Regulation of Polar Auxin Transport by AtPIN1 in Arabidopsis Vascular Tissue

Leo Gäweiler, Changhui Guan, Andreas Müller, Ellen Wisman, Kurt Mendgen, Alexander Yephremov, Klaus Palme*

Polar auxin transport controls multiple developmental processes in plants, including the formation of vascular tissue. Mutations affecting the PIN-FORMED (PIN1) gene diminish polar auxin transport in Arabidopsis thaliana inflorescence axes. The ATPIN1 gene was found to encode a 67-kilodalton protein with similarity to bacterial and eukaryotic auxin carrier proteins, and the ATPIN1 protein was detected at the basal end of auxin transport–competent cells in vascular tissue. ATPIN1 may act as a transmembrane component of the auxin efflux carrier.

Charles Darwin had proposed the concept of translocated chemical messengers in higher plants, which finally resulted in the discovery of polar auxin transport in the 1930s (1). The transport of auxin from the plant tip downward provides directional information, influencing vascular tissue differentiation, apical development, organ regeneration, tropic growth, and cell elongation (2, 3). Polar auxin transport can be monitored by following the movement of radiolabeled auxin through tissues. Auxin transport is specific for the major auxin indoleacetic acid and various synthetic auxins, it requires energy, and it occurs with a velocity of 7 to 15 mm/hour (2).

This transport can be specifically inhibited by synthetic compounds, known as polar auxin transport inhibitors, and by naturally occurring flavonoids (4). The current concept, known as the "chemiosmotic hypothesis," proposes that (i) the driving force for polar auxin transport is provided by the transmembrane proton motive force, and that (ii) the efflux of auxin anions is mediated by saturable, auxin-specific carriers in shoots and root apices. The phenotype of the hpt-1 mutant represents the naked, pin-like phenotype of Arabidopsis thaliana. The phenotype of the hpt-1 mutant can be mimicked by chemical inhibition of polar auxin transport (6). Analysis of auxin transport in hpt mutants suggests that an essential component for auxin transport is affected (6, 7).

To isolate the affected AtPIN1 gene locus, we used the autonomous transposable element Ener from maize to generate mutants in Arabidopsis thaliana. We identified three independent transposon-induced mutants, AtPIN1::En134, AtPIN1::En111, and AtPIN1::En349, that exhibited auxin transport–deficient phenotypes (8). These plants developed naked, pin-shaped inflorescences.

Fig. 1. Phylogenetic and Southern blot analysis of the transposon insertion mutant AtPIN1::En134. (A) The most obvious phenotypic aspect of the homozygous mutant represents the naked, pin-like phenotype of Arabidopsis thaliana. The phenotype of the AtPIN1::En134 mutant population. The Mpo gene of the heterozygous AtPIN1::En134 mutant showed 3:1 segregation for wild-type and mutant phenotype plants (8). The cetyltrimethylammonium bromide method (23) was used to isolate genomic DNA from plants showing the mutant trait (22, 27, 25, 28) and wild-type (12, 43, 45, 46, 47, 52, 56, 60, 75, 78, 79) phenotype and from the ecotype Columbia (Col) plants lacking En-1 insertions. After xbaI digestion, the DNA was separated on a 0.8% agarose gel (2 μg per lane), transferred to a Nylon membrane and hybridized with a [32P]-labeled 3′-end probe of the En-1 transposon (24). Only one fragment of 2.3 kb in length (marked by an arrow) was commonly detected in all 12 tested homozygous AtPIN1::En134 mutants and in 15 heterozygous plants (not all are shown), indicating cosegregation with the AtPIN1::En134 allele. Size bars represent 25 mm (A), 2.5 mm (B), and 10 mm (C) and (D).

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29. G. Chang, R. Spencer, A. Lee, M. Barclay, D. Rees, data not shown.
40. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G; Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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and abnormalities in the number, size, shape, and position of lateral organs (Fig. 1, A to D), similar to those described for the pin-formed mutant (6, 7). In crosses between heterozygous pin-formed and Atpin1::En134 mutants, 25% of the F₂ progeny showed the mutant phenotype, indicating that these mutations were alleles of the same gene (9). Further analysis showed that Atpin1::En111 and Atpin1::En349 were also allelic to Atpin1::En134 (Fig. 2A) (10).

