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AtPIN2 defines a locus of *Arabidopsis* for root gravitropism control

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The molecular mechanisms underlying gravity perception and signal transduction which control asymmetric plant growth responses are as yet unknown, but are likely to depend on the directional flux of the plant hormone auxin. We have isolated an *Arabidopsis* mutant of the *AtPIN2* gene using transposon mutagenesis. Roots of the *Atpin2::En701* null-mutant were agravitropic and showed altered auxin sensitivity, a phenotype characteristic of the agravitropic *wav6-52* mutant. The *AtPIN2* gene was mapped to chromosome 5 (115.3 cM) corresponding to the *WAV6* locus and subsequent genetic analysis indicated that *wav6-52* and *Atpin2::En701* were allelic. The *AtPIN2* gene consists of nine exons defining an open reading frame of 1944 bp which encodes a 69 kDa protein with 10 putative transmembrane domains interrupted by a central hydrophilic loop. The topology of AtPIN2p was found to be similar to members of the major facilitator superfamily of transport proteins. We have shown that the *AtPIN2* gene was expressed in root tips. The AtPIN2 protein was localized in membranes of root cortical and epidermal cells in the meristematic and elongation zones revealing a polar localization. These results suggest that AtPIN2 plays an important role in control of gravitropism regulating the redistribution of auxin from the stele towards the elongation zone of roots.

Keywords: agravitropic mutant/*Arabidopsis thaliana*/auxin transport/gravitropism/transporter

Introduction

All plants exhibit gravitropic growth, but how they sense gravity and transduce this physical stimulus into physiological responses is a central question in plant biology (Firn and Digby, 1980; Pickard, 1985; Moore and Evans, 1986; Moore, 1991; Nedukha, 1997; Sievers *et al*., 1997). Plants respond to gravity by directional growth orientating roots vertically downwards (positive gravitropism) and shoots upwards (negative gravitropism). It is a common view that roots perceive gravity in the columella cells of the root cap through the sedimentation of dense starch grains (amyloplasts or statoliths) onto endoplasmic reticulum complexes, the cytoskeleton or the plasma membrane (Baluska and Hasenstein, 1997). This mechanism is believed to initiate a signal transduction cascade which subsequently transfers the gravity signal to cells of the root elongaton zone inducing differential cell elongation. As a result of asymmetric elongation, root curvature is induced (Young *et al*., 1990; Evans, 1991).

Since Cholodny (1927) and Went (1933) found an involvement of directional auxin transport in gravitropic responses, numerous studies have confirmed that this plant hormone is indeed essential for the mediation of tropic growth responses (Kaufman *et al*., 1995). Polar auxin transport occurs from auxin-synthesizing tissues (e.g. young leaves or the shoot apex) through the vascular tissues of stems towards the root tips (Lomax *et al*., 1995). In roots, auxin is transported within stelar tissues in an acropetal direction towards the root tip. It has been proposed that around the meristematic zone auxin is loaded from the central stele into the cortical and epidermal cell layers where it is transported backwards in basipetal orientation to the elongation zones (Tsurumi and Ohwaki, 1978; Estelle, 1996).

Polar transport of auxin from cell to cell is thought to be primarily mediated by specific efflux carrier proteins polarly located at one end of transport-competent cells. This polar localization of carrier proteins is hypothesized to be responsible for the polarity of auxin transport (Lomax *et al*., 1995). The elucidation of the localization of these auxin carriers in plant cells is expected to provide evidence supporting the Cholodny–Went hypothesis. To understand the mechanisms regulating auxin redistribution and tropic responses, physiological studies have been performed over several decades (reviewed in Kaufman *et al*., 1995), but despite extensive information, we still lack a detailed understanding of the molecular aspects underlying these processes. To dissect genetically the components of this pathway, mutants affected in various steps of gravity perception and transduction including the regulation of polar auxin transport were isolated (Maher and Martindale, 1980; Mirza *et al*., 1984; Kelly and Bradford, 1986; Eason *et al*., 1987; Caspar and Pickard, 1989; Bell and Maher, 1990; Okada and Shimura, 1990; Simmons *et al*., 1995; Yamauchi *et al*., 1997). The gene for one of these agravitropic mutants, *aux1*, has been cloned and shown to encode a permease-like protein that has been proposed to encode a component of the auxin uptake carrier (Bennett *et al*., 1996).

Here we describe the molecular characterization of another agravitropic mutant, *Atpin2::En701*, isolated from an *En-1* transposon mutagenized *Arabidopsis* population. *Atpin2::En701* displays severe defects in root growth and gravitropism, and is allelic to *wav6-52*, a mutant which

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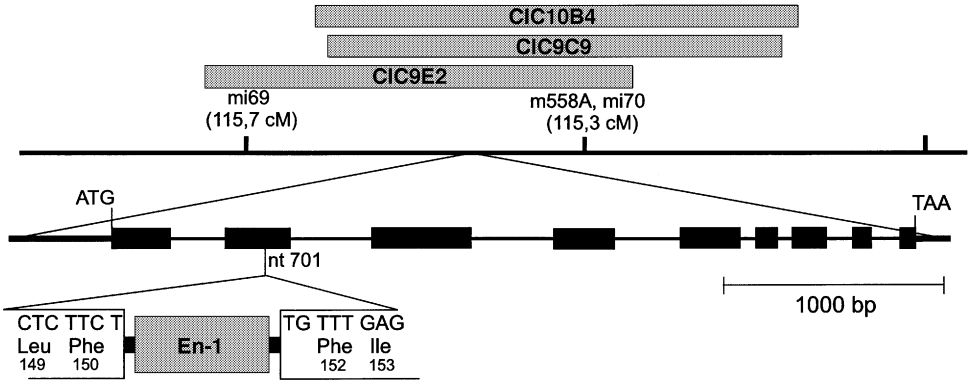

Fig. 1. Structure and physical mapping of *AtPIN2*. *AtPIN2* position on chromosome 5 with adjacent RFLP markers and approximate genetic distances are shown. The approximate positions of YAC clones CIC9E2, CIC10B4 and CIC9C9 (Creusot *et al*., 1995) are indicated. Below, the structure of the *AtPIN2* transcription unit is presented. The open reading frame is terminated by a TAA in-frame stop codon. The diagram depicts introns (thin lines) and exons (black boxes). Bold lines indicate 5′ and 3′ untranslated regions. Sizes are given in bp. The position of the *En-1* transposon insertion is marked. The nucleotide sequences flanking both ends of the transposon insertion in *Atpin2::En701* show the disruption of codon 151 (TTG).

was previously shown to be allelic to *agr1* (Bell and Maher, 1990; Okada and Shimura, 1990). The *AtPIN2* gene encodes a protein showing structural similarity to the major facilitator-like transporter family. The molecular properties and cellular expression profile of AtPIN2p suggested a function in polar auxin transport mediating the gravitropic growth in *Arabidopsis* roots.

