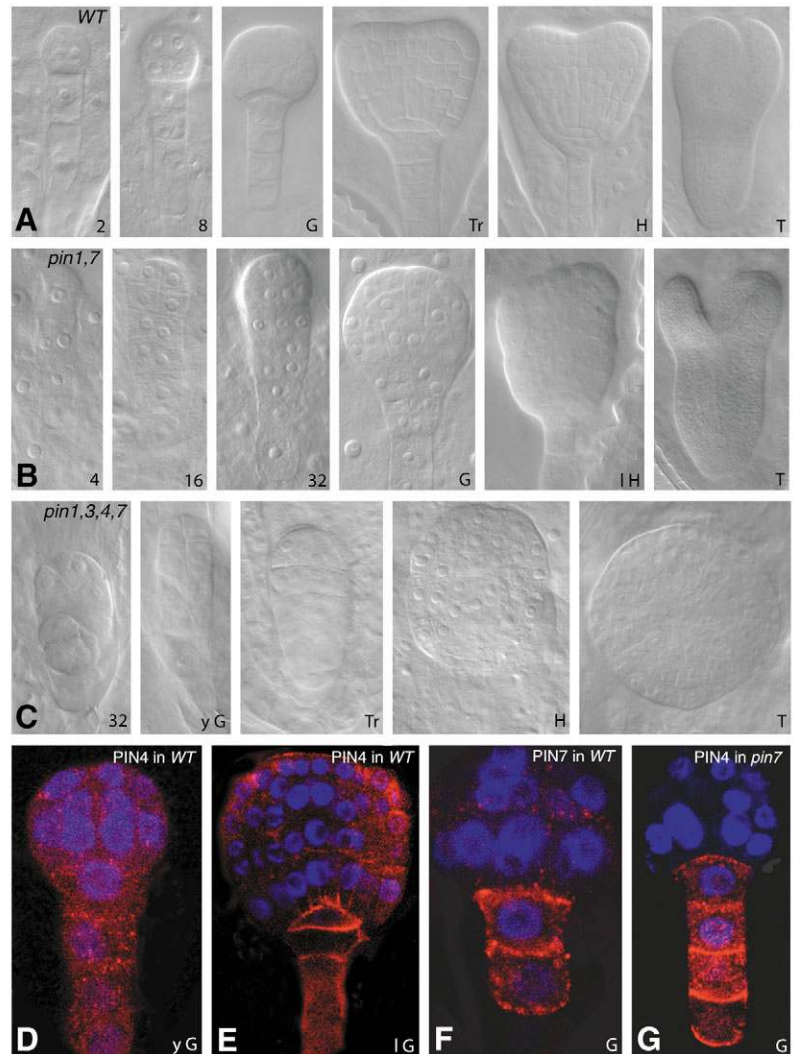


**Fig. 1.** Cross-regulation of PIN expression and function in embryo development. (A-C) Novel embryo phenotypes in *pin1,3,4,7* multiple mutants (C) compared with wild-type (A) and *pin1,7* mutant (B) embryos. (D-G) Immunostaining showing that *PIN4* is ectopically expressed in the suspensor of the *pin7* preglobular embryo (G) in a pattern similar to that of *PIN7* expression in wild type (F). No *PIN4* expression in wild type at this stage (D) and expression restricted to root meristem precursors at the later stages (E). For the embryo stages the numbers indicate the developmental stage according to the actual number of pro-embryo cells of the corresponding wild-type stage. G, globular; H, heart; l, late; T, Torpedo; Tr, triangular; y, young.

## PIN functional redundancy in root development involves cross-regulation of PIN gene expression

In the root meristem, five PIN genes are known to be expressed (Fig. 2A). The *PIN1* expression pattern is somewhat variable, but under our experimental conditions PIN1 could be found predominantly at the basal (lower) side of stele and endodermis cells with occasional weak expression in the quiescent centre and up to the four youngest epidermis and cortex daughter cells (Friml et al., 2002a) (Fig. 2D). *PIN2* is expressed in a non-overlapping pattern in the lateral root cap and older epidermis and cortex cells with apical (upper) polarity in the epidermis and predominantly basal polarity in the cortex (Müller et al., 1998, Friml et al., 2003a) (Fig. 2G). *PIN4* is expressed in the central root meristem with a polar subcellular localization pointing predominantly towards the columella initials (Friml et al., 2002a) (Fig. 2J). By contrast, PIN3 (Friml et al., 2002b) and PIN7 (Blilou et al., 2005) are localized in largely overlapping patterns in columella and stele of the elongation zone. However, with the exception of PIN2, which when mutated causes agravitropic root growth, removal of any of the other PINs causes no, or rather subtle, root phenotypes (Sabatini et al., 1999; Friml et al., 2002a; Friml et al., 2003b). By contrast, *pin1,2* double mutants displayed strong root growth defects reflected in significantly shorter roots and the formation of a smaller root meristem, when compared with either single mutant (Fig. 2B,C) or any other double mutant combination (Blilou et al., 2005). These strong synergistic interactions between PIN1 and PIN2 may indicate a functional cross-regulation similar to that observed with PIN4 and PIN7 in the embryos. Indeed, the analysis of expression and abundance of PIN1 and PIN2 in the respective mutants reveals that *PIN1* became ectopically induced in the *PIN2* expression domain in cortex and epidermis cells of *pin2* (Fig. 2E). Reciprocally, in *pin1* mutants, *PIN2* was ectopically expressed in the endodermis and weakly in the stele (Fig. 2H) along with ectopic upregulation of the *PIN4* expression in the stele (Fig. 2K). Remarkably, ectopically expressed PIN proteins exhibited the polar localization of the PIN protein that had been replaced. PIN2 and PIN4 were basally localized, when upregulated in endodermis or stele of *pin1* (Fig. 2I,L), whereas PIN1 showed apical localization in epidermis and basal localization in cortex

cells when upregulated in roots of the *pin2* mutant (Fig. 2F). These findings demonstrate that the functional redundancy of PIN proteins involves cross-regulation of PIN gene expression and polar targeting in a cell-specific manner, which potentially explains the observed synergistic interactions. Accordingly, ectopic upregulation of PINs, as observed in *pin* mutants, could be sufficient to compensate for the function of missing PIN genes.