**The AtPIN1 gene.** To identify the En-1 transposon insertion responsible for the mutant phenotype, we performed Southern (DNA) blot analysis with the M₂ progeny of a heterozygous Atpin1::En134 mutant. An En-1 probe corresponding to the 3' end of the transposon detected a single 2.3-kb fragment in heterozygous Atpin1::En134 (lane 2) and Atpin1::En349 (Fig. 3B, lane 5). Heterozygous plants (Fig. 3B, lanes 1, 4, and 6) showed AtPIN1 expression, probably from their wild-type allele. Similarly, heterozygous *pin-formed* mutants did not express *AtPIN1* (Fig. 3A, lane 3). We used an *AtPIN1* cDNA probe to identify a yeast artificial chromosome (YAC) contig from the CIC YAC library that represented a region between centimorgan 92.7 and 113.6 in chromosome 1 of *Arabidopsis* similar to the location of the *PIN-FORMED* locus (7, 12). These data from genetic analysis, physical mapping, and gene

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**Fig. 2.** Structural analysis of AtPIN1 alleles and of the deduced AtPIN1 amino acid sequence. (A) Structure of the *AtPIN1* gene (drawn to scale), with black boxes representing exons and mapped En-1 insertion sites in the independent mutant alleles Atpin1::En111 (111), Atpin1::En134 (134), and Atpin1::En349 (349). Numbers in brackets show base pair positions. The positions of the translational start (ATG) and termination codons (TGA) of the predicted open reading frame are depicted. Nucleotide sequences flanking both ends of the En-1 transposon in Atpin1::En134 show the disruption of the coding sequence at codon 45 (F). The duplication of nucleotide triplets (TTT) is characteristic for *En*-1 insertion sites (25). (B) Amino acid sequence (26) deduced from the *AtPIN1* cDNA (accession number AF089084). (C) Hydropathy analysis of AtPIN1. The hydropathy plot was generated with the Lasergene software (DNASTAR, Madison, Wisconsin) and the method of Kyte and Doolittle with a window size of nine amino acids (27).

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**Fig. 3.** *AtPIN1* gene expression analysis. (A and B) Northern blot analysis. Total RNA from different organs and plants were isolated and northern blot analysis was performed (15 μg of total RNA per lane) with a **32**P-radiolabeled *AtPIN1* probe (base pairs, 602 to 1099) probe (26). In (A) various *A. thaliana* ecotype Columbia organs were analyzed: cotyledons (lane 1), flowers (lane 2), roots (lane 3), rosette leaves (lane 4), seedlings (lane 5), inflorescence axes (lane 6), and siliques (lane 7). In (B) different allelic AtPIN1 mutants were analyzed: heterozygous Atpin1::En134 (lane 1), homozygous Atpin1::En134 (lane 2), homozygous *pin-formed* (lane 3), heterozygous *AtPIN1::En349* (lane 5), and wild-type Columbia (lane 7). The RNA was prepared from inflorescence axes of each genotype. (C to E) In situ hybridization analysis of the *AtPIN1* gene expression in wild-type inflorescence axes. Stem segments of plants were fixed, paraffin embedded, cross sectioned (8 μm), and probed with either antisense (C and E) or sense (D), digoxigenin-labeled, in vitro–transcribed *AtPIN1* RNA. The *AtPIN1* transcript signals were indirectly visualized with the help of alkaline phosphatase–conjugated secondary antibodies (29). (E) is a magnified section of a vascular bundle of (C). AtPIN1–specific staining (red) is localized in cambial and xylem tissues. (F and G) Immunocytochemical localization of AtPIN1 protein in cross sections of inflorescence axes. Stem segments of wild-type plants were fixed, paraffin embedded, sectioned (8 μm), and incubated with affinity-purified polyclonal anti-AtPIN1. Bound anti-AtPIN1 was visualized with the help of alkaline phosphatase–conjugated secondary antibodies (18, 30). AtPIN1–specific staining (purple) was found in cambial and in young and parenchymatous xylem cells (G). Size bars represent 100 μm (E) and 200 μm (C), (D), and (F).
expression studies confirmed that the cloned AtPIN1 gene corresponded to the PINFORMED locus. As the phenotypes of both pin-formed and Atpin1::En mutants are based on null mutations and a complete loss of the AtPIN1 expression, we conclude that the pin-formed and Atpin1::En mutants both lack the same component functional in polar auxin transport in Arabidopsis inflorescence axes (13).

The AtPIN1 protein. The predicted AtPIN1 gene product is 622 amino acids long and includes 8 to 12 putative transmembrane segments flanking a central region that is predominantly hydrophilic (Fig. 2C). Similar topologies have been described for proteins that are involved in a wide variety of transmembrane transport processes (14). Database comparisons and screening of libraries with AtPIN1 probes identified several Arabidopsis genes with similarity to AtPIN1 (15). The homologous gene AtPIN2 (also known as EIR1) may encode another catalytic subunit of auxin efflux carrier complexes that performs a similar function in root cells (16). Genes similar in sequence to the AtPIN genes were found in other plant species, even in the evolutionarily distant monocotyledonous species of maize and rice, indicating that AtPIN1 and related genes may be of fundamental importance in plant development (17).

