

# The PINOID protein kinase regulates organ development in *Arabidopsis* by enhancing polar auxin transport

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Accepted 25 July 2001

## SUMMARY

*Arabidopsis pinoid* mutants show a strong phenotypic resemblance to the *pin-formed* mutant that is disrupted in polar auxin transport. The *PINOID* gene was recently cloned and found to encode a protein-serine/threonine kinase. Here we show that the *PINOID* gene is inducible by auxin and that the protein kinase is present in the primordia of cotyledons, leaves and floral organs and in vascular tissue in developing organs or proximal to meristems. Overexpression of *PINOID* under the control of the constitutive CaMV 35S promoter (*35S::PID*) resulted in phenotypes also observed in mutants with altered sensitivity to or transport of auxin. A remarkable characteristic of high expressing *35S::PID* seedlings was a frequent collapse of the primary root meristem. This event triggered lateral root formation, a process that was initially

inhibited in these seedlings. Both meristem organisation and growth of the primary root were rescued when seedlings were grown in the presence of polar auxin transport inhibitors, such as naphthylphthalamic acid (NPA). Moreover, ectopic expression of *PINOID* cDNA under control of the epidermis-specific *LTP1* promoter provided further evidence for the NPA-sensitive action of *PINOID*. The results presented here indicate that *PINOID* functions as a positive regulator of polar auxin transport. We propose that *PINOID* is involved in the fine-tuning of polar auxin transport during organ formation in response to local auxin concentrations.

Key words: Auxin, Signalling, Transport, Protein kinase, AtPIN, Efflux carrier, *Arabidopsis thaliana*

## INTRODUCTION

The plant hormone auxin plays a crucial role in development throughout the life cycle of a plant by directing basic developmental processes such as cell division, cell elongation and differentiation. Experiments with radio-labelled derivatives of indole-3-acetic acid (IAA), the predominant naturally occurring auxin, indicated that IAA is transported downward from its main site of synthesis, the shoot tip, to the root. Treatment of seedlings or plant tissues with inhibitors of this polar auxin transport (PAT), such as naphthylphthalamic acid (NPA) or 2,3,5-triiodo-benzoic acid (TIBA), showed that PAT provides directional and positional information for developmental processes such as vascular differentiation, apical dominance, organ development and tropic growth. To describe PAT, a chemiosmotic model was proposed in which auxin enters the cell in its protonated form through diffusion or through the action of a saturable auxin import carrier. Auxin is deprotonated at the higher pH of the cytoplasm and can only exit the cell through active export by auxin efflux carriers (AECs). The specific location of AECs at the basal side of the cell was hypothesised to be the driving force of PAT (Lomax et al., 1995).

Recent studies using molecular genetic approaches in *Arabidopsis thaliana* have shed new light on the molecular mechanisms behind PAT and auxin action. Important new

insights were obtained through molecular characterisation of *Arabidopsis pin-formed* or *pin1* mutants (Okada et al., 1991). These mutants develop a pin-like inflorescence, which is characteristic of wild-type plants grown in the presence of PAT inhibitors. Occasionally, flowers are produced on the inflorescence of *pin1* mutant plants that have less sepals, more petals, no stamens and abnormal carpels. Some of these flowers consist only of carpelloid structures (Okada et al., 1991). Moreover, *pin1* mutant embryos show defects in cotyledon number and position, a phenotype that can be mimicked by culturing plant embryos with PAT inhibitors (Liu et al., 1993). The *AtPIN1* gene was cloned through transposon tagging and appeared to encode a transmembrane protein with similarity to bacterial-type transporters (Gälweiler et al., 1998). This suggested that the AtPIN1 protein represented the elusive AEC. In agreement with its proposed function as an AEC, the AtPIN1 protein was found to be localised to the basal end of xylem parenchyma and cambial cell files in the *Arabidopsis* inflorescence axis (Gälweiler et al., 1998).

*AtPIN1* was found to be part of a multigene family in *Arabidopsis* comprising 8 members (Friml, 2000). Allelic loss-of-function mutants in another member of this gene family, *AtPIN2*, were independently isolated based on root agravitropism or ethylene resistance phenotypes (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998). Immunolocalisation showed that the AtPIN2 protein is

present at the anti- and periclinal sides of cortical and epidermal cells in the root tip (Müller et al., 1998). The distinct expression and cellular localisation of AtPIN1 and AtPIN2 combined with the phenotypes of the respective loss-of-function mutants suggested that the different members of the AtPIN family each direct distinct processes in plant development, which involve PAT.

The recent finding that lateral organs can be induced on *pin1* inflorescences by exogenous application of IAA (Reinhardt et al., 2000) indicated that the IAA content in the *pin1* inflorescence apex is sub-optimal for organ formation. This result suggested that continuous supply of IAA is essential for proper positioning and development of organs from the inflorescence meristem. The expression of *AtPIN1* in floral organ primordia (Christensen et al., 2000), even at very young stages (Vernoux et al., 2000), suggested that polar transport of IAA is required to guarantee this supply.

Two *Arabidopsis* mutants that share several of the phenotypic characteristics of the *pin1* mutant are *monopteros* (*mp*) and *pinoid* (*pid*) (Berleth and Jürgens, 1993; Bennett et al., 1995). *mp* mutants differ from *pin1* and *pid* in that their vascular strands are disconnected and the root meristem is not formed in the embryo. However, like *pin1* and *pinoid*, cotyledon positioning in the *mp* embryo is aberrant and the inflorescence carries few flowers and terminates prematurely (Przemeck et al., 1996). Flowers have fewer outer whorl organs and have abnormal carpels. In one *mp* allele PAT was found to be reduced, possibly through absence of a continuous vascular strand (Przemeck et al., 1996). The *MONOPTEROS* gene encodes ARF5, a protein with homology to the auxin responsive element binding factor ARF1 (Hardtke and Berleth, 1998). MP/ARF5 was found to be a positive regulator of auxin induced gene expression (Ulmasov et al., 1999) and likely has a function in cell axialisation and vascular development during plant development in response to auxin gradients. The phenotype of *pid* mutants closely resembles that of *pin1* mutant plants, but is less severe. A cross between *pid* and the auxin resistant mutant *axr1* suggested that AXR1 and PID have overlapping functions and that PID plays some role in an auxin-related process (Bennett et al., 1995). The *PID* gene was recently cloned and found to encode a protein-serine/threonine kinase (Christensen et al., 2000). Based on the phenotypes induced by constitutive expression of *PID*, Christensen and co-workers (Christensen et al., 2000) concluded that the protein kinase is a negative regulator of auxin signalling.

Here we show that *PID* is an auxin-responsive gene and that the main site of *PID* expression is the vascular tissue in young developing organs. Based on these results, and on detailed analyses of *35S::PID* overexpression phenotypes, we propose that *PID* functions as a positive regulator of polar auxin transport.

## MATERIALS AND METHODS

### Plant growth conditions

Plant transformations were performed by floral dip (Clough and Bent, 1998). *Arabidopsis* seeds were surface sterilised and plated on solid M-A medium (Masson and Paszkowski, 1992), where needed containing 25 mg/l kanamycin, 15 mg/l phosphinothricine or 20 mg/l hygromycin for selection, and germinated at 21°C under a 16-hour

photoperiod with 3000–4000 lux. Two- to three-week-old plants were transferred to soil and grown at 21°C under a 16-hour photoperiod and 60% relative humidity.