Results

Isolation and structure of the AtPIN2 gene

The *AtPIN2* gene and the corresponding cDNA were isolated using a probe derived from the *AtPIN1* gene which is involved in the control of polar auxin transport in *Arabidopsis* plants (L.Gälweiler, C.Guan, A.Müller, E.Wisman, K.Mendgen, A.Yephremov and K.Palme, submitted). A genomic clone was obtained spanning a region of ~4 kb. Screening of several cDNA libraries resulted only in truncated *AtPIN2* cDNAs. Therefore, we reconstructed a full-length cDNA clone by PCR amplification of 269 bp from the genomic clone and ligation of this PCR fragment to the 5′ end of an incomplete cDNA. Sequence comparison of the genomic clone with the cDNA showed that the coding sequence of the *AtPIN2* gene was interrupted by eight introns (Figure 1). Several in-frame stop codons upstream of the coding region of 1944 bp and polyadenylation signals located at cDNA positions 1967, 2088 and 2135 indicated that the cDNA contained the full coding region. In addition, the sequence around the initiation codon confirmed Kozak's rules (Kozak, 1991). To determine the map position of the *AtPIN2* gene, we hybridized the CIC YAC library with an *AtPIN2* probe and identified a YAC contig consisting of CIC9E2, CIC10B4 and CIC9C9 (Creusot *et al*., 1995) located in the region of 115.34 cM (m558A) and 115.26 cM (mi70) on the lower arm of *Arabidopsis* chromosome 5 (Figure 1).

AtPIN2p is a protein with structural similarity to transmembrane transporters

The *AtPIN2* gene encodes a protein of 647 amino acids with a predicted molecular mass of 69 kDa (Figure 2A). The predicted AtPIN2 protein sequence consisted of >35% hydrophobic amino acids, yielding a calculated isoelectric point of 9.37 and a net charge of +12.36 at pH 7.2. The protein showed high similarity to the AtPIN1 protein (64% identity; L.Gälweiler, C.Guan, A.Müller, E.Wisman, K.Mendgen, A.Yephremov and K.Palme, submitted), and to other members of the *PIN* gene family (unpublished data). Hydropathy analysis revealed three characteristic regions: a hydrophobic region with five to six transmembrane segments (residues 1–163), a predominantly hydrophilic core extending from residue 164 to 482 which was followed by another hydrophobic region with about four to five transmembrane segments between residues 483 and 647 (Figure 2B and C). The position of the transmembrane segments was equally detected by programs PSORT, PredictProtein, Tmpred and PeptideStructure. PSORT data suggested at a probability of 64% that AtPIN2p was a type-III membrane protein anchored through internal sequences in the plasma membrane (Singer, 1990).

The Atpin2::En701 mutants exhibit agravitropism and altered auxin physiology

For functional analysis of the *AtPIN2* gene, we screened for a knock-out mutant in a collection of *Arabidopsis* plants mutagenized by the maize transposon *En-1* (E.Baumann, J.Lewald, H.Saedler, B.Schultz and E.Wisman, submitted; Wisman *et al*., 1998). We identified a mutant, *Atpin2::En701*, in which the *En-1* transposon was inserted into the second exon of the *AtPIN2* gene (between nucleotide positions 701 and 702) thereby disrupting the codon for Leu151 (Figure 1).

To characterize the *Atpin2::En701* mutant with respect to growth of aerial and root organs, homozygous progeny were grown on agar plates. Roots of mutant plants displayed an agravitropic phenotype coupled to reduced root elongation growth (Figure 3A and B). Physical mapping revealed that the chromosomal position of *AtPIN2* corresponded to the *WAV6* locus. Since the *wav6-52* EMS mutant exhibited a similar agravitropic phenotype as *Atpin2::En701* (Okada and Shimura, 1990), we performed crosses between the homozygous *wav6-52* and *Atpin2::En701* mutants to test for allelism. Analysis of the F1 progeny indeed showed that *wav6-52* and *Atpin2::En701* were allelic (data not shown). We have