### Cross-regulation of PIN gene expression is related to changes in polar auxin transport

In order to investigate how the observed cross-regulation of PIN gene expression might be regulated, we modified auxin distribution by blocking auxin transport with the auxin transport inhibitor NPA. To exclude possible cross-reaction of anti-PIN antibodies with related PIN proteins, we performed the experiments on the *PIN1::PIN1:GFP*, *PIN4::PIN4:GFP* and *PIN2::PIN2:HA* transgenic lines and analysed either GFP or HA-tag expression. In *PIN1::PIN1:GFP* (Benková et al., 2003), the GFP fluorescence was detected in the stele and endodermis cells of primary roots with occasional weak expression in the quiescent centre and a few youngest cortex and epidermis cells (Fig. 3A), corresponding to anti-PIN1 immunolocalization results. Following NPA treatment, ectopic upregulation of PIN1:GFP fluorescence was observed in the epidermis (Fig. 3B). In untreated *PIN4::PIN4:GFP* roots, the expression of PIN4:GFP was restricted to the central root meristem and columella with only a faint signal or no signal in the stele (Fig. 3E), whereas PIN2:HA expression in *PIN2::PIN2:HA* plants was detected only in cortex, epidermis and lateral root cap cells (Fig. 3C). Following NPA treatment, PIN4:GFP as well as PIN2:HA expression became strongly upregulated in the stele (Fig. 3F,D). In *PIN1::GUS*, *PIN2::GUS* and *PIN4::GUS* transgenic plants, a similar ectopic upregulation of GUS activity following NPA treatment could be observed (Fig. 3G,H and not shown), demonstrating an effect on the PIN promoter activity rather than post-transcriptional regulations.

These data demonstrate that the chemical inhibition of auxin transport can modulate PIN gene expression in a way similar to that observed in *pin* mutants, suggesting a link between NPA-sensitive auxin transport and the regulation of PIN gene expression.

### Auxin-dependent signalling controls PIN gene expression in a tissue-specific manner

Next we addressed potential mechanisms

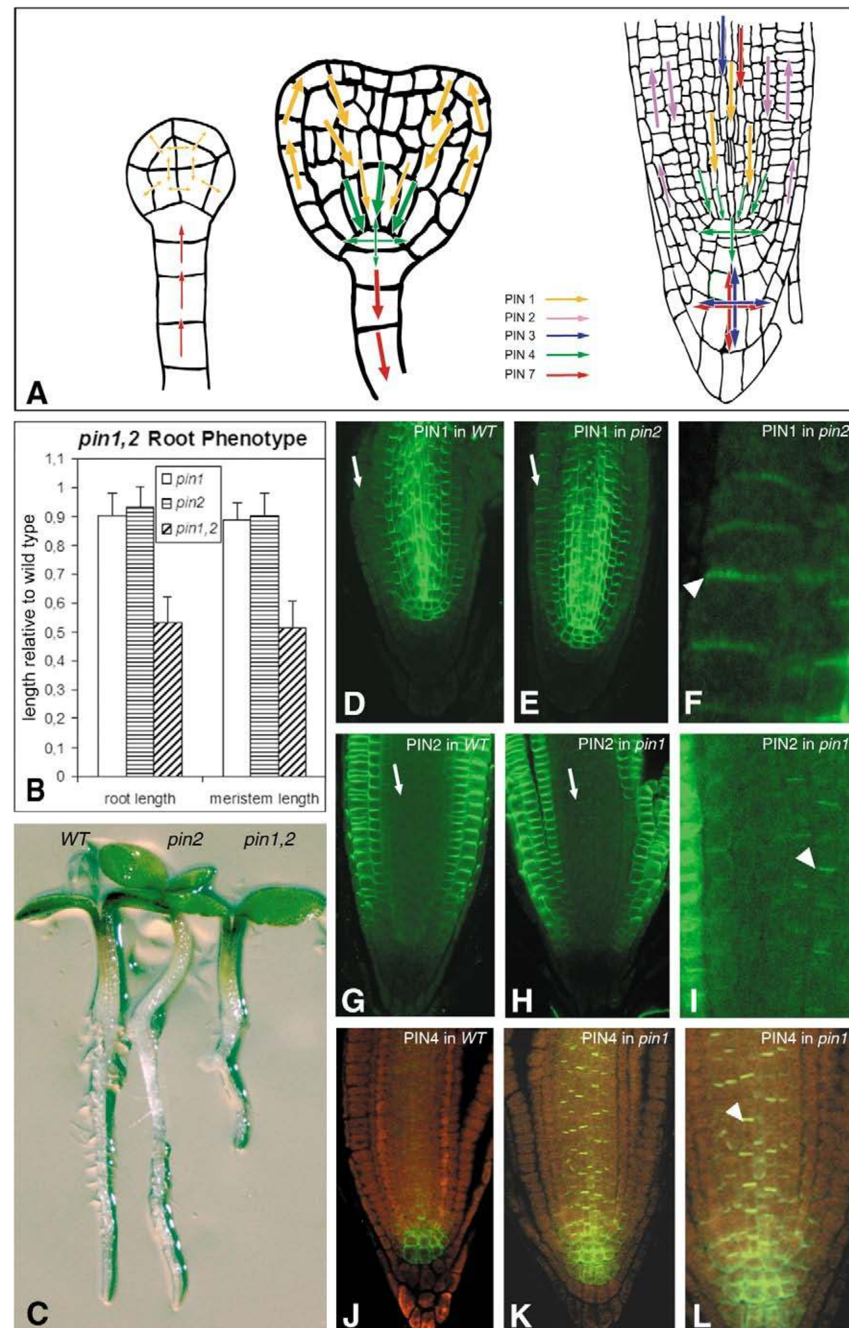


**Fig. 2.** Cross-regulation of PIN gene expression in root. (A) Symbolic depiction of PIN gene expression and polar localization in preglobular, early heart embryos and in seedling root. Arrows indicate presumed directions of auxin flow based on subcellular PIN polarity. (B) Comparison of root and meristem length between *pin1* and *pin2* single mutants and *pin1,2* double mutants. Standard deviations are depicted. (C) Seedling phenotypes of wild type, *pin2* single mutant and *pin1,2* double mutants. (D-L) Immunostaining showing the cross-regulation of PIN expression. PIN1 is upregulated in the epidermis of *pin2* root (E) compared with wild type (D). Detail showing polar PIN1 localization in epidermis of *pin2* root (F). PIN2 is ectopically expressed in the stele of *pin1* root (H) compared with wild type (G). Detail showing polar PIN2 localization in *pin1* stele (I). PIN4 is upregulated in the stele of *pin1* root (K,L) compared with wild type (J). Arrows indicate corresponding expression domains; arrowheads polarity of PIN localization.