### RNA expression analysis

Poly(A) enriched RNA was isolated from root tips using the Quick Prep *Micro* mRNA Purification Kit (Pharmacia). Total RNA isolation and RNA blot analysis were performed as described previously (Memelink et al., 1994) and signal detection was performed by Phosphor-Image analysis (Molecular Dynamics). Whole-mount in situ localisation of *PID* mRNA was performed as described previously (de Almeida Engler, 1998; Friml, 2000), using a 337 bp 5' fragment of the *PID* cDNA and T3- and T7-polymerase (Promega) for digoxigenin-labelled sense and anti-sense probe synthesis, respectively.

### Identification of *En1* transposon-induced *pid* alleles and detection of the *pid-2* allele

The *pid::En197* and *pid::En310* mutants were identified from a collection of *En-1* transposon mutagenized lines by a PCR-based screen using the *En-1* specific primers En205 and En8130 (Wisman et al., 1998) and *PINOID* specific primers PKV (5'-TCCTTTCTCTCAAACCTCACCGATCC-3') and PKIII (5'-CGTAGAGAAACACTCCAAAGGCCAC-3'). The presence of the *pid-2* allele was detected by amplification of the *PID* locus using primers 1D.3 (5'-CATGCATTGACTCTGTTCAC-3') and 1D.4 (5'-TAACATTATCTATCGTTACAGTG-3') and digestion of the PCR product with *DdeI*.

### Bacterial strains, DNA libraries and cloning procedures

General cloning and molecular biology procedures were performed as described previously (Sambrook et al., 1989) using *E. coli* strain DH5 $\alpha$ . For plant transformation, binary vectors were transferred to *Agrobacterium* strain LBA1115 by tri-parental mating (Ditta et al., 1980) or through electroporation (Den Dulk-Ras and Hooykaas, 1995).

The *PID* cDNA was isolated from a cDNA library of auxin-treated root cultures of *Arabidopsis* ecotype C24 (Neuteboom et al., 1999). DNA sequencing was performed by Eurogentec (Belgium).

The fusion between *PID* and *gusA* was created by cloning the *SphI*-*MspAI* genomic fragment containing 3.6 kb of 5' untranslated region and the complete *PID* gene, excluding the last six codons, in-frame with the *gusA* gene in pCAMBIA1381Xb (McElroy et al., 1995). For the sense overexpression constructs, the *PINOID* cDNA was cloned into the expression cassette of pART7, which was subsequently introduced as a *NotI* fragment onto binary vector pART27 (Gleave, 1992).

The pACT and pEF constructs containing the *mGAL4:VP16* gene and *UAS* promoter, respectively, will be described in detail elsewhere (D. W., J. Haseloff, E. van Ryn, P. H. and R. O., unpublished). The *DR5::GUS* reporter was obtained by cloning a synthetic fragment containing 7 copies of the CCTTTTGTCTC sequence (Ulmasov et al., 1997) upstream of the -47 35S promoter and fusing the resulting promoter to the *GFP::GUSA* reporter gene (Quaedvlieg et al., 1998).

### Histochemical staining and microscopy

Starch granule staining was performed as described previously (Sabatini et al., 1999). To detect *gusA* expression, plant tissues were fixed in 90% acetone for 1 hour at -20°C, washed three times in 10 mM EDTA, 50 mM sodium phosphate (pH 7.0), 2 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, and subsequently stained for up to 16 hours in 10 mM EDTA, 50 mM sodium phosphate (pH 7.0), 1 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 1 mM K<sub>4</sub>Fe(CN)<sub>6</sub> containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-glucuronide (AG Biosynth). Tissue was cleared using chloral hydrate after fixation in ethanol:acetate (3:1). GUS-stained tissues were embedded in Technovit 7100 (Heraeus, Germany) and 5  $\mu$ m sections were stained with Safranin (0.05% in water) for 10 seconds, washed with excess water and mounted in Epon. GUS expression and starch staining were

visualised using a Zeiss Axioplan2 imaging microscope with DIC optics. For confocal laser scanning microscopy (CLSM) roots were stained for 10 minutes in 10 mg/l propidium iodide and visualised using a Zeiss Axioplan microscope equipped with a BioRad MRC 1024 confocal laser. Microscopic images were recorded using a Sony DKC 5000 3CCD digital camera and Adobe PhotoShop software. Angles of hypocotyls and root tips towards the horizontal axis were determined using Adobe PhotoShop. Root and hypocotyl lengths were measured using NIH Image.

## RESULTS

### *PINOID* is a primary auxin response gene encoding a protein kinase

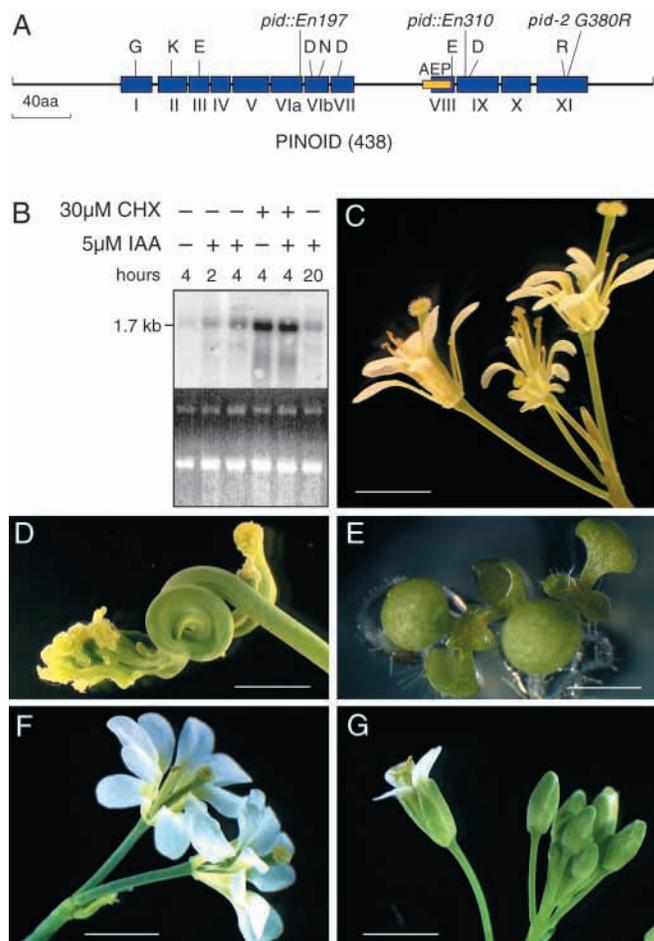
As part of our studies on auxin regulated gene expression, we analysed the expression and function of the putative protein-serine/threonine kinase gene *PINOID* (*PID*). This gene had previously been defined through loss-of-function mutants that form a pin-like inflorescence (Bennett et al., 1995), and was recently cloned (Christensen et al., 2000). The *PID* protein kinase shows significant similarity in the catalytic domain to members of the flowering plant-specific AGC group VIII of protein-serine/threonine kinases (Hanks and Hunter, 1995) (Fig. 1A). The closest member of this group in *Arabidopsis* shares less than 50% overall similarity with *PID*, indicating that *PID* is a single copy gene in *Arabidopsis*.

Northern blot hybridisation of poly(A) enriched RNA from *Arabidopsis* roots showed that the *PID* protein kinase gene is upregulated 4 hours after auxin treatment (Fig. 1B). Furthermore, induction of expression by cycloheximide and decline of expression after 20 hours of incubation with auxin (Fig. 1B) suggested that the gene belongs to the group of primary auxin response genes. Because of the auxin inducibility of the gene we looked for previously characterised auxin responsive elements in the promoter region of the gene. A single auxin responsive TGTCTC element (Ulmasov et al., 1997) was found within 500 bp of the transcription start (at position -33).