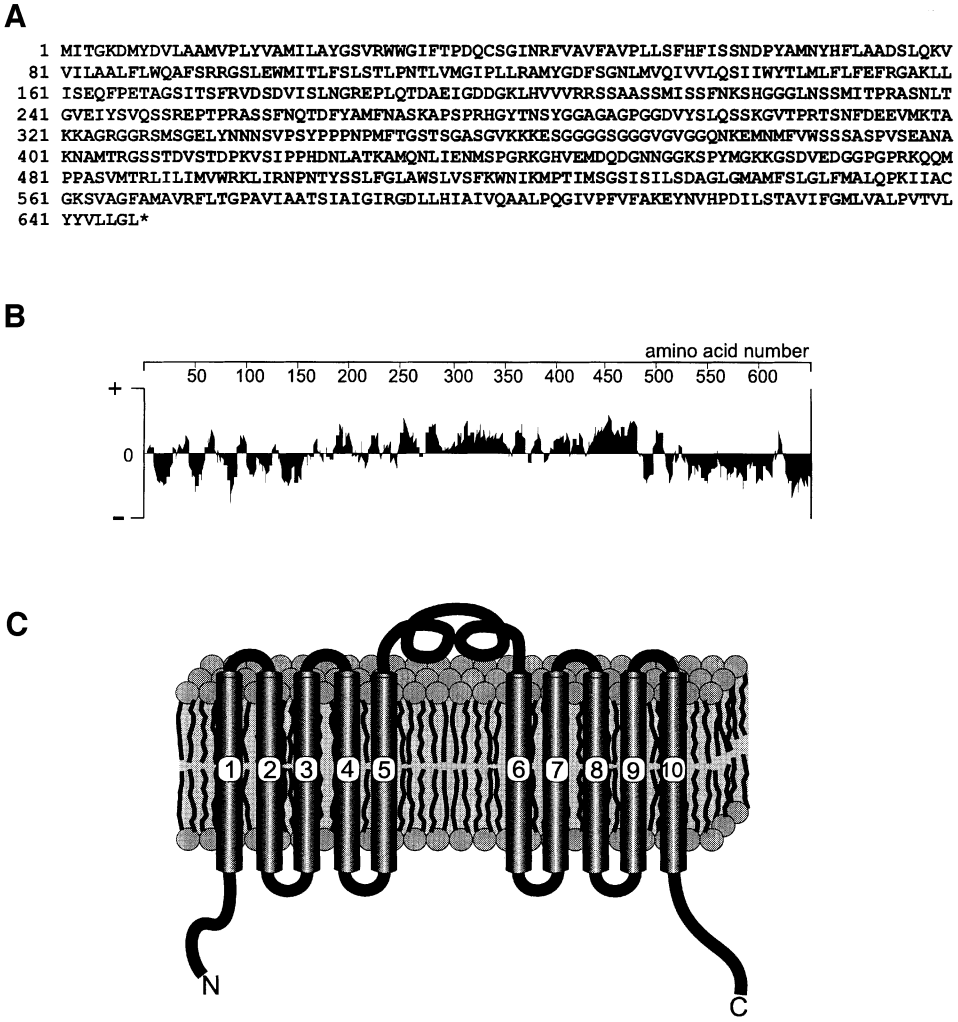

Fig. 2. (**A**) Sequence of AtPIN2 protein (DDBJ/EMBL/GenBank accession nos AF086906 and AF086907). (**B**) Hydrophilicity plot of the AtPIN2 protein. The hydropathy analysis was performed by the Lasergene software DNASTAR according to Kyte and Doolittle (1982). Hydrophilicity (+) and hydrophobicity (–) are indicated. (**C**) Predicted topology of the AtPIN2 polypeptide containing ~10 putative transmembrane segments (1–10) and a loop-forming region between transmembrane segments five and six. Predictions have been performed using the servers: http://psort.nibb.ac.jp (Psort; Nakai and Kanehisa, 1992), http://www.embl-Heidelberg.de/predictprotein/predictprotein.html (PHD topology; Rost, 1996); http://ulrec3.unil.ch/software/TMPRED_form.html (Tmpred; Hofmann and Stoffel, 1993); and Peptidestructure (GCG program package; Devereux *et al*., 1984).

tested the auxin sensitivity of root elongation for the *Atpin2::En701* and *wav6-52* alleles, since several agravitropic mutants have been observed to exhibit altered auxin response (Hobbie and Estelle, 1995; Simmons *et al*., 1995). Seeds were first germinated on hormone-free Murashige and Skoog (MS) agar medium, then transfered onto MS agar plates containing various concentrations of 1-naphthylacetic acid (1-NAA; Figure 4A and B), indole-3-acetic acid (IAA; Figure 4C and D) or 2,4-dichlorophenoxyacetic acid (2,4-D; Figure 4E and F). Additional root growth following transfer was measured after 3 days and the percentage of root elongation was calculated relative to growth on media containing no auxin. The results revealed that elongation of *Atpin2::En701* mutant roots was more sensitive than wild-type at elevated concentrations of 1-NAA ($p < 0.0001$ student *t*-test at 3.3×10^{-7} M; Figure 4A), and IAA ($p < 0.0001$ Student's *t*-test at 10^{-7} M; Figure 4C), but no difference in 2,4-D sensitivity was found between them (Figure 4E). Similar responses have been observed for the *wav6-52* allele (Figure 4B, D and F). The greater sensitivity of *Atpin2::En701* and *wav6-52* mutant roots towards the auxins 1-NAA > IAA > 2,4-D (Figure 4) is consistent with the known substrate specificity of the auxin efflux carrier (Delbarre *et al*., 1996).

Expression analyses revealed a polar localization of AtPIN2p in root cells

Northern blot analysis showed that *AtPIN2* was expressed in young seedlings and roots of plants grown on agar and in liquid culture, respectively (Figure 5A). This was confirmed by Western blot analyses using affinity-purified polyclonal anti-AtPIN2p antibodies raised against a polypeptide extending from residues 275–487 of AtPIN2p. The anti-AtPIN2p antibodies detected a specific signal corresponding to the expected size of 69 kDa in membrane fractions of *Arabidopsis* roots (Figure 5B). In order to localize *AtPIN2* expression, the spatial distribution of its mRNA was determined by whole-mount *in situ* hybridization using 3-day-old seedlings (Figure 6). *AtPIN2* expression was observed in a region spanning meristematic and

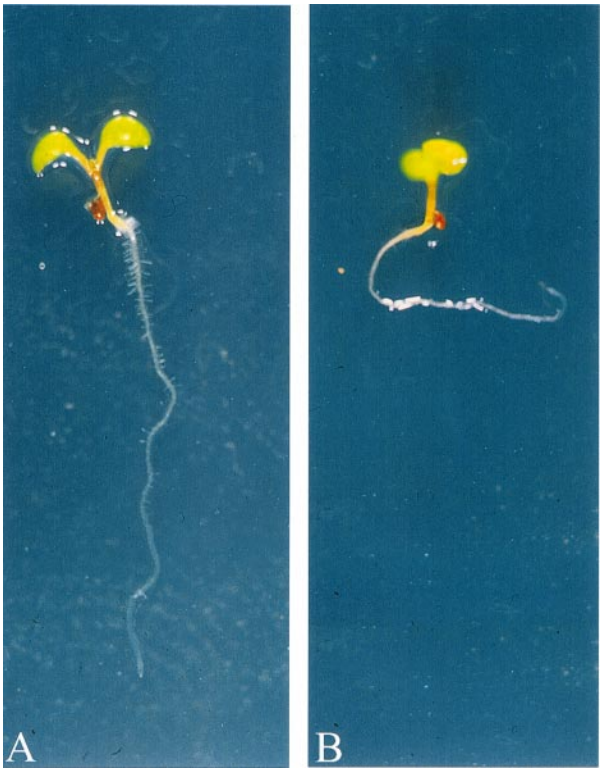

Fig. 3. Mutations in the *AtPIN2* gene alter root growth and gravitropism. Homozygous 5-day-old Columbia-0 wild-type seedlings (**A**) and *Atpin2::En701* mutant seedlings (**B**) were grown vertically on agar plates.