Development

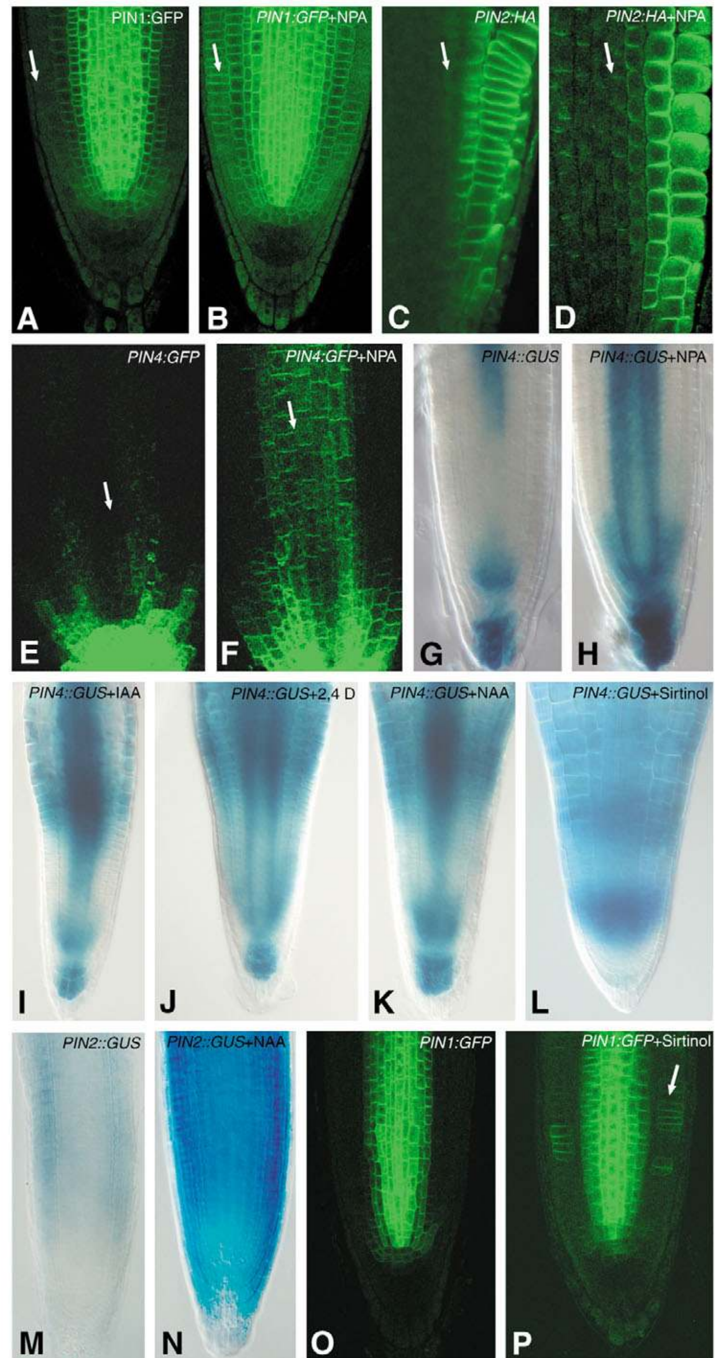


**Fig. 3.** Manipulation of auxin homeostasis leads to ectopic PIN gene expression. (A-H) Inhibition of auxin transport by NPA (50 μmol/l for 24 hours) leads to upregulation of *PIN1::PIN1:GFP* in epidermis and cortex (B); *PIN2::PIN2:HA* (D), *PIN4::PIN4:GFP* (F) and *PIN4::GUS* (H) in the stele compared with untreated controls (A,C,E,G). (I-L) Treatment for 24 hours with biologically active auxins such as IAA (50 μmol/l, I), 2,4-D (0.1 μmol/l, J) and NAA (10 μmol/l, K) or for 5 days with auxin precursor sirtinol (20 μmol/l, L) leads to an upregulation of *PIN4::GUS* expression compared with control (G). (M,N) Upregulation of *PIN2::GUS* expression in root following treatment with 50 μmol/l NAA for 24 hours (N) compared with untreated control (M). (O,P) Treatment for 5 days with auxin precursor sirtinol (20 μmol/l) leads to upregulation of *PIN1::PIN1:GFP* in epidermis and cortex cells (P) compared with control (O).

underlying the observed cross-regulation of PIN gene expression. As NPA treatment and various *pin* mutants change the pattern of auxin distribution in roots and embryos (Luschnig et al., 1998; Sabatini et al., 1999; Friml et al., 2002a; Friml et al., 2003b), we tested whether auxin itself can directly influence PIN gene expression. Treatments with different biologically active auxins such as IAA, NAA and 2,4-D led to an increase in GUS activity in *PIN4::GUS* (Fig. 3I-K) and *PIN2::GUS* (Fig. 3M,N and not shown) roots. Importantly, both NAA and 2,4-D, which differ in their transport properties (Delbarre et al., 1996), induced PIN gene expression in a similar way, indicating that auxin influences PIN gene expression without the need of the active auxin transport. This was further confirmed by analysis of the effects of sirtinol – a compound that is not a substrate of the auxin transport system but is converted to a substance with auxin effects (Zhao et al., 2003; Dai et al., 2005). The effect of sirtinol seemed to be somewhat delayed when compared to auxin effects, but prolonged treatments had the same impact on the induction of PIN gene expression as auxins, as shown, for example, by the upregulation of *PIN4::GUS* and PIN1:GFP (Fig. 3L,P).