To analyze the function of the AtPIN1 protein in plants, we raised polyclonal antibodies to a portion (amino acid 155 to 408) of recombinant AtPIN1 with an NH2-terminal His6 affinity tag. The affinity-purified antibody to AtPIN1 (anti-AtPIN1) identified on protein immunoblots a protein from Arabidopsis microsomes matching the molecular

Fig. 4. AtPIN1 immunolocalization in longitudinal Arabidopsis tissue sections. (A to F) Indirect immunofluorescence analysis by laser scanning confocal microscopy. Stem segments of plants were fixed, sectioned, and incubated with polyclonal anti-AtPIN1 (18). Bound anti-AtPIN1 was indirectly visualized with the help of fluorescent (FITC) secondary antibodies (30). The immunofluorescent cells (green-yellow signals) formed continuous vertical cell strands in vascular bundles (A). The AtPIN1 signals are found at the basal end of elongated, parenchymatous xylem cells in the neighborhood of vessel elements, which are distinguished by secondary cell wall thickening structures (C). The red tissue autofluorescence [(A), (C), (E), and (F)] and comparison with the corresponding differential interference contrast (DIC) images [(B) and (D)] facilitated the histological localization of the AtPIN1-specific signals. The arrows point to the AtPIN1-specific fluorescence at the basal end of the xylem cells (C) or to the corresponding positions in the DIC image (D). They also indicate the direction of polar auxin transport in the tissue studied. In (C) two fluorescent signals of three cells forming a vertical cell strand are shown. The upper signal is found at the basal end of the cell extending out of the top of the picture. The cell underneath is fully shown in vertical extension, also fluorescently labeled at its basal end. The fluorescent signal of its basally contacting cell is not shown, because its basal end is out of the picture. A longitudinal hand section of an Arabidopsis stem is shown in (E). AtPIN1 immunofluorescence is primarily localized to the basal side of the cells extending slightly up the lateral walls. A control with a longitudinal section from the Atpin1::En134 mutant is shown in (F). No AtPIN1-specific fluorescent signals were detected. (G) Ultrathin tissue sections were incubated with the polyclonal anti-AtPIN1 and gold-coupled secondary antibodies and examined with an electron microscope (18, 31). Gold grains (marked by arrows) were detected only in one membrane of two contacting cells and were absent at the opposite plasma membrane. ep, epidermis; co, cortex; cw, cell wall; cy, cytoplasm; pm, plasma membrane; pi, pith; v, vessel; vb, vascular bundle. Size bars represent 25 μm [(C), (E), and (F)], 100 μm (A), and 0.1 μm (G).

Fig. 5. Analysis of vascular patterning in Atpin1::134 mutants (32). Inflorescence of a wild-type Columbia Arabidopsis plant (A), an Atpin1::En134 mutant (B), and a wild-type plant (C), grown in the presence of auxin transport inhibitor NPA (15 μM). Cross sections were cut as indicated by arrows in (A), (B), (C). The sections presented were cut just above the first cauline leaf (1, 4, 7) and directly below the first (2, 5, 8) and second cauline leaves (3, 6, 9). Arrows on the cross sections (5, 6, 8, 9) indicate the position of the leaves above. Abnormal xylem proliferation was observed in the inflorescence axis below cauline leaves, adjacent to the leaf attachment site. The diameters of the stem sections are ~1 to 2 mm.
AtPIN1 mutants and its effects on plant development indicate a role of AtPIN1 in polar auxin transport, most likely in supporting auxin efflux from the cell. On the basis of the predicted topology of AtPIN1, its homology to carrier proteins, and its polar localization in auxin transport–competent cells, we propose that AtPIN1 might act as a catalytic auxin efflux carrier protein in basipetal auxin transport.