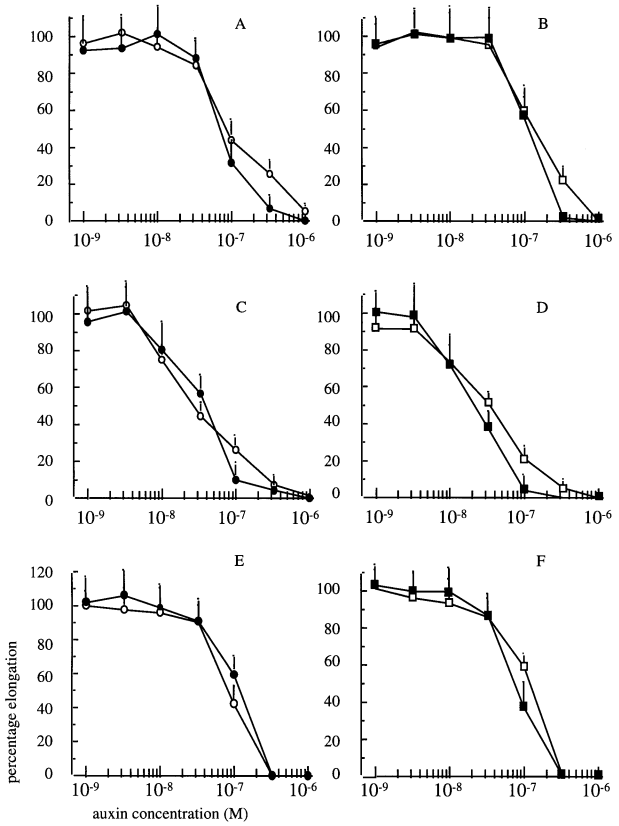

Fig. 4. *AtPIN2* alleles exhibit altered auxin responses. Auxin-resistance profiles for Columbia (open circles), *Atpin2::En701* (closed circles), Landsberg (open squares) and *wav6-52* (closed squares) were determined by germinating seeds on MS agar and then transferring seedlings to agar plates containing various concentrations of 1-NAA (**A** and **B**), IAA (**C** and **D**) or 2,4-D (**E** and **F**). Additional root growth following transfer was measured after 3 days (15 seedlings per auxin concentration) and the percentage of root elongation was calculated relative to growth on media containing no auxin. Error bars represent standard deviations.

distal elongation zones of wild-type seedlings (Figure 6), whereas no signals were detected in the *Atpin2::En701* mutant (data not shown). To localize the AtPIN2 protein within root cells, whole-mount *in situ* immunostaining analysis of 4-day-old seedlings was performed using affinity purified anti-AtPIN2p primary antibodies with fluorescein isothiocyanate (FITC)-coupled secondary antibodies. Immunohistochemical staining of wild-type plants revealed AtPIN2p-specific signals in a region spanning meristematic and distal elongation zones, consistent with the *AtPIN2* mRNA *in situ* hybridization results (Figure 7). In roots of homozygous *Atpin2::En701* mutant plants we observed only diffuse background staining as expected for a loss-of-function mutant (Figure 7C). In wild-type seedlings, the AtPIN2p-specific FITC fluorescence was localized exclusively in membranes of root cortical and epidermal cell files, starting three to five cells distant from the quiescent center (Figure 7B and D). No signal was observed in the stele, pericycle or endodermis. The fluorescence was very weak, if not completely absent, at the periclinal side of cortical cells contacting the endodermal cells. Stronger signals were observed at the anti- and periclinal sides of cortical cells adjacent to other cortical and epidermal cells (Figure 7B, D and E). In the epidermal cell file AtPIN2p was preferentially localized to the anticlinal side of the cells (Figure 7D and E). As the epidermal cell file extended towards the elongation zone, the signal was present at the basal side of cells oriented towards the elongation zone (Figure 7F). This striking cellular polarity of AtPIN2p is highly reminiscent of the basal localization of the homologous AtPIN1 protein

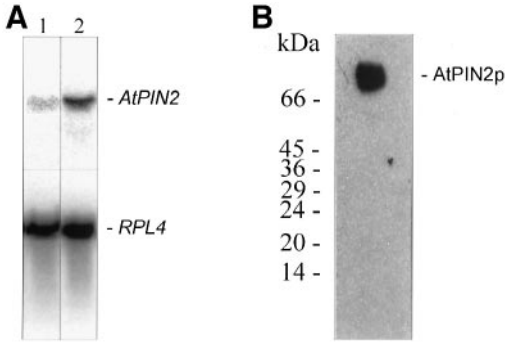

Fig. 5. (**A**) Northern blot analysis of *AtPIN2*. RNA was isolated from seedlings (lane 1) and roots (lane 2) grown on agar. A band of 2.4 kb was detected using a ^{32}P-labeled *AtPIN2* cDNA probe. The blot was subsequently hybridized with the *RPL4* gene to demonstrate equal loading. (**B**) Western blot analysis of AtPIN2p. Roots from *Arabidopsis* plants grown in liquid medium or on agar plates were used to isolate microsomal membranes by differential centrifugation. After fractionation by SDS–PAGE and blotting to PVDF cross-reacting proteins were identified using an affinity-purified antibody raised against a recombinant protein spanning a region corresponding to amino acids 275–487 of AtPIN2p. A 69 kDa signal corresponding to AtPIN2p was indirectly visualized with the help of peroxidase-conjugated antibodies.