By screening *En-1* transposon mutagenized lines (Wisman et al., 1998) for insertion mutations in the protein kinase gene, we obtained two new *pid* alleles in which a transposon disrupted the region encoding the conserved catalytic domain of the protein kinase. We named the mutants *pid::En197* and *pid::En310* according to the codon that was disrupted by the *En-1* insertion (Fig. 1A). Mutant plants develop an inflorescence that ends in a pin-like structure and carries only a few aberrant flowers. Flowers generally contain few or no sepals and stamens, more petals, have a trumpet shaped pistil and produce no or only few seeds (Fig. 1C,F). Occasionally flowers develop with only carpelloid structures (Fig. 1D). Approximately 50% of the mutant seedlings showed abnormal cotyledons, with three cotyledons being the most common phenotype (Fig. 1E). The penetrance of the abnormal cotyledon phenotype indicated that the *pid::En* mutants represent strong loss-of-function alleles (Bennett et al., 1995; Christensen et al., 2000).

### *PINOID* is expressed in young vascular tissue and aerial organ primordia

*PID* mRNA is most abundant in young flower buds (not shown). Expression in both roots and shoots of seedlings is low but can be induced by auxin and cycloheximide treatment (Fig.



**Fig. 1.** The *PINOID* gene encodes a plant-specific protein-serine/threonine kinase with a role in organ development. (A) Schematic presentation of the *PINOID* protein showing the positions of the 12 conserved protein kinase subdomains, the newly identified AEP domain characteristic for ACG-group VIII members, the amino acid residues that are conserved in 95% of all protein kinases and the positions of the mutations in respectively *pid-2* (codon 380), *pid::En197* (codon 197) and *pid::En310* (codon 310). (B) *PID* is expressed at low levels, but expression is upregulated 4 hours after treatment with IAA and/or cycloheximide (CHX), as shown by an autoradiograph of a RNA gel blot containing poly(A) enriched mRNA from roots after hybridization with a genomic fragment containing the second exon of the *PID* gene. The ethidium bromide-stained gel in the lower panel shows that equal amounts of RNA were loaded. Flowers of *pid::En310* (C,D), *pid-2* (F) and wild-type *Arabidopsis* (G). (E) The three cotyledon phenotype observed in the loss-of-function mutants *pid::En197* and *pid::En310*. Scale bars, 2.5 mm (C,E-G) and 1.5 mm (D).

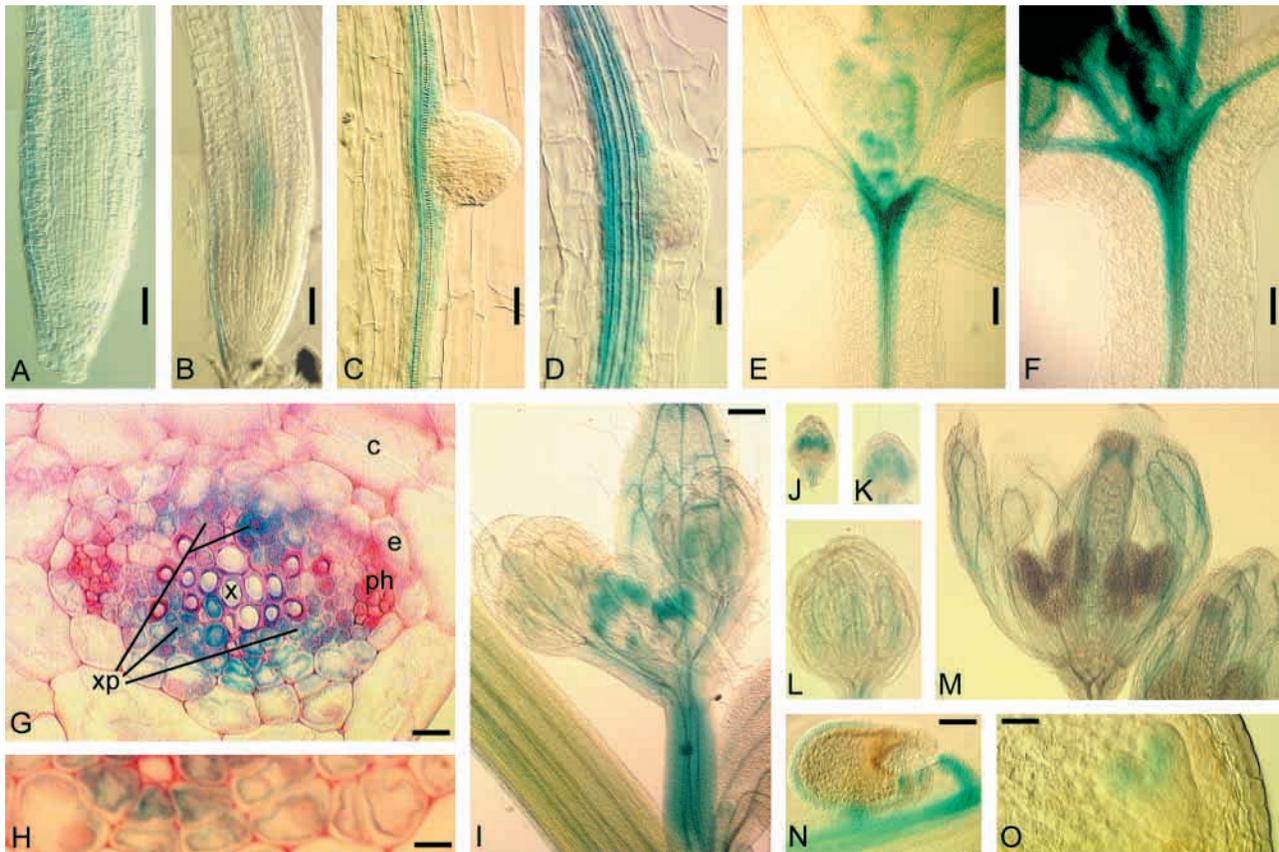
1B and not shown). To obtain a reliable impression of the spatial and temporal distribution of *PID* gene expression and the cellular localisation of the *PID* protein, we fused the complete gene, including the 3.8 kb 5' untranslated region, but excluding the last six codons, in-frame to the *gusA* reporter gene on pCAMBIA1381Xb (*PID::GUS*). Multiple lines were generated in ecotype Columbia (Col) that expressed the 4 kb *PID::GUS* transcript and showed the same  $\beta$ -glucuronidase (*GUS*) expression pattern. Transformation of the empty pCAMBIA1381Xb vector did not result in a detectable *GUS*

expression. One representative line, *PID:GUS-18*, was selected for further analysis.

The *pid-2* mutant (Bennett et al., 1995) was crossed with the *PID:GUS-18* line and F<sub>2</sub> progeny were tested for complementation of the intermediate *pid-2* allele by the *PID:GUS* gene. Among 301 F<sub>2</sub> seedlings only 5 developed abnormal cotyledons, a phenotype that showed 20% penetrance in the *pid-2* mutant. The data were significant for goodness of fit ( $\chi^2=0.4$ ,  $P>0.5$ ) with the 1:80 ratio expected for complementation of the *pid-2* mutation by the *PID:GUS* transgene. After transfer to soil, F<sub>2</sub> individuals were checked by PCR for the presence of the *pid-2* allele and the *PID:GUS* construct. Plants homozygous for the *pid* allele developed the typical *pid* inflorescence, whereas plants that were homozygous for the *pid* allele but also contained the *PID:GUS* construct developed a wild-type inflorescence (not shown). These results proved that the *PID:GUS* fusion protein restores normal growth to *pid* mutant plants and therefore has wild-type PINOID function. More importantly, the complementation of *pid-2* by *PID:GUS* showed that the GUS activity in line

*PID:GUS-18* is likely to reveal the spatial and temporal expression and cellular localisation of the endogenous *PID* protein kinase.

