# MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development

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**Abstract Previous data have suggested an involvement of MDR/PGP-like ABC transporters in transport of the plant hormone auxin and, recently, AtPGP1 has been demonstrated to catalyze the primary active export of auxin.**

**Here we show that related isoform *AtPGP4* is expressed predominantly during early root development. *AtPGP4* loss-of-function plants reveal enhanced lateral root initiation and root hair lengths both known to be under the control of auxin. Further, *atpgp4* plants show altered sensitivities toward auxin and the auxin transport inhibitor, NPA. Finally, mutant roots reveal elevated free auxin levels and reduced auxin transport capacities. These results together with yeast growth assays suggest a direct involvement of AtPGP4 in auxin transport processes controlling lateral root and root hair development.**



*Keywords:* Auxin transport; ABC transporter; MDR; *p*-Glycoprotein; Lateral root; Root hairs

## 1. Introduction

Despite its controversially discussed action as hormone, morphogen or neurotransmitter-like substance [1] auxin has proven as unique signaling molecule virtually controlling all plant developmental processes [2–4]. Recent research has concentrated on the polar transport of auxin, principally indole-3-acetic acid (IAA). Polar auxin transport (PAT) is regulated at the cellular level and is apparently both a product and determinant of cellular polarity [1,2,5]. Auxin loading and unloading is thought to be mediated by protein complexes that are characterized by members of the AUX and PIN protein families; the latter have been suggested to function as gradient-driven auxin influx and efflux carriers, respectively, but a clear biochemical verification is still lacking [5,20].

Previous findings have provided intriguing evidence that ABC transporters of the multidrug resistance (MDR)-like *p*-glycoprotein (PGP) subclass [6,7] are involved in auxin transport [8–11]. First, several MDR/PGPs (hereafter referred to as PGPs) found in *Arabidopsis* specifically bind the auxin efflux inhibitor 1-N-naphtylphtalamic acid (NPA, [12]). Second, the phenotype of *atpgp19* (*atmdr1*) and *atpgp1 atpgp19* T-DNA insertional null mutants and *atpgp1* antisense plants show a pleiotropic auxin-related phenotype [10,30]. Additionally, basipetal auxin transport in the hypocotyl of *atpgp1* and *atpgp19* single and double mutants is reduced [8,11]. Finally, loss of an *AtPGP1* ortholog in maize *br2* and sorghum *dw3* mutants leads to compact stalks and reduced basipetal IAA transport [13].

PIN1 has been shown to be mislocalized in Arabidopsis *pgp* mutants [9]. Therefore, in mechanistic models, it has been proposed that PGPs function in PIN vesicular trafficking [9,10], PIN regulation, or direct transport of auxin [9]. Very recently, AtPGP1 has been demonstrated by heterologous expression in yeast and mammalian cells to catalyze the primary active efflux of natural and synthetic auxin and oxidative breakdown products [11]. AtPGP1-mediated efflux is sensitive to auxin efflux and ABC transporter inhibitors. Moreover, AtPGP1 exhibits non-polar expression in the root and shoot apices and polar (mainly basal [36]) localization in mature cortical and endodermal cells in the distal elongation zone suggesting a function in polar or reflux movement of auxin [11,14]. In agreement, basipetal auxin transport from the root tip is strongly reduced. As a result auxin levels are reduced in *atpgp1* mutant plants as shown by gas chromatography–coupled mass spectrometry (GC–MS) analysis and reduced expression of the auxin responsive *DR5::GUS* reporter gene [11,15].

Genetic and physiological evidence suggests that auxin is required at several specific developmental stages to facilitate root hair (RH) development and lateral root (LR) formation [16,22,23,25]. While an interpretation for root hair development is complicated by interaction among hormone response pathways [21], a shoot-derived auxin pulse has been suggested to promote the emergence of LR primordia [17]. Lately, in a microarray-based approach to profile early lateral root initiation, a homologue of *AtPGP1*, *AtPGP4*, was shown to be induced 2 h after external lateral root induction [18]. Here we