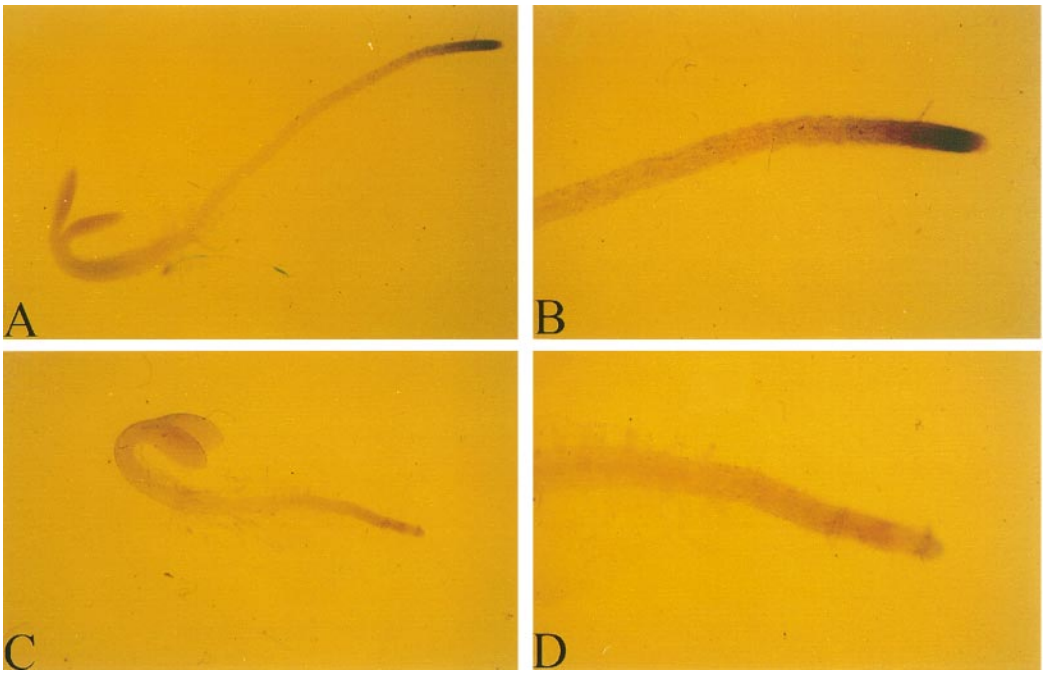

Fig. 6. Whole-mount *in situ* hybridization analysis of 3-day-old *Arabidopsis* seedlings using antisense (**A** and **B**) and sense probes (**C** and **D**) of *AtPIN2*. Seedlings were grown on agar medium. Signals were detected in the root tip indirectly visualized with the help of alkaline phosphatase-conjugated antibodies. Images shown in (B) and (D) are magnifications of seedlings shown in (A) and (B), respectively.

in *Arabidopsis* inflorescence axes (L.Gälweiler, C.Guan, A.Müller, E.Wisman, K.Mendgen, A.Yephremov and K.Palme, submitted). This common basal localization of AtPIN1 and AtPIN2 proteins in different auxin-transport-competent cells is consistent with the proposed direction of auxin flux in each of these tissues (Lomax *et al*., 1995).

Discussion

The mechanisms underlying gravitropic growth have been intensively investigated since Cholodny and Went independently proposed their elegant models, leading to the formulation of the Cholodny–Went hypothesis (Evans, 1991; Estelle, 1996). This hypothesis predicts that gravitropic curvature of plant organs results from redistribution of the plant hormone auxin. By differential transport processes, auxin accumulates at one side of the organ thereby affecting the differential elongation growth at one side of the tissue causing directional curvature (Harrison and Pickard, 1989; Young *et al*., 1990). Direct demonstration of a gravity-induced auxin gradient in roots has been problematic, mainly due to artifacts which can occur when radioactively labeled auxin is applied exogenously to roots (Pernet and Pilet, 1976) and the lack of better analytical techniques.

To study the mechanisms involved in gravitropism control, mutants altered in their response to gravity provide important tools. Thus, several mutants impaired in distinct steps of gravitropic control have been isolated. Interestingly, most of these mutants were derived from genetic screens for auxin resistance (Hobbie and Estelle, 1995; Bennett *et al*., 1996), suggesting a strong link between defects in root auxin physiology and control of gravitropism. Although most available mutants still require a detailed functional characterization, physiological experiments using roots incubated with either exogenous auxin or polar auxin transport inhibitors such as *N*-1-naphthylphthalamic acid (NPA) have demonstrated that auxin transport plays an essential role in the regulation of gravitropism. Roots of some agravitropic mutants, such as *axr4* (*rgr1*), are resistant to natural and synthetic auxins like IAA and 2,4-D as well as to polar auxin transport inhibitors (Hobbie and Estelle, 1995; Simmons *et al*., 1995).

In our study, we describe the isolation and characterization of an *Arabidopsis* mutant, *Atpin2::En701*, and the corresponding *AtPIN2* gene. We demonstrate that this agravitropic mutant displays reduced root elongation growth. Reduced root elongation of *Atpin2::En701*, even on medium without exogenous auxin, mimics the effect of high auxin concentrations or polar auxin transport inhibitors such as NPA on wild-type root growth (Muday and Haworth, 1994), consistent with a lack of auxin efflux carrier function in cortical and epidermal cell files. Furthermore, NPA causes agravitropic root growth as has been observed for *Atpin2::En701* and several other auxin-transport-deficient mutants (Muday and Haworth, 1994; Bennett *et al*., 1996; Garbers *et al*., 1996). The altered auxin response of *Atpin2::En701* roots and the agravitropism can therefore be rationalized as a result of a defect in auxin transport processes.