GUS expression in *PID:GUS-18* seedlings was mainly localised to the vascular tissue and was strongest in regions of vascular differentiation proximal to the meristems and lateral root primordia (Fig. 2A,C,E). A cross section of the hypocotyl just below the shoot apical meristem showed that GUS activity is present in the xylem parenchyma cells and in the endodermis around the vasculature (Fig. 2G). Moreover, closer examination of the sub-cellular localisation of the GUS signal in untreated and auxin-treated seedlings suggested that PINOID does not accumulate in the nucleus (Fig. 2H and data not shown). Treatment of seedlings with 5  $\mu$ M IAA induced a significant increase of expression in vascular tissue and leaf primordia (Fig. 2B,D,F). In the inflorescence, expression was detected in anther primordia, in the vasculature of the growing flower stalk, of young pedicels and bracts (Fig. 2I,J,K,L) and of developing sepals, but not in petals (Fig. 2M). In pistils, *PID* was transiently expressed in the vasculature of the style and the



**Fig. 2.** Histochemical staining of plant tissues of line *PID:GUS-18* for GUS activity shows that in 7-day-old seedlings *PID:GUS* is mainly localised in the vascular tissue proximal to the root meristem (A), the shoot apical meristem (E) or lateral root primordia (C). Expression is also present in developing cotyledons and leaves (E) and is significantly increased when the seedlings are incubated overnight in the presence of 5  $\mu$ M IAA (B,D,F). (G) A transverse section through the hypocotyl, just below the shoot apex, shows that *PID:GUS* is localised in the cytoplasm (see detail in H) of xylem parenchyma (xp) and endodermis (e) cells but not in the xylem plate (x) or in cells of the cortex (c) or the phloem (ph). Histochemical staining of (I) an inflorescence stem segment with a bract and a secondary inflorescence and (J-M) of flower buds in progressive stages of development shows that *PID:GUS* is transiently expressed in anther primordia (J-L) and in the vascular tissue of young pedicels and bracts (I) and of developing sepals (M). In the pistil, expression is detected in the vascular tissue of the style and septum (M) and in the integument and funiculus of fertilised ovules (N). During embryogenesis *PID:GUS* expression is localised to the cotyledon primordia (O). Scale bars, 30  $\mu$ m (A-D), 80  $\mu$ m (E), 60  $\mu$ m (F,N), 15  $\mu$ m (G,O), 5  $\mu$ m (H) and 0.3 mm (I-M).

septum (Fig. 2M), in the integuments and funiculus of the developing ovule (Fig. 2N) and in the cotyledon primordia of embryos (Fig. 2O).

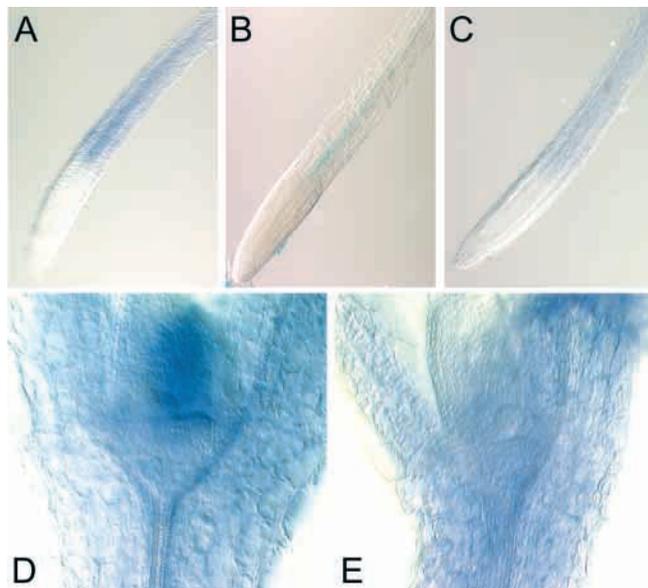
*PID:GUS* expression in flowers and embryos corroborates the in situ localisation of *PID* mRNA in these tissues by Christensen and colleagues (Christensen et al., 2000). This expression pattern correlates with the phenotypic changes observed in the loss-of-function mutants, i.e. altered cotyledon and floral organ numbers and altered pistil morphology (Fig. 1C,D,E,F), and confirms the importance of *PID* for the development of these organs. The localisation of *PID* expression in the vasculature and in leaf primordia was confirmed by whole-mount mRNA in situ hybridisation on seedlings (Fig. 3A,D), although expression of *PID*, as detected by in situ hybridisation in the shoot, is less pronounced compared to the expression of the *PID:GUS* fusion gene.

***PID* overexpression phenotypes corroborate involvement of *PID* in auxin action**

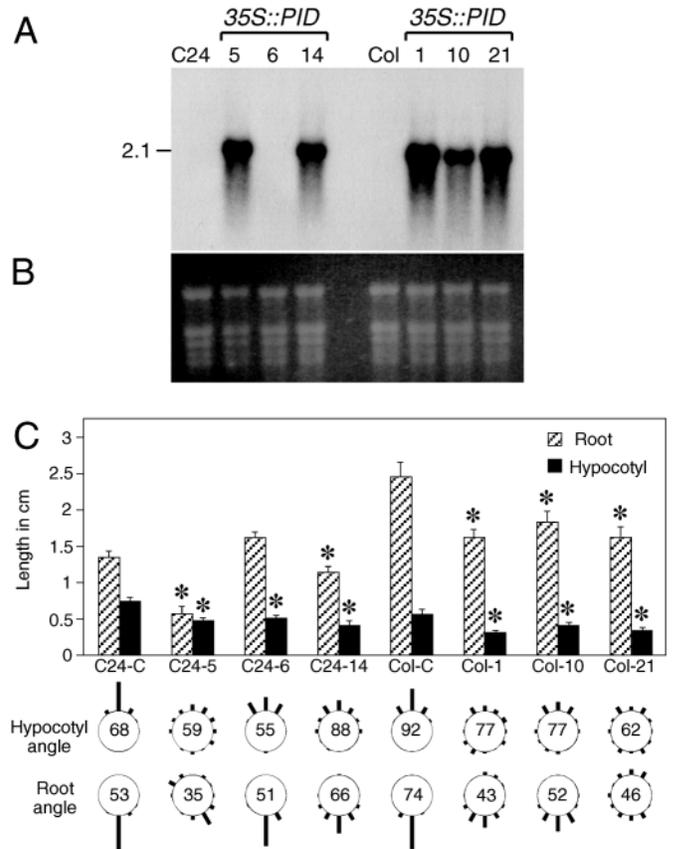
The phenotype of the *pid* loss-of-function mutants (Bennett et al., 1995) and the auxin responsive expression pattern of *PID* imply a role for the *PID* protein kinase in regulating auxin action. To further define this role, the *PID* cDNA was cloned in sense orientation behind the strong Cauliflower Mosaic Virus 35S promoter (*35S::PID*) and introduced into *Arabidopsis thaliana* ecotypes Col and C24. Of each ecotype, three lines with different levels of *PID* overexpression were selected for further analysis (Fig. 4A,B). One of these lines, C24-6, was exceptional in that it did not express *PID* at a detectable level (Fig. 4). Instead of a clear overexpression phenotype, adult plants developed inflorescences typical for *pid* mutants and 8% of the seedlings of this line developed abnormal cotyledons. The *pid* mutant phenotypes combined

with the non-detectable overexpression indicated that this line represented a partial loss-of-function mutant due to silencing of the endogenous *PID* gene by the multiple transgene locus present in this line.