To quantitatively assess the effect of auxin on PIN gene expression, we performed a quantitative real-time RT-PCR (Q-RT-PCR) following a treatment with NAA. To address possible differences in the effect of auxin on PIN gene expression in different parts of the seedling, we examined PIN gene expression in cotyledons, hypocotyl and roots separately. The expression of all tested PIN genes (*PIN1,2,3,4,6,7*) clearly responded to auxin treatments but showed prominent differences in different parts of seedlings (Fig. 4A). In cotyledons, the response to auxin was more divergent and varied from strong upregulation of *PIN1*, *PIN7*; to a somewhat weaker response of *PIN6* and to no upregulation of *PIN2*, *PIN3* and *PIN4* expression. In hypocotyls, hardly any effect of auxin on PIN gene expression was detected, whereas in roots, all PIN genes showed a clear increase in expression (up to 22-fold in the case of *PIN7*) following auxin treatment. The apparent downregulation of PIN2 and PIN6 in hypocotyls is not significant and due to the very low expression levels of these genes in this tissue. The auxin effect on PIN gene expression was further confirmed by testing PIN gene promoter activity in GUS transcriptional fusions. Indeed, *PIN1,2,3,4,6,7::GUS* transgenic plants responded to auxin treatment by upregulation of GUS expression in a tissue-specific manner (Fig. 4B-D and data not shown). For example, in cotyledons, the *PIN1::GUS*, but not *PIN3::GUS* or *PIN4::GUS* seedlings showed increased GUS activity following auxin treatment, but neither of the transgenic lines showed any upregulation in hypocotyl (Fig. 4B-D) under the same conditions. However, the GUS activity in *PIN1::GUS*, *PIN3::GUS* and *PIN4::GUS* was clearly increased in roots. Closer examination of the induction pattern (e.g. in *PIN3::GUS* and *PIN4::GUS* roots) confirmed tissue- and cell-specific response to auxin treatment, as in some cells more upregulation occurred than in others (Fig. 4C,D).

In summary, these results show that the expression of *PIN*s is directly or indirectly controlled by auxin in a tissue-specific manner, which provides a plausible mechanism for the observed cross-regulations in PIN functional redundancy.

### The auxin effect on PIN gene expression is time- and concentration-dependent

Analyses of GUS activity in *PIN1::GUS*, *PIN3::GUS*, *PIN4::GUS* and *PIN7::GUS* roots revealed that the auxin effect on the activity of PIN promoters is time- and concentration-dependent (Fig. 5A-D). Staining conditions here were chosen to maximize the dynamic range of staining intensities in order to better resolve the differences in GUS expression levels after auxin treatments rather than to obtain optimal overall staining patterns. Thus, for example, untreated *PIN7::GUS* seedlings, when optimally stained, also showed GUS signal in the stele (Fig. 5D inset), which is in accordance with earlier observations of PIN7 expression (Blilou et al., 2005). Interestingly, independently of the time and concentration of the auxin treatment, the upregulation remained largely confined to the same tissues, further confirming the cell-type-specific effect on PIN gene expression.

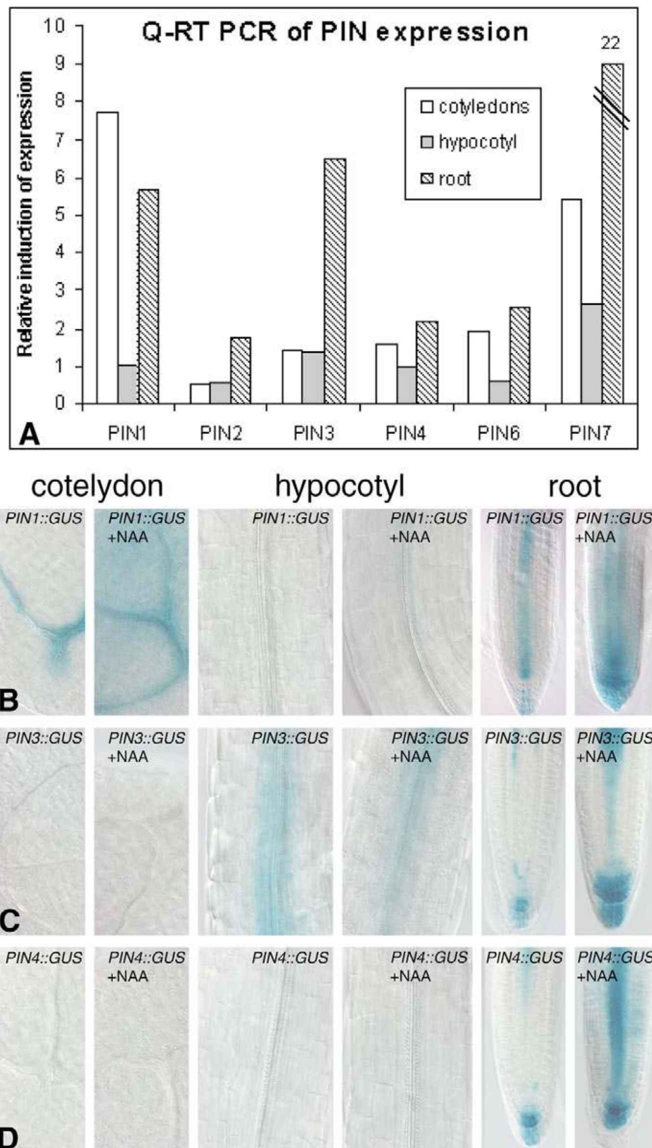


**Fig. 4.** Tissue-specific regulation of PIN gene expression by auxin. (A) Quantitative RT-PCR showing upregulation of PIN gene expression in cotyledons, hypocotyls and roots following auxin treatment (10 μmol/l NAA for 3 hours). Induction of PIN gene expression is depicted relative to the non-induced controls. (B-D) Induction of *PIN1::GUS* (1 μmol/l, B), *PIN3::GUS* (0.5 μmol/l, C) and *PIN4::GUS* (0.5 μmol/l, D) expression in cotyledons, hypocotyls and roots after growing the plants for 4 days on medium containing NAA.