The predicted structure of the AtPIN2 protein suggests that it performs a transport function, sharing a similar topology with carrier proteins from the major facilitator superfamily, a widely conserved class of transport proteins in bacteria and eukaryotes. These transporter proteins typically contain 12 transmembrane domains which are interrupted by a central and apparently dispensable hydrophilic loop resulting in a 6 + 6 transmembrane segment pattern. Members of this transporter superfamily catalyze the transport of a wide variety of drugs such as quinolone

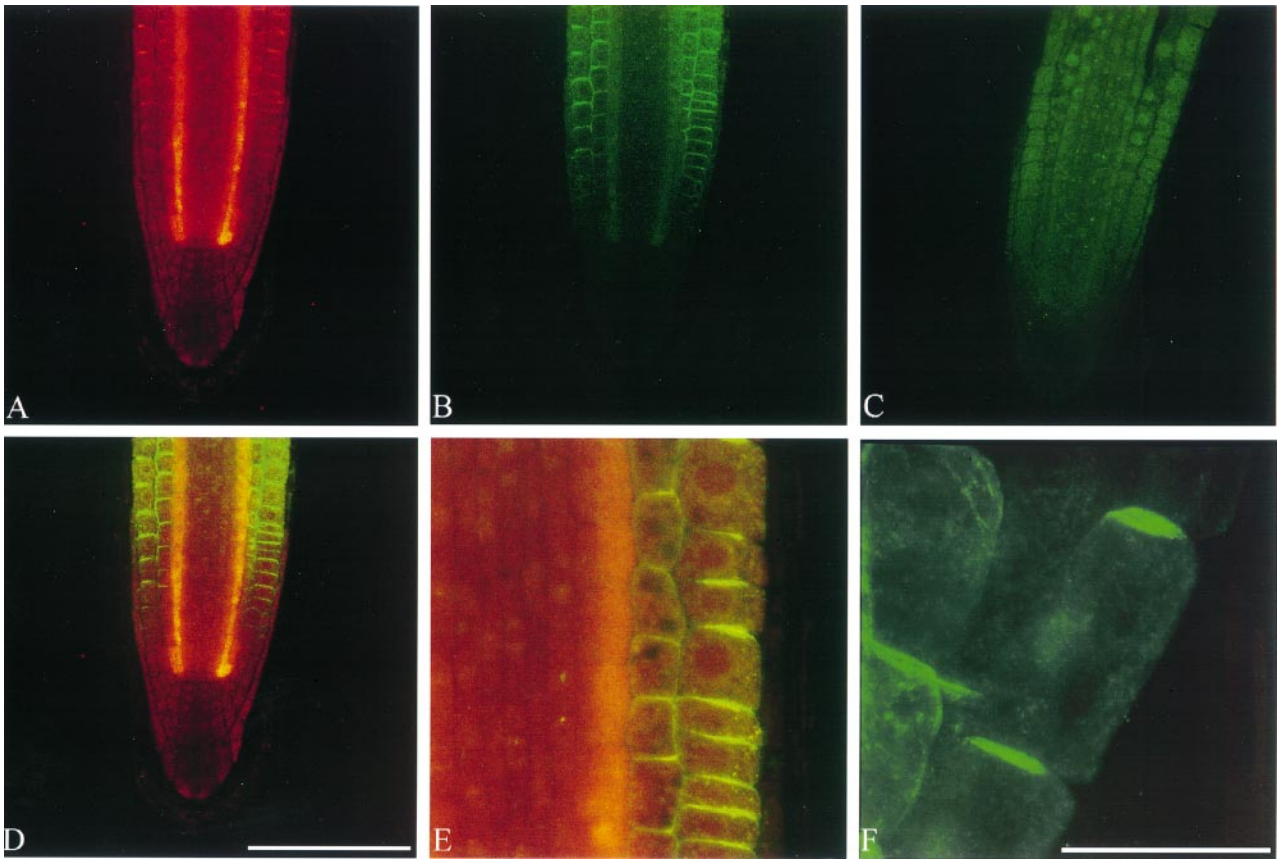

Fig. 7. Localization of AtPIN2p in 4-day-old *Arabidopsis* seedling root tips. Immunocytochemical analysis was performed using primary antibodies raised against AtPIN2p and a FITC-conjugated secondary antibody. Fluorescent staining was imaged by laser-scanning confocal microscopy. Indirectly visualized signals of the FITC-conjugated antibody are indicated in green. The red tissue autofluorescence image (**A**) of wild-type roots as well as the composition image (D), overlaying images (A) and (B), facilitate the histological localization of the AtPIN2p specific signals (green) in cortical and epidermal wild-type root cells (**B**, **D** and **E**). Magnification of cortex and epidermis cells (E) revealed preferential AtPIN2p localization at the anti- and periclinal sides of cortical cells adjacent to other cortical or epidermal cells as indicated already in (B) and (D). The image of single epidermis cells (**F**) presented the AtPIN2p signals preferentially at the basal cell side, oriented towards the elongation zone. Fluorescent staining of *Atpin2::En701* mutant roots (**C**) revealed diffuse background staining only. (D) Bar = 100 μm; (E) bar = 25 μm.

or tetracycline, sucrose, lactose, arsenic acid and organic phosphate esters (Marger and Saier, 1993; Diorio *et al*., 1995; Hoenke *et al*., 1997).

As a member of the *PIN* gene family, AtPIN2p shares sequence and topology similarity with AtPIN1p. Mutations within the *AtPIN1* gene result in a drastically reduced level of basipetal auxin transport within the inflorescence tissues, leading to the proposal that the AtPIN1 protein plays an important role in the control of cellular auxin efflux (L.Gälweiler, C.Guan, A.Müller, E.Wisman, K.Mendgen, A.Yephremov and K.Palme, submitted; Goto *et al*., 1991; Okada *et al*., 1991). Mutations within *AtPIN2* confer an auxin-sensitive phenotype which is consistent with a loss of auxin efflux carrier function in root cells (Figure 4). We rationalize that exogenous auxin will accumulate within elongating root cells in the absence of an auxin efflux carrier, resulting in an auxin-sensitive phenotype and subsequently causing inhibition of root growth. Furthermore, *Atpin2* mutant roots exhibit greater sensitivity towards the auxins 1-NAA > IAA > 2,4-D (Figure 4), which is consistent with the known substrate specificity of the auxin efflux carrier (Delbarre *et al*., 1996). The characteristics of the *Atpin2* mutant contrast with that described for the putative auxin influx carrier mutant *aux1* which confers reduced auxin sensitivity, consistent with an impaired ability of the mutant to accumulate inhibitory levels of auxin (Bennett *et al*., 1996).