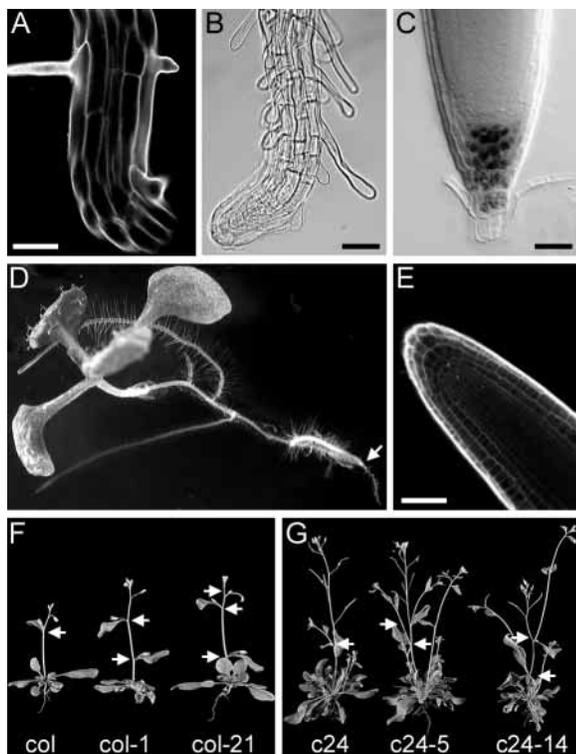
Seedlings of *35S::PID* lines showed agravitropy and reduced elongation growth of roots and hypocotyls (Fig. 4C). The severity of the phenotype corresponded to the level of overexpression in each case (Fig. 4A,B) and was reproducible in subsequent generations. Lateral root formation was delayed in *35S::PID* seedlings and interestingly, we observed collapse of the main root meristem within a few days after germination. The remaining root tip consisted of only a few layers of large elongated cells, several of which showed epidermal identity as evidenced by the development of root hair structures (Fig. 5A,B). Staining for starch granules indicated the absence of cells with columella identity (Fig. 5B,C). Lateral roots emerged following the disintegration of the primary root meristem (Fig. 5D). These lateral roots were again



**Fig. 3.** Localisation of *PID* mRNA by whole-mount in situ hybridisation in roots (A,C) and in the shoot apex (D,E) of 4-day-old seedlings. (A,D) Anti-sense *PID* probe. (C,E) Sense *PID* probe. (B) Root of a seedling of line *PID:GUS*-18 stained for GUS activity.



**Fig. 4.** CaMV 35S promoter-controlled expression of the *PID* cDNA in *Arabidopsis* ecotypes C24 and Columbia (Col). (A) Autoradiograph of an RNA gel blot containing total RNA from 7-day-old seedlings of wild-type (C24 and Col) and *35S::PID* after hybridization with the *PID* cDNA probe. (B) Ethidium bromide-stained gel. (C) Root and hypocotyl lengths and gravitropy, as measured in 7-day-old seedlings. Histogram bars indicate average lengths of 20 roots or hypocotyls. The bars marked with an asterisk differ significantly from the wild-type control (Student's *t*-test:  $P < 0.05$ ). The gravitropic response is depicted as the percentage of hypocotyls or root tips that was classified into each of twelve 30° sectors on a circle. The number of seedlings scored per line is indicated in the middle of each circle.



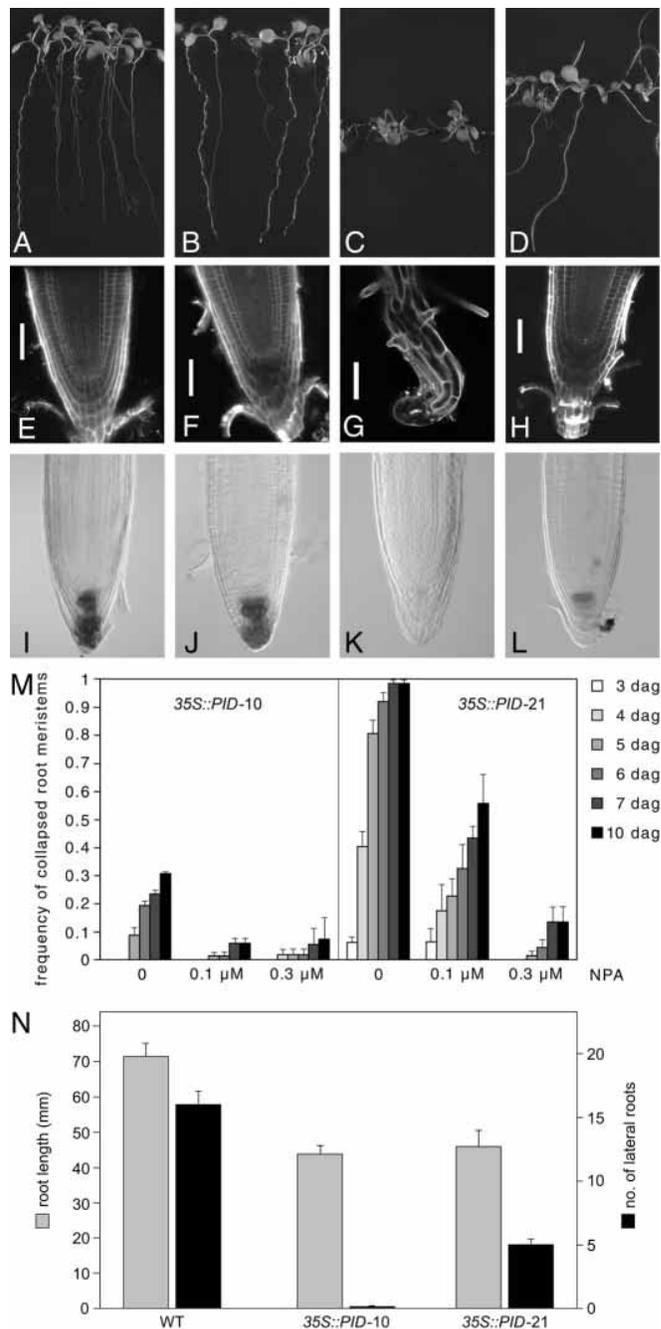
**Fig. 5.** High levels of *35S::PID* expression result in frequent disintegration of the primary root meristem and reduced apical dominance. (A) CLSM optical section of propidium iodide-stained collapsed primary root tip of a 4-day-old *35S::PID* Col-21 seedling. No cells with columella identity are detected after staining for starch granules (B), whereas columella cells are clearly stained in a root meristem of a 6-day-old wild-type seedling (C). (D) Lateral roots have emerged from the primary root of a 10-day-old *35S::PID* Col-21 seedling following collapse of the root meristem (white arrow). (E) CLSM optical section of a propidium iodide-stained lateral root meristem from a seedling as in D. (F,G) Wild-type and *35S::PID* plants photographed 5 and 12 days after bolting, respectively. The white arrows indicate the positions where lateral branches emerge on the primary inflorescence. Scale bars, 25  $\mu\text{m}$  (A,E) and 35  $\mu\text{m}$  (B,C).

agravitropic, but their meristems showed normal patterning (Fig. 5E) and collapse of these meristems was not observed.

Flowering plants of lines with high levels of *35S::PID* expression showed reduced apical dominance, developing a primary inflorescence with two or three lateral inflorescences (Fig. 5F,G). Also, the emergence and development of axillary inflorescences was significantly enhanced in these plants.