The kinetics and concentration-dependence of the auxin effect on PIN gene expression were evaluated by Q-RT-PCR for *PIN1*, *PIN2*, *PIN3*, *PIN4* and *PIN7* in roots. The experiment revealed that expression of different *PIN*s displays different kinetics in their auxin-dependent responses. Following auxin (2,4-D) application, *PIN1* (*t*-test: $P<0.01$), *PIN3* ($P<0.01$) and *PIN7* ($P<0.005$) showed a significant upregulation by 15 minutes and a steady increase in RNA levels up to 6 hours after induction. By contrast, *PIN2* ($P<0.005$) and *PIN4* ($P<0.005$) showed a delayed reaction with significant upregulation only after 1 hour of 2,4-D incubation and *PIN4* showed a maximal response after 2 hours (Fig. 6A). Also, concerning the maximal effective auxin concentrations that lead to the upregulation of expression, differences between PINs were found. *PIN2* ($P<0.01$) expression was induced by hormone concentrations as low as at 10 nmol/l 2,4-D, whereas for the induction of *PIN1* ($P<0.01$), *PIN3* ($P<0.005$), *PIN4* ($P<0.01$) and *PIN7* ($P<0.005$) expression, concentrations as high as 100 nmol/l 2,4-D were needed to obtain significant changes in expression (Fig. 6B). For the induction of PIN1 and PIN7 expression, the optimal concentration was around 1 μmol/l 2,4-D, whereas for PIN2, PIN3 and PIN4 it was higher than 10 μmol/l. Northern blot analysis and quantitative analysis of GUS activity confirmed the Q-RT-PCR results, as shown, for example, by analysis of *PIN2* expression in response to NAA (Fig. 6E,F). These results show that while expression of PIN proteins is induced by auxins, induction kinetics and effective concentrations exhibit a variability within the PIN gene family.

### Auxin regulates PIN gene expression through an Aux/IAA-dependent pathway

We then addressed the molecular mechanism by which auxin regulates PIN gene expression. Even when the protein synthesis was inhibited by cycloheximide, auxin induced the expression of PIN proteins (not shown), demonstrating that the auxin-dependent PIN upregulation does not require de novo synthesis of any factors and thus *PIN*s are primary response genes. Significantly, a treatment with cycloheximide alone was sufficient to induce expression of *PIN1*, *PIN2*, *PIN3* and *PIN4* to roughly maximum levels (Fig. 6C), implying that PIN gene expression is controlled by an unstable transcriptional repressor. The auxin effect on gene expression is known to involve a rapid, auxin-dependent degradation of the Aux/IAA transcriptional repressors (Gray et al., 2001). Indeed, in the *solitary-root-1* (*slr-1*) mutant, which carries the stabilized version of the IAA14 repressor (Fukaki et al., 2002), auxin-dependent upregulation of PIN gene expression is severely compromised (Fig. 6C), suggesting that auxin utilizes Aux/IAA-dependent signalling to regulate PIN gene expression. In addition, we used transgenic plants harbouring a stabilized version of IAA17 (AXR3) under

the control of a heatshock promoter (*HS::axr3-1*) (Knox et al., 2003). The induction of axr3 expression by 2 hours of 37°C treatment concomitantly abolished the auxin-dependent upregulation of *PIN1*, *PIN2*, *PIN3*, *PIN4* and *PIN7* expression (Fig. 6D), directly linking the regulation of PIN gene expression to the Aux/IAA signal transduction pathway. These conclusions gained additional support from global expression analysis following auxin-dependent induction of lateral root formation (Vanneste et al., 2005). Microarray-based analysis was performed at different time points after auxin application to seedlings that were grown under inhibited auxin transport (by NPA). Only the differentiated part of the primary root was analysed to minimize the influence of different tissue- and developmental-stage-specific factors (Vanneste et al., 2005). Under these conditions, the expression of *PIN1*, *PIN3* and *PIN7* was rapidly and strongly induced by auxin, along with a number of well-known primary auxin response genes as well as *PINOID* and related genes (Fig. 6G). Expression of other PIN genes was also analysed (e.g. PIN genes were also on the ATH1 Affymetrix chip) but were not induced in this experiment (data not shown). Importantly, the observed auxin-dependent induction of PIN gene expression was completely abolished when the expression profiling experiment was performed in the *slr-1* mutant (Fig. 6G). In summary, these experiments demonstrate that tissue-specific PIN gene expression is regulated by auxin through AUX/IAA-dependent signalling.