References and Notes
3. G. W. van der Weij, ibid. 31, 810 (1934).
15. Crosses between the heterozygous transposon inser- tional mutants yielded ~25% mutant phenotypes in the F1 generation, indicating allelism. Using En-1 and AtPIN1-specific primers amplified by reverse transcription (R. Zettl, J. Schell, K. Palme, EMBO J. 13, 717 [1994]) with linker-specific oligonucleotides after Csp6 I restriction of genomic DNA and ligation of compatible linker DNA. The isolated flanking DNA was used as a probe to screen a cDNA library, prepared from suspension cells, for homologous clones that were then used to screen a genomic library of A. thaliana. The λ libraries were prepared from the ecotype Columbia and provided by the Arabidopsis DNA Centre, Cologne. Sequence analysis of the longest AtPIN1 cDNA (2276 base pairs) identified an open frame encoding 632 amino acids. An in-frame stop codon located upstream to the ATG suggested that the cDNA encodes a full-length protein. GenBank accession numbers are as follows: AF098904 (AtPIN1 cDNA) and AF098905 (AtPIN1 genomic DNA).
16. By screening the CIC YAC library [F. Creusot et al., Plant J. 8, 763 (1995); provided by the Arabidopsis DNA Centre, Cologne], we identified a genomic clone carrying the AtPIN1 gene. Using a genomic library and primer pairs to identify a contig consisting of the overlapping clones CIG61H, C12G10, C12H9D, and C12H4C. Physical mapping was performed with the probe http://cbil.humgen.upenn.edu/~atgcphysical.html.
17. Repeating auxin transport measurements with stem segments, we confirmed the reduction of polar auxin transport in pin-formed mutants (6, 7) and found a reduction of polar auxin transport in AtPIN1::En134 mutants as well.
19. GenBank accession numbers of homologous clones in Arabidopsis thaliana are as follows: ACOO2293, AF056026 (EIR1), AF086906 (AtPIN2 cDNA), AF086907 (AtPIN2 genomic DNA), AC002291, AC005560, AB017068, AC004260, AC003979, AF087016, AF087818, AF087819, AF087820, 861585, T43633, T44648, Z38075, and R41517.
27. The 3’-End probe DNA was generated by PCR with the 5’-specific primers En 1—CATCGATGATCTCATCGTATG-3’ and En 8141—5’-GGAC-GGCAGCTCTTATGGTAAACG-3’. In Southern blot analysis, this probe hybridized to the 3’-ends of Xba I-digest En 1 DNA, detecting fragments of 198-kb En 1 DNA plus flanking plant DNA.
29. Single-letter abbreviations for amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
31. P. Chomczynski and N. Sacchi, Anal. Biochem. 162, 156 (1986). To check for equal RNA loading we rehybridized the Northern blots with ribosomal protein large subunit 4 (RPL4) and ubiquitin carrier (UBC) probes.
32. Segments of inflorescence axes of 3- to 4-week-old A. thaliana ecotype Columbia grown in a greenhouse at 18° to 24°C, with 16 hours of light were fixed, paraffin embedded, and analyzed by in situ hybridization as described (22), with the following modifications. To generate AtPIN1-specific RNA probes, we inserted the Bgl II–Hind III fragment of the AtPIN1 cDNA (base pairs 602 to 1099) into the Bam HII–Hind III–cleaved vector pBluescript SK– (Stratagene).

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generating pin23HIX. After linearizing pin23HIX (Hind III for antisense and Xba I for sense transcription), we performed in vitro transcription and digoxigenin labeling using the DIG RNA Labeling Kit (Boehringer Mannheim). The RNA hybridization was performed overnight at 42°C with a probe concentration of 30 ng per 100 μL. The slides were then washed with 4× standard saline citrate (SSC) containing 5 mM diethy- thoacetate (DTT) [10 min, room temperature], 2× SSC containing 5 mM DTT (30 min, room temperature), and 0.2× SSC containing 5 mM DTT (30 min, 65°C). After blocking with 0.5% blocking agent (Boehringer Mannheim), we detected signals using anti-digoxi- gin (1:3000, Boehringer Mannheim) coupled to alkaline phosphatase followed by a nitroblue tetrazolium, brome-chloro-indolyl phosphate staining reaction.