#### ***PID* overexpression phenotypes are rescued by polar auxin transport inhibitors**

The phenotype of *pid* loss-of-function mutants most closely resembles that of the *pin1* mutants, which are proposed to be blocked in PAT owing to the absence of a functional AEC (Gälweiler et al., 1998). This led us to hypothesise that the PID protein kinase acts by regulating PAT. To further examine this possible role for PID, we studied the effect of PAT inhibitors on root meristem collapse. Seeds of wild-type *Arabidopsis* and the *35S::PID* lines Col-10 and Col-21 were germinated on medium containing 0.1 or 0.3  $\mu\text{M}$  naphthylphthalamic acid



**Fig. 6.** Rescue of primary root growth of *35S::PID* seedlings by polar auxin transport inhibitors. Seedlings of Columbia wild type (A,B) and line *35S::PID* Col-21 (C,D) 7 days after germination (dag) on vertical plates. CLSM optical sections of propidium iodide-stained root tips of wild-type (E,F) and *35S::PID* Col-21 (G,H) seedlings, 7 dag. Root tips of 4-day-old wild-type (I,J) or *35S::PID* Col-10 (K,L) seedlings containing the *DR5::GUS* reporter gene after staining for GUS activity. (B,D,F,H,J,L) On 0.3  $\mu\text{M}$  NPA. (M) Timing and frequency of primary root meristem collapse in *35S::PID* lines Col-10 and Col-21. Frequencies are averages from counting phenotypes in three independent populations of 20 seedlings. (N) Length and number of lateral roots on primary roots of 14-day-old wild-type, *35S::PID* Col-10 and *35S::PID* Col-21 seedlings when rescued with 0.3  $\mu\text{M}$  NPA. Mean values are based on at least 15 independent roots. Error bars indicate the standard error of the mean. Scale bars, 50  $\mu\text{m}$ .

(NPA). These specific concentrations were used because such treatments exerted only mild effects on wild-type root development, as observed by root growth, lateral root initiation (Fig. 6A,B) and patterning of the root meristem (Fig. 6E,F). Moreover, the expression of an auxin responsive *DR5::GUS* reporter indicated that only minor changes occurred with regard to auxin distribution, or sensitivity, in roots treated with these NPA concentrations (Fig. 6I,J). Growth of *35S::PID* seedlings on NPA significantly increased root elongation (Fig. 6C,D) and prevented collapse of the primary root meristem (Fig. 6G,H). Similar results were obtained when seedlings were grown on TIBA, which belongs to a different class of PAT inhibitors. Clearly, suppression of PAT by inhibitor concentrations that only mildly interfere with the development of wild-type seedlings was sufficient to rescue the organisation of the primary root meristem in *35S::PID* lines.

We introduced the auxin responsive *DR5::GUS* reporter gene into the *35S::PID* background and confirmed the observation by Christensen and co-workers (Christensen et al., 2000) that GUS expression in root tips of young *35S::PID* seedlings is significantly reduced (Fig. 6K). In view of the auxin efflux enhancing activity of PID, this result can be explained by lower auxin levels in root tips of *35S::PID* seedlings. Interestingly, reduction of auxin efflux by NPA treatment partially restored the level of *DR5::GUS* expression in root tips of these seedlings (Fig. 6L).

More detailed analysis showed that root meristem collapse in the strong overexpression line *35S::PID* Col-21 was observed by 3 days after germination in some seedlings, and after 7 days in most (Fig. 6M). Meristem disintegration also occurred in line *35S::PID* Col-10, which expresses an intermediate level of the *35S::PID* transgene, but in only up to 50% of the seedlings and with significantly delayed timing. Primary root meristems of the majority of the *35S::PID* Col-21 seedlings were rescued with 0.3  $\mu$ M NPA. This concentration was critical, since at a slightly lower concentration (0.1  $\mu$ M) the rescue was only partial for high expressing line *35S::PID* Col-21, whereas rescue was almost complete for the intermediate expressing line *35S::PID* Col-10.

The *p35S*-driven *PID* overexpression typically inhibits lateral root development in young seedlings, but the inhibition is overcome by collapse of the primary root meristem. Since root meristem disintegration occurs earlier and more frequently in the high expressing *35S::PID* lines, seedlings of these lines generally develop a more vigorous root system. In the presence of NPA however, the meristem is rescued and lateral root formation remains suppressed. To quantify this prolonged suppression by NPA, we grew wild-type and *35S::PID* Col-10

and Col-21 seedlings for 14 days on 0.3  $\mu$ M NPA and determined the length and the number of lateral roots per primary root. NPA-rescued primary roots of both *35S::PID* lines were similar in length, but significantly shorter than those of wild-type seedlings ( $P < 0.001$ ; Fig. 6N). Although the inhibition of lateral root formation was maintained for both *35S::PID* lines in the presence of NPA, it was apparent that Col-21 seedlings developed significantly more lateral roots than those of line Col-10 ( $P < 0.001$ ; Fig. 6N). Apparently, high, as opposed to intermediate, levels of *p35S*-driven *PID* expression promote branching of primary roots. These findings clearly do not fit with a role of PID as a negative regulator of auxin signalling (Christensen et al., 2000), which implies a negative correlation between root branching and the level of *35S::PID* expression. Instead, our observations are more readily explained by general enhancement of auxin efflux due to ectopic PID expression, which results in dynamic changes in auxin distribution during development of the *35S::PID* seedlings.

### Ectopic *PINOID* expression in the shoot results in more lateral roots

Participation of PID in the regulation of PAT implies that PID does not only act locally in tissues where it is expressed, but also exerts its effect over a distance. To test this, we analysed the effects of tissue-specific *PID* expression using a *GAL4*-based transactivation-reporter system (D. W., J. Haseloff, E. van Ryn, P. H. and R. O., unpublished). This system enabled us to study the effects of the ectopic expression of *PID* and to simultaneously localise PID expression through the observation of *GFP::GUS* reporter transactivation. An activator plant line *ACT-LTP1* containing the epidermis-specific promoter of the *Arabidopsis thaliana* *Lipid Transfer Protein 1* (*LTP1*) gene fused to the *GAL4::VP16* gene was crossed with an effector line (*EF-PID*) harbouring both the *PID* cDNA and the *GFP::GUS* reporter under control of the *GAL4*-dependent *UAS* promoter.  $F_2$  seedlings were germinated without selection and 11- to 12-day-old seedlings were stained for GUS expression. Segregating at the expected 9:7 ratio for GUS-positive and GUS-negative seedlings, the  $F_2$  population clearly displayed significantly enhanced lateral root formation by *LTP1* promoter-driven *PID* expression (Table 1, exp. 1). A striking observation, especially since *LTP1* promoter activity is confined to the aerial parts of seedlings (Fig. 7A), except for expression in the young epidermis of lateral roots. Since *pLTP1* activity in lateral roots is not detectable before a stage VI primordium (Malamy and Benfey, 1997; Fig. 7B,C), it is unlikely that this local *pLTP1*-

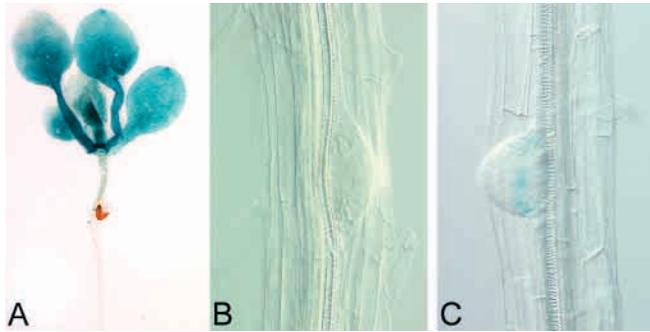
**Table 1. Effect of ectopic *pLTP1*-driven *PID* expression on lateral root development**