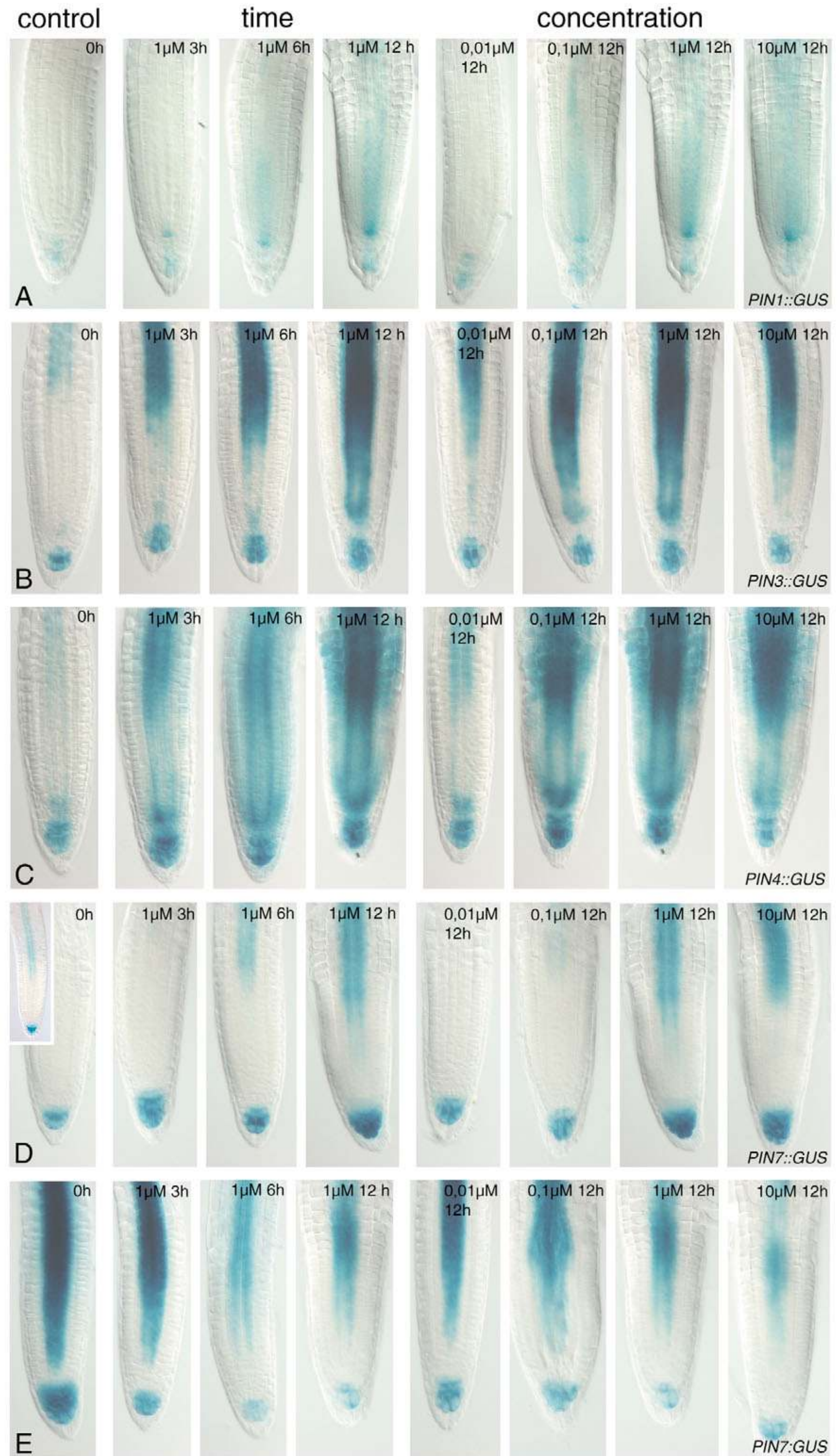


**Fig. 5.** Time- and concentration-dependence of auxin-regulated PIN gene expression. (A-D) Upregulation of *PIN1::GUS* (A), *PIN3::GUS* (B), *PIN4::GUS* (C) and *PIN7::GUS* (D) expression after different times and different concentrations of 2,4-D incubation. Inset (D) shows untreated *PIN7::GUS* roots after prolonged GUS staining. (E) Downregulation of PIN7:GUS fusion protein abundance in *PIN7::PIN7:GUS* seedlings.

## Auxin-dependent post-transcriptional downregulation of PIN proteins

Our results suggest that auxin is able to modulate PIN levels by regulating PIN gene expression in a highly specific way. Additional levels of regulations might occur due to effects on PIN protein stability, as at least PIN2 degradation was shown to be regulated by auxin levels (Sieberer et al., 2000). To address the post-transcriptional effects of auxin on the abundance of PIN proteins, we utilized GUS and GFP translation fusions with PIN1, PIN2, PIN4 and PIN7. Comparisons of the auxin effects on *PIN7::GUS* and *PIN7::PIN7:GUS* transgenic plants clearly showed a time- and concentration-dependent transcriptional upregulation of *PIN7* promoter activity (Fig. 5D), but a downregulation of PIN7:GUS levels (Fig. 5E). Similarly, PIN7:GFP (Fig. 7A) and PIN2:GFP (Fig. 7B) abundance decreased at higher auxin concentrations (higher than 100 nmol/l 2,4-D). However, at lower concentrations, the PIN2 and PIN7 protein amount increased (best at 10 nmol/l), suggesting that both the transcriptional and the post-transcriptional auxin effects on PIN expression overlap. In support of this notion, the transcriptional upregulation of *PIN2* expression in stele, which occurs in

