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 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 (4). 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 cellular efflux of auxin anions is mediated by saturable, auxin-specific carriers in shoots presumably located at the basal end of transport-competent cells (2). Immunocytochemical work with monoclonal antibodies to pea stem cell fractions indicated that the auxin efflux carrier is located at the basal end of auxin transport–competent cells (5).

Gene tagging. The phenotype of the pin-formed mutant of Arabidopsis can be mimicked by chemical inhibition of polar auxin transport (6). Analysis of auxin transport in pin-formed 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 En-1 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...
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 F1 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 M3 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 of Xba I–digested genomic DNA cosegregating with plants showing the mutant phenotype. This fragment was also detected in heterozygous plants, which segregated the mutant phenotype in about 25% of their M3 progeny, as expected for a recessive mutation (Fig. 1E). DNA flanking the tagged locus was isolated from the genomic DNA of homozygous Atpin1::En134 mutant plants with the use of a ligatation-mediated polymerase chain reaction (PCR). The resulting PCR fragment was sequenced and used as a probe to isolate homologous clones from wild-type Arabidopsis genomic and complementary DNA (cDNA) libraries (11). DNA sequence analysis revealed that the AtPIN1 gene consisted of five exons with lengths of 1246, 235, 244, 77, and 64 nucleotides (Fig. 2A). Analysis of mutant Atpin1 transposon insertion alleles showed that the En-1 element was inserted into the first exon of the AtPIN1 gene (Fig. 2A). Excision of the En-1 transposon from the Atpin1::En134 and Atpin1::En349 alleles resulted in revertant alleles that restored the wild-type phenotype. Sequence analysis of the revertant alleles confirmed that the En-1 element had excised from the first AtPIN1 exon, resulting in an exact restoration of the AtPIN1 open reading frame (9).

Northern (RNA) blot hybridizations with an AtPIN1–specific probe showed that the gene was transcribed in all wild-type organs tested, yielding a transcript signal of 2.3 kb in length (Fig. 3A). AtPIN1 gene expression was absent in the homozygous transposon insertion mutants Atpin1::En134 (Fig. 3B, 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, homozygous 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 expression 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 AF089804). (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).

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 AF089804). (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).

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 32P-radiolabeled AtPIN1 probe (bases 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 Atpin1::En134 (lane 3), heterozygous pin-formed (lane 4), homozygous Atpin1::En349 (lane 5), homozygous Atpin1::En349 (lane 6), 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 [G]) and 200 μm ([C], [D], and [F]).
expression studies confirmed that the cloned AtPIN1 gene corresponded to the PIN-FORMED 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

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**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.
Polar localization of AtPIN1. To localize the AtPIN1 gene products in situ, we probed cross sections of Arabidopsis inflorescence axes with antisense AtPIN1 RNA and polyclonal anti-AtPIN1. In both cases parenchymatous xylem and cambial cells were labeled (Fig. 3, C to G). Probing longitudinal sections from Arabidopsis inflorescence axes with affinity-purified anti-AtPIN1, we observed labeling at the basal end of elongated parenchymatous xylem cells (Fig. 4, A to E). The basal-apical orientation of the cells was identified with the help of angled razor cuts and residual leaf bases on the excised stem segments. AtPIN1-specific fluorescent signals were primarily located to the basal side of the plasma membrane, with some signal extending beyond the basal side forming a U-shaped fluorescent zone (Fig. 4E). Immuno- ngold labeling and electron microscopy of longitudinal tissue sections revealed gold grains exclusively at the upper membrane of two contacting cells (Fig. 4G). The polar localization of AtPIN1 in these tissues is consistent with the proposed distribution of auxin efflux carriers that mediate shoot-basipetal transport (Fig. 5A).

AtPIN1 mutants and its effects on plant development indicate a role of AtPIN1 in polar auxin transport, most likely in supporting efflux of auxin 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
A Free-Fall Determination of the Newtonian Constant of Gravity

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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 free-falling 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 \( G = (6.6873 \pm 0.0094) \times 10^{-11} \text{ m}^3 \text{kg}^{-1} \text{s}^{-2} \).

Here we report a method for determining the Newtonian gravitational constant, \( G \), by measuring 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 provides a unique as well as valuable measurement challenge that sharpens and prepares experimental skills to better deal with a variety of precision and null experiments. Yet additional problems arise from the difficulty of generating a sufficiently large signal for accurate measurement. Additional 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 investigate 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 difficulties that center on the need to calibrate precisely the restoring force. Indeed, the subtle 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 \( G \) because of its small error is the dominant contributor to the value that is accepted today. More recently, Fitzgerald and Armstrong (5) developed a compensated torsion balance in which electrostatic forces cancel out the gravitational force of the source mass, and Michaelis and co-authors (6) experimented with a compensated torsion balance using a fluid mercury bearing instead of a fiber as a support. Walesch, 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 supported by two pendulums at different distances from the mass. Finally, Schurr, Nolting, and Kündig (9) recently published the experimental 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—di-
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