Exp. no.	$F_2$ population*	NPA <sup>‡</sup>	No. of lat. roots/mm $\pm$ s.e.m. (no. of plants)		Statistics <sup>§</sup>	
			GUS <sup>+</sup>	GUS <sup>-</sup>	Difference	<i>P</i>
1	ACT- <i>pLTP1</i> $\times$ EF- <i>PID</i>	–	0.33 $\pm$ 0.02 (24)	0.23 $\pm$ 0.01 (21)	Significant	<0.001
2	ACT- <i>pLTP1</i> $\times$ EF- <i>PID</i>	0 $\mu$ M	0.24 $\pm$ 0.01 (19)	0.20 $\pm$ 0.01 (15)	Significant	<0.05
		5 $\mu$ M	0.17 $\pm$ 0.01 (16)	0.15 $\pm$ 0.01 (13)	Not significant	>0.05
		20 $\mu$ M	0.08 $\pm$ 0.01 (17)	0.07 $\pm$ 0.01 (18)	Not significant	>0.05

\* $F_2$  seeds were germinated and 11- to 12-day-old seedlings were tested for GUS expression by histochemical staining. The root length and number of lateral roots was subsequently determined.

<sup>‡</sup>5  $\mu$ l droplets of agarose containing the indicated concentration of NPA were applied to the transition zone of 4-day-old seedlings.

<sup>§</sup>The difference between samples was tested using the Student's *t*-test.



**Fig. 7.** Expression pattern of the epidermis-specific *LTP1* promoter in an F<sub>2</sub> seedling from the cross ACT-*LTP1* × EF-*PID* after histochemical staining for activity of the co-expressed *GFP::GUS* gene. The reporter gene is predominantly expressed in the shoot (A). Expression is also observed in the young epidermis of lateral root primordia, but only after a stage VI primordium has been formed. Stage V (B) and stage VI (C) lateral root primordia of a GUS-positive F<sub>2</sub> seedling.

driven *PID* expression is the cause of the enhanced lateral root formation.

Lateral root induction is known to rely on PAT from the shoot into the root (Reed et al., 1998; Casimiro et al., 2001). We therefore hypothesised that the *pLTP1* driven *PID* expression would result in enhanced auxin efflux from the epidermis in the shoot, thereby inducing an increased transfer of auxin from the shoot into the root. Local application of the auxin transport inhibitor NPA to the transition zone of the seedlings reduced the difference between GUS+ and GUS- seedlings to a non-significant level, confirming that the increase in lateral roots is caused by enhanced efflux of auxin from the shoot into the root (Table 1, exp. 2). These data support a function of the protein kinase in enhancing polar auxin transport.

## DISCUSSION

The *PID* locus in *Arabidopsis thaliana* was initially defined by a series of 8 allelic mutants exhibiting the inflorescence phenotype of few aberrant flowers and a terminal pin-like structure. Depending on the severity of the mutant allele, up to 50% of mutant seedlings show aberrations in cotyledon number and/or separation (Bennett et al., 1995). Recently, Christensen et al. (Christensen et al., 2000) reported that the *PID* gene encodes a protein-serine/threonine kinase that regulates auxin response. We show that *PID* is an auxin-responsive gene, we provide important new insights into the expression of *PID* throughout *Arabidopsis* development and we present data that further our understanding of the role of the *PID* protein kinase as a mediator of auxin action in plant development.

### PINOID expression reveals a role in organ development

*PID* expression was observed in the cotyledon primordia of embryos, in young leaves and in young floral organs (Christensen et al., 2000; our observations). This expression pattern corroborates the function of *PID* in the regulation of

aerial organ development and cotyledon positioning and separation. *PID* expression in vascular tissues initially suggested that *PID* might direct vascular development in young developing organs. However, mild vascular defects were only observed in the aberrant flowers and not in other organs of *pid* loss-of-function mutants (Christensen et al., 2000; our observations), indicating that the regulation of vascular development is not the primary task of *PID*. More likely, based on the interpretation of the loss-of-function mutant phenotype and gene expression, *PID* is probably involved in determining the position and outgrowth of cotyledon, leaf, flower and floral organ primordia. The importance of auxin and the involvement of PIN1 in positioning and outgrowth of lateral aerial organs was demonstrated recently (Reinhardt et al., 2000; Vernoux et al., 2000).

In contrast to the clear developmental defects observed in aerial organs, the effects of *pid* loss-of-function mutations on root development were not very obvious. However, we did observe an irregular root waving pattern in *pid* loss-of-function mutants (not shown), while *35S::PID* overexpressors had strong alterations in root gravitropism and development. This suggests that some *PID* functions do indeed influence auxin-mediated processes in roots.

### PINOID: a negative regulator of auxin signalling?

Based on two phenotypes of the *35S::PID* lines, decreased expression of the auxin responsive *DR5::GUS* reporter and reduced lateral root initiation even upon exogenous application of auxin, Christensen et al. (Christensen et al., 2000) concluded that *PID* acts as a negative regulator of auxin signalling. However, the new observations that we have made argue against such a function for *PID*. *DR5::GUS* expression is reduced in young roots of the *35S::PID* lines, but we found that expression of the reporter was clearly present in the root vasculature after collapse of the primary root meristem or in roots of older plants (not shown), suggesting that auxin signalling to this promoter is not impaired, but rather, prevented in young primary roots. Another auxin-dependent process that is initially perturbed in *35S::PID* lines is lateral root formation. However, 14-day-old seedlings of the high expressing *35S::PID* line, Col-21, developed more lateral roots than those of the intermediate expressing line, Col-10, indicating that auxin signalling leading to lateral root induction is not repressed by *PID* (over)expression. Moreover, roots of *35S::PID* lines are clearly as sensitive to exogenously applied auxins as wild-type roots (Christensen et al., 2000; our observations). Reduced sensitivity of root elongation to exogenously applied auxin has been one of the major criteria for distinguishing mutations in components or regulators of auxin signalling (e.g. *axr1*). In conclusion, none of the observations provides sufficient evidence that auxin signalling in *35S::PID* roots is perturbed and, although we do not exclude an involvement of the *PID* protein kinase in auxin signalling, we consider the available data indicating a role for the protein kinase as a positive regulator of PAT.

### PINOID acts as a positive regulator of auxin efflux

The pin-shaped inflorescences and the aberrant cotyledons and flowers of the *pid* mutants closely resemble those of *pin-formed* mutants. The cotyledon and inflorescence phenotypes can be mimicked by treatment with auxin transport inhibitors

of globular stage embryos or mature plants, respectively (Okada et al., 1991; Liu et al., 1993; Hadfi et al., 1998). Recently it was shown that some aspects of the aberrant flower development in *pid* and *pin1* mutants can be mimicked by spraying flowers with the same inhibitors (Nemhauser et al., 2000). Moreover, expression studies show that PID localises with AtPIN1 in the xylem parenchyma cells of the vascular tissue (our observations; Gälweiler et al., 1998). All these data strongly suggest that PID acts in concert with AtPIN1 in the vasculature, or with other AtPINs in other tissues, to positively regulate PAT. If so, loss-of-function *pid* mutants would be expected to show reduced levels of PAT and PID function would be sensitive to PAT inhibitors.