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*Abbreviations:* PAT, polar auxin transport; IAA, indole-3-acetic acid; NPA, 1-*N*-naphtylphtalamic acid; LR, lateral root; RH, root hair; ACC, aminocyclopropane carboxylic acid; AVG, ethylene biosynthesis inhibitor aminovinylglycine; GC–MS, gas chromatography-coupled mass spectrometry; MS, Murashige and Skoog basal medium; SE, standard error; ORF, open reading frame

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show that loss-of-function mutant alleles of *AtPGP4* reveal more lateral roots and longer root hairs at early stages. Moreover, mutant plants show elevated auxin levels but reduced auxin uptake capacities suggesting that AtPGP4 is involved in auxin transport processes that trigger plant lateral root and root hair initiation.

## 2. Materials and methods

### 2.1. Isolation of atpgp4 mutants

In order to identify mutant lines carrying T-DNA insertions in the *AtPGP4* gene (At2g47000), two T-DNA insertion lines SALK_010207 and SALK_067557 were ordered from the Arabidopsis Stock Center at Ohio State University (ABRC). Segregation of T-DNA insertion was shown by PCR employing the following combinations of gene- and T-DNA-specific primers: gene-specific primers *AtPGP4* (for SALK_010207 fw: 5′-tagtttacctttgcatcagcg; rev: 5′-aaaggacccggaaggagctta; for SALK_067557 fw: 5′-ccaagcgacaaattaatgtcgag; rev: 5′-acgcgctagaccgtgttatgg). PCR reactions were performed on genomic DNA using these primers in combination with LBb1 5′-GCGTGGACCGCTTGCTGCAACT which anneals to the left border of the T-DNA insertion, according to the protocol published at http://signal.salk.edu/tabout.html. T-DNA insertion numbers were determined by Southern Blot analysis. Genomic DNA from wt and pgp4 mutants was digested with *Hin*dIII and *Bam*HI. The resulting blot was probed with a [α-$^{32}$P]dCTP-labeled T-DNA specific DNA fragment corresponding to the left border region of the T-DNA with a size of 266 bp. The probe was obtained by PCR amplification using the following pairs of primers: pROK2fw (5′-AACAGCTGATTGCCCTTCAC) and pROK2rev (5′-TTTGGGTGATGGTTCACGTA).

### 2.2. Semi-quantitative RT-PCR

For tissue-specific expression analysis, RNA was extracted from leaves, roots, flowers and siliques of pot-grown plants. Seedlings were grown on bactoagar for seven days and each day seedlings were collected. Total RNA was purified from plants using the RNeasy Plant Mini Kit (Qiagen). cDNAs were prepared using M-MLV reverse transcriptase (Promega) as indicated by the manufacturer. Semi-quantitative RT-PCRs were performed as described in [32] using gene-specific sense (S) and anti-sense (AS) primers for *PGP4* (PGP4-S: 5′-ttc atc agt ggt ctg caa cag; PGP4-AS: 5′-tga agc tga act aac gaa gca), ACTIN2 (actin2-S: 5′-tggaatccacgagacaaccta; actin2-AS: 5′-ttctgtgaacgattcctggac), and S16 (S16-S: ggcgactcaaccagctactga and S16-AS: cggtaactcttctggtaacga).

### 2.3. Plant growth conditions

Seeds were surface sterilized for 5 h in a chamber containing vapor of HCl and Na-hypochlorite and stratified in a 0.1% agar solution for two days at 4 °C. Subsequently, the seeds were plated on sterile half-strength Murashige and Skoog (MS) medium containing 2% sucrose solidified with 0.6% phytagel (Sigma, Buchs, Switzerland), and grown vertically as described in [35]. For phenotypes of adult plants, mutants were grown under short day conditions: 8 h, 100 μmol $m^{-2}$ $s^{-1}$ white light, 22 °C.