Auxin is unique amongst plant hormones in demonstrating a polarity in its movement (Goldsmith, 1977). Rubery, Sheldrake and Raven have proposed within their chemiosmotic hypothesis that the polarity of IAA transport reflects the asymmetric subcellular distribution of auxin efflux (and possibly auxin influx) carrier proteins (Rubery and Sheldrake, 1974; Raven, 1975).

The AtPIN1 protein has been localized to the basal side of auxin-transport-competent cells within root and shoot vascular tissues (L.Gälweiler, C.Guan, A.Müller, E.Wisman, K.Mendgen, A.Yephremov and K.Palme, submitted). The cellular immunolocalization of AtPIN2p revealed a basal distribution within epidermal root cells in the meristematic and the elongation zone (Figure 7). In the epidermis, auxin is thought to be transported basipetally from meristematic tissues towards the elongation zone of roots (Ohwaki and Tsurumi, 1976; Lomax *et al*., 1995). The asymmetric cellular localization of AtPIN1p in inflorescence tissues (L.Gälweiler, C.Guan, A.Müller, E.Wisman, K.Mendgen, A.Yephremov and K.Palme, submitted) and AtPIN2p in the root epidermis is consistent with a role in mediating directional auxin fluxes within these cell types.

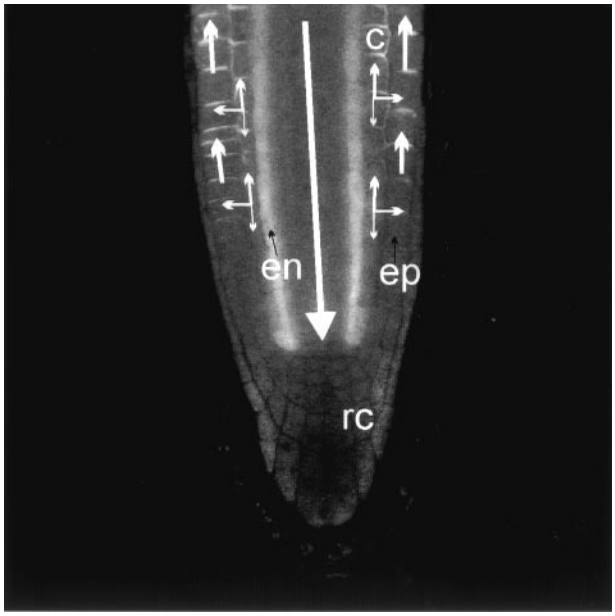

Fig. 8. A scheme for AtPIN2p function mediating auxin fluxes in *Arabidopsis* roots. Arrows indicate the major routes for auxin flow in the root. In the stele, auxin is transported acropetally towards the root tip. In the tip region auxin redistribution from the stele into cortical (c) and epidermal (ep) cell layers occurs and, due to the function of the AtPIN2 protein, subsequent basipetal transport towards the elongation zone follows. A weaker acropetal movement of auxin in the cortex is also shown (thin arrows) which can not be excluded based on our data. Endodermis (en) and root cap (rc) are indicated.

The transporter-like topology of the AtPIN2 protein, its homology to AtPIN1p as well as to several other transporters, the remarkable polarized localization of AtPIN2p in root cells and the *Atpin2* mutant phenotype suggest that AtPIN2p influences root gravitropism by functioning as a transmembrane component of the auxin efflux carrier complex. Morris *et al*. (1991) proposed that the auxin efflux carrier complex consists of at least three components: a transmembrane carrier protein, an NPA-binding protein and a third component of high turnover. Ruegger *et al*. (1997) have described the isolation of the *tir3* mutant which exhibits significantly reduced levels of NPA binding, leading to the suggestion that *TIR3* may encode (or regulate the activity of) the NPA-binding protein associated with the efflux carrier. AtPIN2p is likely to represent the transmembrane component of the auxin efflux carrier complex.

In summary, our results have led us to formulate a model featuring AtPIN2p regulating directional auxin efflux in cortical and epidermal root cells. As depicted schematicaly in Figure 8, auxin, supplied from the shoot to the root tip via the stele into cortical and epidermal cells, is basipetally transported backward towards the elongation and differentiation zone by AtPIN2p (Lomax *et al*., 1995; Estelle, 1996). This hypothesis is supported by various physiological measurements in maize roots which demonstrate the existence of such basipetal auxin transport (Pernet and Pilet, 1976; Martin *et al*., 1978). We propose that the loss of AtPIN2p function impairs basipetal auxin transport, hence disrupting the response of the elongation zone upon gravistimulation.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* Heynh., ecotype Columbia (Col-O) or Landsberg erecta (Ler), were grown on soil under white light in a controlled environment chamber (irradiance 150 μE/m^2/s, 16 h light, 60% humidity, 20–25°C day temperature, 10–15°C night temperature). For *in vitro* culture, seeds were surface sterilized using 5% sodium hypochloride and plated on agar MS medium (4.3 g/l MS salts, 1–3% sucrose, 1% bactoagar pH 6.0 with 1 M KOH; Murashige and Skoog, 1962). To break dormancy, seeds were incubated for 1–2 days at 4°C. The hormones NAA and IAA were dissolved in 1.0 M NaOH, whilst 2,4-D was resuspended in 100% dimethyl sulfoxide (DMSO). The resuspended hormones were diluted with water (30:70) to provide stock solutions which were later added at various dilutions to plant growth media.

Auxin resistance assays

Seedlings were grown on MS agar in constant white light at 22°C for 3 days. Seedlings of similar size were transferred to MS agar containing various concentrations of auxins and the position of primary root tips was marked. The seedlings were grown vertically for 3 days under the same growth conditions, then additional root growth was measured. The percentage growth relative to the control growing on media with no auxin addition was calculated.