Indeed, auxin transport seemed reduced in the inflorescence stems of loss-of-function *pid* alleles (Bennet et al., 1995; our observations). However, PAT levels in inflorescence stems are determined by many indirect factors, such as the presence and number of developing aerial organs (Bennett et al., 1995; Oka et al., 1998) and proper development of the vascular tissue (Carland and McHale, 1996; Przemeck et al., 1996), which indicates that conclusions from direct transport measurements should also be confirmed by other functional tests. Two important observations support the role of PID as a regulator of PAT: (i) the PAT inhibitor sensitivity of PID action and (ii) the fact that, spatially, *PID* expression and the resulting effects on plant development do not necessarily overlap.

The NPA/TIBA-sensitivity of PID action was first demonstrated by the rescue of root growth of *35S::PID* seedlings by low doses of these PAT inhibitors. We propose a model to explain the *35S::PID* phenotype (Fig. 8), in which the *35S* promoter-mediated *PID* expression preferentially enhances downward-directed PAT through the axis of the seedlings. This canalisation of auxin depletes the more peripheral tissue layers of auxin, which leads to reduced elongation and agravitropy of the hypocotyl and delays lateral root formation. Indeed, increasing endogenous auxin levels by growing the seedlings at 28°C (Gray et al., 1998) resulted in enhanced hypocotyl elongation of *35S::PID* seedlings (not

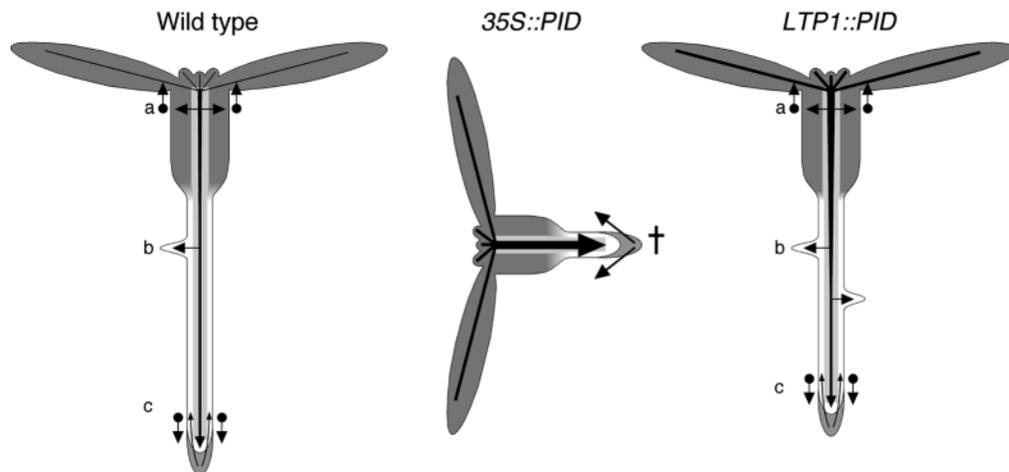
shown), thereby confirming that IAA concentrations are sub-optimal for hypocotyl elongation. In our model, ectopic *PID* expression results in enhanced efflux of auxin from the root meristem (Fig. 8), thereby resolving the proposed auxin maximum as organiser of the root meristem (Sabatini et al., 1999) and thus leading to collapse of the meristem. The sub-optimal IAA levels in the root tip explain the reduced *pDR5::GUS* expression in *35S::PID* background roots and the slow and agravitropic growth of these roots. The collapse of the primary root meristem in *35S::PID* seedlings alleviates auxin depletion of the root and thereby releases the initial delay in lateral root formation. This is in line with previous findings that removal of the root meristem or the root cap enhances the formation of lateral root primordia and the emergence of lateral roots (Torrey, 1950; Reed et al., 1998; Tsugeki and Fedoroff, 1999). In the presence of NPA at sub-micromolar concentrations, elevated PAT is slowed down in roots of *35S::PID* seedlings, allowing for maintenance of the organising auxin maximum (Sabatini et al., 1999), rescue of the root meristem and partial rescue of the gravitropy and growth of the root. The recent finding that PAT inhibitor treatments induce an accumulation of IAA in the root tip (Casimiro et al., 2001) is consistent with this model.

Both the PAT inhibitor-sensitivity of PID action and the fact that *PID* expression does not necessarily have a local effect on development were demonstrated by the *LTP1* promoter-mediated ectopic expression of *PID* in the aerial part of seedlings (Thoma et al., 1994; Fig. 7). The resulting significant increase in lateral root formation could be reduced by application of NPA at the transition zone between shoot and root, indicating that *pLTP1*-mediated *PID* expression increases auxin transfer from the shoot into the root (Fig. 8).

## Conclusion

Based on the data presented above we propose that the protein kinase PID is a positive regulator of PAT. The inducibility of the *PID* gene by auxin suggests that PAT is enhanced by accumulation of PID in the cell in response to an increase in

**Fig. 8.** A schematic model to explain the observed seedling phenotypes caused by ectopic *PID* expression. In wild-type seedlings auxin is transported from its location of synthesis in the shoot downward to the root. Lateral transport of auxin is essential for hypocotyl elongation and tropic growth (a) and lateral root formation (b). Redistribution of auxin at the root tip by basipetal transport toward the elongation zone is essential for growth and gravitropy of the root (c). *35S::PID* expression preferentially stimulates downward directed PAT to the



root apex, thereby reducing lateral transport. From the root tip auxin is rapidly transported to a location where it is metabolised or secreted into the medium. This deprives the peripheral cell layers and root apex of auxin and results in reduced gravitropy and growth of hypocotyl and root and reduced lateral root formation. Removal of auxin from the root apex eventually leads to collapse (†) of the primary root meristem. *LTP1::PID* expression only enhances PAT from the shoot into the root, thereby increasing lateral root formation. The directions and level of PAT is indicated by black arrows. Elongation growth is indicated by black arrows that start with a dot.

local IAA levels. Indications for regulation of PAT by auxin are provided by the fact that PAT levels in inflorescence stems are significantly reduced when developing flower buds or siliques, the presumed source tissues of IAA, are removed or absent (Bennett et al., 1995; Oka et al., 1998), by the report that auxin efflux is enhanced in the auxin overproducing *sur1* mutant (Delarue et al., 1999) and from the observed twofold increase of acropetal [<sup>3</sup>H]IAA transport through the root in the presence of cold IAA (Rashotte et al., 2000). The phenotypic characteristics of the *pid* loss-of-function mutants suggest that the PID function is essential for proper cotyledon positioning and development, for maintenance of the inflorescence meristem, for whorl definition during flower development and it is important for wild-type root growth. Enhancement of PAT may be necessary to prevent feed-back regulation of auxin biosynthesis due to accumulation of auxin, and may possibly guarantee a continuous source-to-sink transport of auxin that is essential for the organisation of, and differentiation in, these developing organs. One important step in elucidating the mechanism of the PID-mediated enhancement of PAT will be to investigate interactions between PID and the putative AECs.

The *pid-2* mutant was kindly provided by the Arabidopsis Biological Resource Center at Ohio State University. We thank Bert van der Zaal for helpful comments and for providing the root-specific cDNA library, Sacco de Vries for providing the *LTP1* promoter, Andrew Gleave for sending the constructs pART7 and pART27, Elvira Baumann and Ellen Wisman for help and advice during screening of the collection of *En-1* mutants (MPI, Cologne), Gerda Lamers and Celia Vogel for technical assistance, Peter Hock, Adri 't Hooft and Peter van Mulken for artwork, Jiri Friml and Enrico Scarpella for helpful discussions and for help with in situ mRNA hybridisations and transformation of pCAMBIA1381Xb respectively, and Keithanne Mockaitis, Jan Traas, Klaus Palme and Kim Boutilier for useful comments on the manuscript. D. W. was supported by the Research Council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organisation for Scientific Research (NWO).

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