30. Inflorescence axes of 3- to 4-week-old Arabidopsis wild-type and mutant plants (grown in a greenhouse at 18° to 24°C, with 16 hours of light) were cut and fixed in ice-cold methanol/acidic acid (3:1). Paraffin embedding, sectioning, and mounting were done as described (22). Antibody incubation and immunohistochemical staining was performed as described [S. Reinold and K. Hahlbrock, Plant Physiol. 112, 131 (1996)], with the following modifications: 8-μm cross sections and 30-μm longitudinal sections of inflorescence axes were incubated with affinity-puri- fied anti-AtPIN1 ([18], 4°C, overnight), diluted 1:100 in buffer [3% (w/v) milk powder in phosphate-buff- ered saline (PBS), pH 7.4]. Incubation with secondary antibodies coupled to fluorescein isothiocyanate (FITC) or alkaline phosphatase (Boehringer Mannheim, 1:100) was done at room temperature for 2 to 3 hours. After antibody incubation, washing was per- formed three times (10 min) with PBS containing 0.2% Tween 20. For hand sectioning, stem segments were fixed in 4% paraformaldehyde, diluted in MTBS (50 mM piperazine ethanesulfonic acid, 5 mM ethyl- ene glycol tetraacetic acid, 5 mM MgSO₄, pH 7.0), treated with 2% Driselase (Sigma, in MTBS, 0.5 hour), and permeabilized with 10% dimethylsulfoxide and 0.5% NP-40 (in MTBS, 1 hour). After hand sectioning with razor blades, antibody incubation was performed as described above. Alkaline phosphatase staining reactions were carried out for several hours to overnight, and the results were analyzed micro- scopically. Fluorescent signal analysis was performed with a confocal laser scanning microscope (Leica DMIRBE, TCS 4D with digital image processing) with 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 overlaid, resulting in red autofluorescence and green-yellow APiN1-specific fluorescence. DIC images were gener- ated to determine the exact cellular signal localiza- tion. Controls with preimmune serum and secondary antibodies alone yielded no specific signals. Tissue orientation of the longitudinal stem sections was determined with the help of residual traces of lateral leaves and by cutting stem segments apically and basally with different angles. Polar signal localization was also obvious in cells in which the immunostained cytoplasm was detached from the basal cell wall (9). The APiN1 localization results were reproduced by several experiments.

A Free-Fall Determination of the Newtonian Constant of Gravity
Joshua P. Schwarz, Douglas S. Robertson, Timothy M. Niebauer, James E. Faller

Recent determinations of the Newtonian constant of gravity have produced values that differ by nearly 40 times their individual error estimates (more than 0.5%). In an attempt to help resolve this situation, an experiment that uses the gravity field of a one-half metric ton source mass to perturb the trajectory of a free-falling mass and laser interferometry to track the falling object was performed. This experiment does not suspend the test mass from a support system. It is therefore free of many systematic errors associated with supports.

The measured value was \( G = (6.6873 \pm 0.0094) \times 10^{-11} \text{ m}^3 \text{kg}^{-1} \text{sec}^{-2} \).

Here we report a method for determining the Newtonian gravitational constant, \( G \), by measure- ing the perturbation to the acceleration of a free-falling object due to a well-known source mass. A precise knowledge of \( G \) is of considerable metrological interest, for it pro- vides a unique as well as valuable measure- ment challenge that sharpens and prepares experimental skills to better deal with a vari- ety of precision and null experiments. Yet despite two centuries of experimental effort, the value of \( G \) remains poorly known; recent determinations of \( G \) differ by as much as 40 times their individual estimates of uncertain- ty, suggesting the presence of significant sys- tematic errors. The difficulty in measuring \( G \) stems in part from the extreme weakness of the gravitational force and the conse- quent difficulty of generating a sufficiently large signal for accurate measurement. Ad- ditional problems arise from the difficulty of eliminating spurious forces because of such things as electromagnetic fields and thermal gradients. In 1798 Henry Cavendish performed the first experiment specifically designed to in- vestigate the gravitational attraction between small masses using a torsion balance to match the tiny gravitational force produced by local

source masses against the restoring torque of a fiber support. This was the first laboratory measurement of this elusive fundamental constant. In the 1930s Heyl reintroduced the “time-of-swing” measurement, in which source masses modulate the oscillation frequency of a torsion pendulum. Both types of torsion methods introduce experimental dif- ficulties that center on the need to calibrate precisely the restoring force. Indeed, the sub- tle properties of torsion fibers are still being investigated (1–3).

In 1982 Luther and Towler (4) used the time-of-swing method to achieve a value of \( G \) that because of its small error is the dominant contributor to the value that is accepted to- day. More recently, Fitzgerald and Arm- strong (5) developed a compensated torsion balance in which electrostatic forces cancel out the gravitational force of the source mass- es, and Michaelis and co-authors (6) experi- mented with a compensated torsion balance using a fluid mercury bearing instead of a fiber as a support. Walsec, Meyer, Piel, and Schurr (7,8) introduced a dual pendulum method in which the gravitational gradient of a source mass is measured through its effect on the length of a Fabry-Pérot cavity support- ed by two pendulums at different distances from the mass. Finally, Schurr, Nolting, and Kündig (9) recently published the experimen- tal results obtained using a beam-balance method [see (10) for discussion of these and other experiments].

The values for \( G \) determined from these experiments differ by more than 40 times the quoted standard errors. This situation—dis-
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