Mutant isolation

The *Atpin2::En701* mutant was identified by reverse genetic screening of an *En-1* mutagenized collection of *Arabidopsis* plants. PCR-based screens were performed according to a three dimensional grid as described (Baumann *et al*., submitted). The following primers specific for the *AtPIN2* gene were used: P2-En4: 5′-GAAGAAAAGG-GCATGTGGAAA-3′; P2-En5: 5′-CCTCTGGTCATAGCATTCTT-3′.

General cloning procedures and database searches

All cloning procedures were performed according to standard methods (Ausubel *et al*., 1994). *Arabidopsis* genomic and cDNA libraries were screened using a probe derived from the *AtPIN1* gene (DDBJ/EMBL/GenBank accession No. AF089084; Gälweiler *et al*., submitted). Sequencing was performed using an ABI sequencer (377) according to the manufacturer's instruction. Sequences were aligned and analyzed using Gap, Map and Pileup programs of the GCG program package (version 8; Genetics Computer Group, Madison, WI; Devereux *et al*., 1984). The nucleotide and predicted amino acid sequences were compared with the database using BLASTN and TFASTA algorithm (Altschul *et al*., 1990; Pearson, 1990). Oligonucleotides were designed using the Oligo4S program and obtained from commercial suppliers (MWG, Ebersbach). For polypeptide topology prediction, the following servers were used: http://psort.nibb.ac.jp (Psort; Nakai and Kanehisa, 1992), http://www.embl-Heidelberg.de/predictprotein/predictprotein.html (PHD topology; Rost, 1996), http://ulrec3.unil.ch/ software/TMPRED_form.html (Tmpred; Hofmann and Stoffel, 1993) and Peptidestructure (GCG program package; Devereux *et al*., 1984).

Northern analysis

Arabidopsis plants were grown either *in vitro* on agar plates for 3 days, in liquid culture for 2 weeks or in the soil for 3 weeks. Organs were collected, frozen in liquid nitrogen and stored at –80°C. Isolation of mRNA and Northern blot hybridizations were performed as described (Teichmann *et al*., 1997). Expression of *RPL4* (ribosomal protein large subunit 4; Kim *et al*., 1990) was used to monitor uniformity of loading of RNA.

In situ hybridization

A region of *AtPIN2* cDNA corresponding to nucleotides 841–1461 was cloned in pBluescript and used as a probe. Digoxigenin-labeled sense and antisense probes were prepared by *in vitro* transcription (Boehringer Mannheim). Three-day-old seedlings were fixed, permeabilized and hybridization performed as described (Ludevid *et al*., 1992). After hybridization, the samples were washed once with 4× SSC for 10 min at 20°C, 2× SSC for 30 min at 20°C, and then with 0.2× SSC at 65°C for 30 min.

Immunolocalization and Western blot analysis

To generate polyclonal AtPIN2p-specific antibodies, a PCR fragment carrying the cDNA sequence coding for a peptide between amino acids

275–487 was synthesized and inserted into pT7H6(GS)$_2$ (Studier *et al*., 1990). Expression of the recombinant protein containing an N-terminal His_6 tag was performed in *Escherichia coli* BL21 after induction with CE6 phages. The 24 kDa recombinant protein was extracted from bacteria, purified by Ni^{2+}–NTA chromatography and used for immunization of rabbits. The rabbit polyclonal anti-AtPIN2p antiserum was affinity purified according to standard procedures (Ausubel *et al*., 1994) and analyzed by Western blotting using the recombinant antigen and *Arabidopsis* microsomal fractions. Microsomal fractions of *Arabidopsis* roots from plants grown in liquid medium or on agar plates were isolated by differential centrifugation (Zettl *et al*., 1994), incubated in SDS–PAGE sample buffer for 5 min at 42°C, and separated by electrophoresis on 10% SDS–PAGE gels. Proteins were electrotransferred to PVDF membranes, the blots were blocked with phosphate-buffered saline/4 % low fat milk powder and incubated with anti-AtPIN2p antibodies. Signal detection was achieved using peroxidase-conjugated secondary antibodies and by performing chemiluminescence reaction (ECL kit; Pierce) followed by X-ray film exposure.

Immunofluorescence analysis

Four-day-old *Arabidopsis* seedlings were incubated for 1 h under vacuum in 50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$ pH 7.0 (MTSB). Seedlings were subsequently washed three times for 15 min in MTSB/0.1% Triton X-100, three times in water/0.1% Triton X-100 and then incubated in MTSB/2% Driselase (Sigma, Deisenhofen) for 15–30 min. After washing three times with MTSB, seedlings were permeabilized by incubation in 0.5% nonident P-40/10% DMSO in MTSB for 1 h, washed again three times with MTSB and incubated in blocking buffer (3% bovine serum albumin/MTSB) for 30 min. Permeabilized seedlings were incubated with affinity-purified anti-AtPIN2p antibodies in a humid chamber for 1 h, washed three times for 5 min in MTSB and further incubated for 1–2 h at 20°C with FITC-conjugated anti-rabbit IgG antibodies (Boehringer Mannheim) diluted in blocking buffer. Before mounting, seedlings were washed three times for 5 min with MTSB followed by washing twice with water. Fluorescent signal detection and documentation was performed with the help of a confocal laser scanning microscope (Leica DMIRBE, TCS 4D with digital image processing) using a 530 ± 15 nm band-pass filter for FITC-specific detection and a 580 ± 15 nm band-pass filter for autofluorescence detection. For histological signal localization, both images were electronically overlayed resulting in a red autofluorescence and green–yellow AtPIN2p-specific fluorescence. Images were further processed by Photoshop 4.0 for graphic presentation.

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During revision of this manuscript, Lusching *et al*. reported the isolation and characterization of the *Arabidopsis EIR1* gene (*Genes Dev.*, **12**, 2175–2187). The agravitropic mutation eirl-3 was shown to be allelic to the previously described mutations wav6-52/agr1.