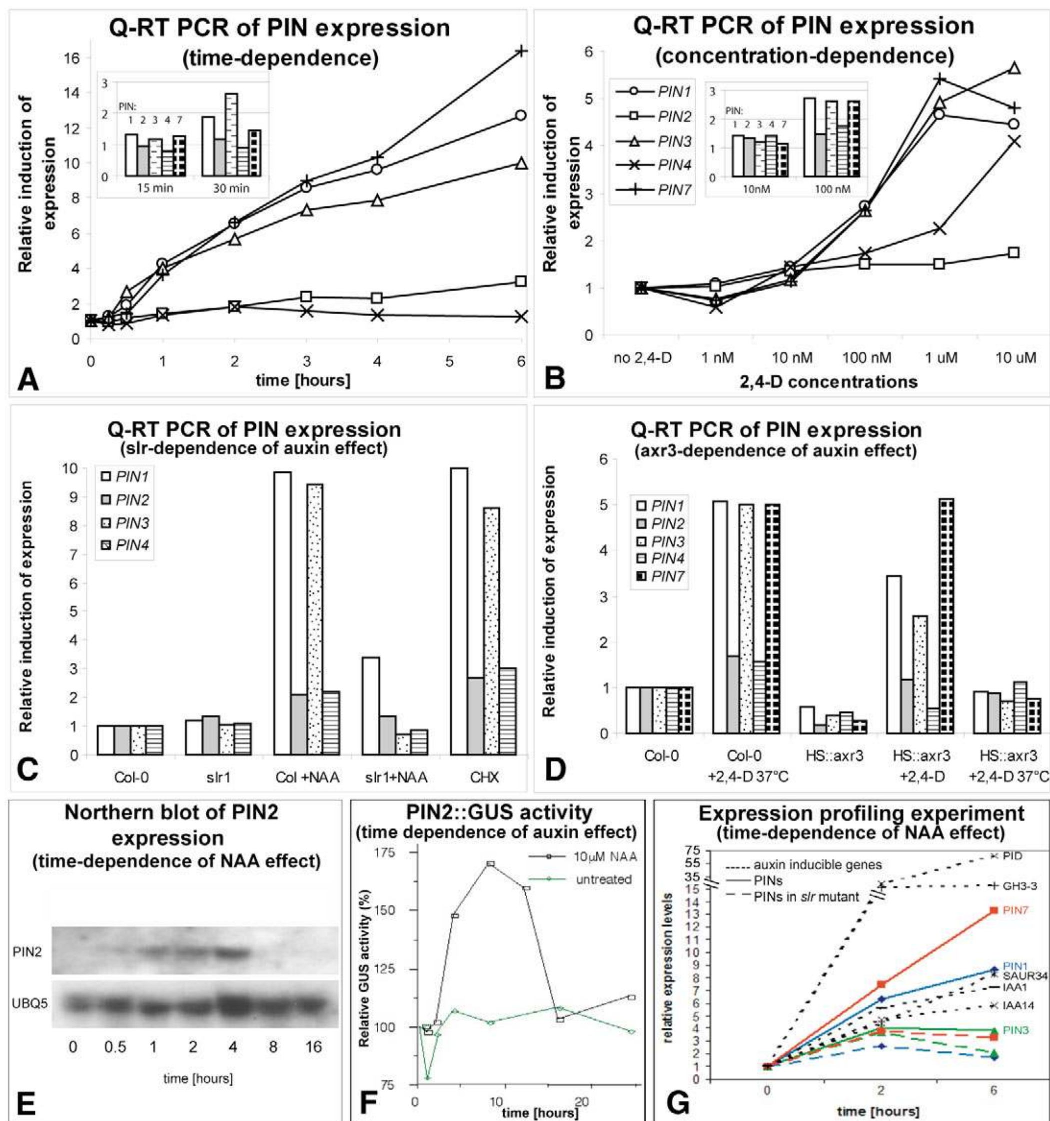


**Fig. 6.** Quantitative evaluation of auxin-regulated PIN gene expression. (A,B) Quantitative RT-PCRs showing time-dependence (A) and concentration-dependence (B) of the effect of auxin (1 μmol/l 2,4-D in A) on PIN expression. Insets show higher magnifications of early time points (A) and low concentrations (B). The legend in B also applies to A. (C,D) Auxin (1 μmol/l 2,4-D) does not induce PIN expression in *slr-1* mutants (C) or after induction of axr3 expression in *HS::axr3-1* lines (D), as shown by quantitative RT-PCR. Cycloheximide alone induces PIN expression (C). (E,F) Northern blot (E) and quantitative GUS assays (F) show the time-dependence of auxin (10 μmol/l NAA) effect on *PIN2* expression. (G) An expression profiling experiment shows auxin-dependent upregulation of PIN genes, *PID* and selected primary auxin response genes in differentiated parts of the root grown on NPA. The auxin-dependent upregulation of PIN gene expression is abolished in the *slr1* mutant. Induction of PIN gene expression is depicted relative to the non-induced controls.

*PIN2::GUS* seedlings following auxin treatment (Fig. 3N), cannot be observed in *PIN2::PIN2:GFP* seedlings (Fig. 7B). In *PIN1::PIN1:GFP* roots, the optimal 2,4-D concentration for the PIN1:GFP upregulation in epidermal cells was 100 nmol/l. At higher concentrations, the PIN1:GFP level decreased also in its stele expression domain (Fig. 7C), albeit to a lesser extent than in the case of PIN2 and PIN7 reporter proteins. However, there was no visible decrease in the PIN4:GFP amount following auxin treatment (not shown). These results show that higher auxin concentrations, besides modulating PIN gene expression, post-transcriptionally downregulate the abundance of specific PIN proteins. This provides an additional level of regulation for modulating of different PIN protein amounts in different cells.

## Discussion

### Functionally redundant PIN-dependent auxin distribution as a common mechanism in plant development

The local accumulation of the plant signalling molecule auxin in certain cells (auxin gradients) underlies an unexpected variety of developmental processes. Embryo development, postembryonic formation of various organs, such as lateral roots, leaves, flowers, floral organs and ovules, vascular tissue differentiation, the regulation of root meristem activity and directional growth responses – all these processes are accompanied by, and require, locally elevated auxin activities (e.g. Friml et al., 2003b; Benková et al., 2003; Reinhardt et al., 2003; Mattson et al., 2003; Sabatini et al., 1999). The current model proposes that increased auxin levels in different cells activate a signalling cascade, which via a network of AUX/IAA transcriptional repressors and ARF transcription factors leads to the expression of a specific set of genes and to the activation of a specific developmental programmes (Weijers and Jürgens, 2004). The importance of contributions of tissue-specific auxin synthesis and degradation are not entirely clarified yet (Ljung et al., 2002), but it seems that the major mechanism by which auxin accumulates in given cells is an intercellular, directional flow of auxin. The auxin distribution network is molecularly characterized by polarly localized PIN auxin efflux facilitators (Friml and Palme, 2002). Whether or not different PIN proteins

Development

have the same molecular function is still unclear, as they mediate different developmental processes. However, the synergistic effects of multiple *pin* mutant combinations on embryo and root development demonstrate functional redundancy (Friml et al., 2003; Blilou et al., 2005). Also the findings that different *PIN*s get ectopically expressed in *pin* mutants and thus can at least partially compensate for the function of the missing PIN protein suggest that different PIN proteins are to some extent functionally interchangeable (Blilou et al., 2005). Thus it seems that the molecular function of different PIN proteins in auxin transport is similar, although they obviously differ in the regulation of their expression, as shown by the differential expression pattern as well as by their different responsiveness to increased auxin levels. It is likely that PIN proteins will differ also in the regulation of their proteasome-dependent turnover (Sieberer et al., 2000) and regulation of their subcellular polarity in different cell types. Such properties of a functionally redundant PIN-dependent auxin distribution network would allow for the integration of various signals at different levels, thereby determining local auxin distribution in different parts of plants.

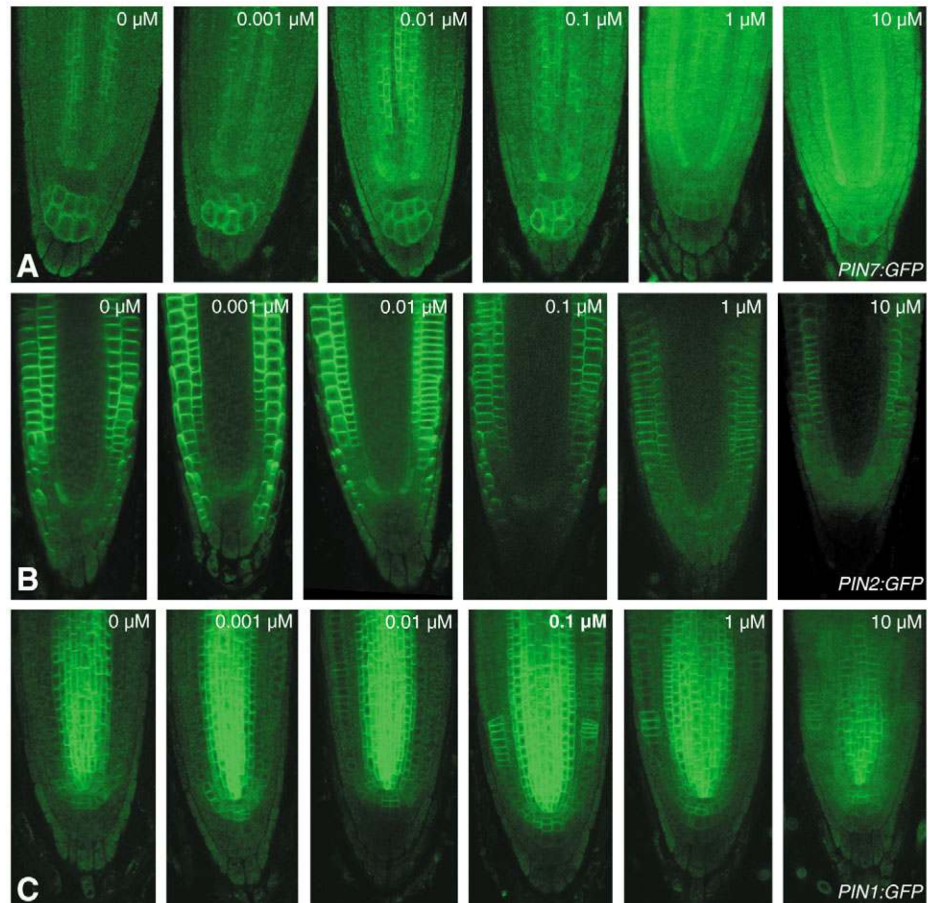


**Fig. 7.** Increased auxin levels lead to a decrease in PIN levels in *PIN::PIN:GFP* roots. (A-C) The PIN7:GFP (A), PIN2:GFP (B) and PIN1:GFP (C) protein levels decrease at higher auxin concentrations. Four-day-old roots were treated with different concentrations of 2,4-D for 24 hours.

## Model for feedback regulations and compensatory properties in auxin distribution

Plant development is characterized by its flexibility and adaptability, which allow the optimal adjustment of plant shape according to the environment. The auxin distribution network is supposed to enable the integration of multiple environmental and developmental signals to allow the flexible changes in auxin accumulation patterns that underlie the adaptive nature of plant development. The regulation of PIN polar targeting, degradation and differential regulation of expression are potential upstream control points for mediating the dynamic auxin gradients. For example, the PIN3 polarity can be rapidly modulated by environmental signals such as gravity, which through asymmetric auxin distribution ultimately leads to gravitropic bending (Friml et al., 2002a). Also, developmental signals can mediate dynamic changes in PIN polarity and thus mediate apical-basal axis specification in embryos (Friml et al., 2003b), trigger specific patterns of organ positioning (Reinhardt et al., 2003) or perpetuate organ primordium development (Benková et al., 2003).

Under conditions of an ever-changing environment and constant stimulation, a dynamic system such as the PIN-dependent auxin transport network requires a mechanism(s), which would at some point stabilize and perpetuate its readjustments. For this purpose, biological systems typically accommodate feedback regulatory loops. Long-standing physiological models, such as the canalization hypothesis, proposed that auxin itself can modulate its own transport and its polarity (Sachs, 1988) and thus mediate regenerative properties of plant development, especially the de novo formation of vascular strands (Sachs, 2000; Berleth and Sachs, 2001). The canalization hypothesis assumes, besides positive feedback on transport activity, a directional polarization of auxin flow relative to the position of the auxin source. Our data show that auxin itself, together with cell-type-specific factors, can positively control PIN transcription, which involves the activity of Aux/IAA transcriptional repressors. The complementary evidence for the influence of auxin distribution on PIN gene expression came from the expression profiling experiments in poplar (Schrader et al., 2003) and from analysis of flavonoid mutants, where both the auxin transport and the distribution of PIN proteins are affected (Peer et al., 2004). However, the effect of auxin on PIN polarity or on polarity of auxin flow has not been demonstrated so far. Our data show that ectopically expressed PIN proteins in various *pin* mutants always adopt the correct polar localization, suggesting a tight cell-type-based control, apparently requiring direct or indirect regulation by auxin. Such a functional link is also provided by

the recent analysis of regulators of PIN polarity, such as the Ser/Thr protein kinases of the PINOID type (Friml et al., 2004). It has been reported previously that *PINOID* is a primary auxin response gene (Benjamins et al., 2001). Also our expression profiling data show that *PINOID* and homologous genes are upregulated along with the PIN genes in the same tissues. It is thus conceivable that auxin mediates changes of cellular PIN polarity via control of *PINOID* expression. In such a scenario, both cellular PIN levels and PIN localization can be influenced by auxin itself. Such feedback regulations may contribute to the compensatory properties of the auxin distribution network. In the simplest model, the defect in auxin flow caused, for example, by a mutation in a specific PIN protein, would lead to auxin accumulation within affected cells. This in turn would lead to the upregulation of expression and polar retargeting of other PIN family member(s), which, in this manner, could functionally compensate. This unique, so far undescribed, type of regulatory redundancy explains observed genetic redundancy and provides a possible mechanism for the stabilization of changes in auxin distribution. The fine interplay between the modulating external signals and the stabilizing internal feedback in the PIN-based auxin transport network might thus contribute to both the flexible and robust nature of plant development.

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