### 2.4. Morphometric analysis

Seedlings were grown vertically as described above on nutrient media optionally supplemented with 10 nM IAA, 5 μM NPA, 1 μM AVG or 200 nM ACC. Five dag seedlings were photographed using a Leica DMR microscope equipped with a Leica DC300 F charge coupled device (CCD) camera and root hair lengths all along the primary root were measured from digital pictures using the tool "Image Manager" of the Leica IM1000 software (Leica, Heerbrug, CH). Lateral roots number of 40 seedlings per each genotype was counted after 5, 7, 9 or 11 dag under a dissecting microscope. Values were used to determine the means (±S.E.). The measurement was repeated two times showing similar results. *P* values were analyzed by Student's *t* test. Root hairs and lateral roots were photographed using a Nikon SMZ1500 binocular, equipped with a Nikon Coolpix 5000 camera. Digital pictures were processed using Adobe Photoshop 7.0 (Adobe Systems).

### 2.5. Auxin measurements

For free IAA quantification, root and shoot segments of 30–50 seedlings were collected, pooled and their fresh weight was determined. Samples were methanol extracted and analyzed by GC–MS. Calculation of isotopic dilution factors was based on the addition of 100 pmol $^2H_2$-IAA to each sample. Measurements are presented as means of two individual samples.

### 2.6. Root auxin transport assays

Arabidopsis seedlings were grown in hydroponic culture as described in [35]. Two weeks later seedlings were collected and incubated in 10 ml transport buffer (5 mM HEPES, pH 7.0, and 1 mM $CaCl_2$) supplemented with 5-$^3$H-IAA (specific activity 20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO, 1 μl/10 ml). At 2, 8, 16, 24, 32, 40, 48 min, seedlings were washed in transport buffer containing 10 μM cold IAA and the amount of radioactivity in the first 1 cm of root was quantified by scintillation counting. Two-minute treatments were considered as initial loading and used to calculate relative transport kinetics. Experiments were repeated at least three times, representative results are shown.

### 2.7. Recording of root apex auxin fluxes using an IAA-specific microelectrode

A platinum microelectrode as a substrate electrode immobilizing multiwalled carbon nanotubes was used to monitor IAA fluxes as described in [33]. For measurements, plants were grown in hydroponic cultures and used 5 dag.

### 2.8. Yeast yap1-1 complementation assays

*AtPGP4* was cut out from pBSKII-AtPGP4 (kindly provided by A. Murphy) and inserted *Kpn*I/*Not*I into yeast shuttle vector pYES2 (Invitrogen) resulting in pMG89. pMG89 was introduced into *S. cerevisiae* strains JK93da and *yap1-1* [31], respectively, and single colonies were grown in synthetic minimal medium without uracil, supplemented with 2% glucose (SD-URA). For detoxification assays, transformants grown in SD-URA to an $OD_{600}$ = 0.8 were washed and resuspended in water to $OD_{600}$ = 1 in water. Cells were 5-times 10-fold diluted and each 5 μl were spotted on minimal media galactose plates (SG-URA) containing either 250 μM 5-fluoro-indol (5-FI, JK93da) or 10 μM IAA (*yap1-1*). Pictures were taken after 3–5 days of growth at 30 °C. Assays were performed with three independent transformants.

## 3. Results and discussion

### 3.1. Expression of AtPGP4

Based on microarray transcript profiling, AtPGP4 has been suggested to be involved in early lateral root initiation [18]. Additionally, Digital Northern analysis (www.genevestigator.ethz.ch, [26]) supported a predominant expression of *AtPGP4* in the root while expression in aerial parts of the plant is significantly lower (Suppl. Fig. 1A). Interestingly, expression is high both in the root elongation zone and in lateral roots. Furthermore, again based on a microarray surveys *AtPGP4* seems to be indeed expressed at early stages of the plant development (Suppl. Fig. 1B). We confirmed these *in silico* data by semi-quantitative RT-PCR. Using *AtPGP4* gene-specific primers we found high expression in roots while expression in other parts of the plants was low (Fig. 1A). Likewise, *AtPGP4*-specific transcripts accumulate at early stages of plant development with a maximum expression at day 3 (Fig. 1A).

### 3.2. atpgp4 mutants show increased lateral root formation and reduced sensitivity toward auxin transport inhibitor NPA

For phenotypic analysis, two SALK T-DNA lines [19] were obtained and insertions in homozygous plants were verified by PCR (Fig. 1C). *atpgp4-1* (SALK_010207) carries a T-DNA

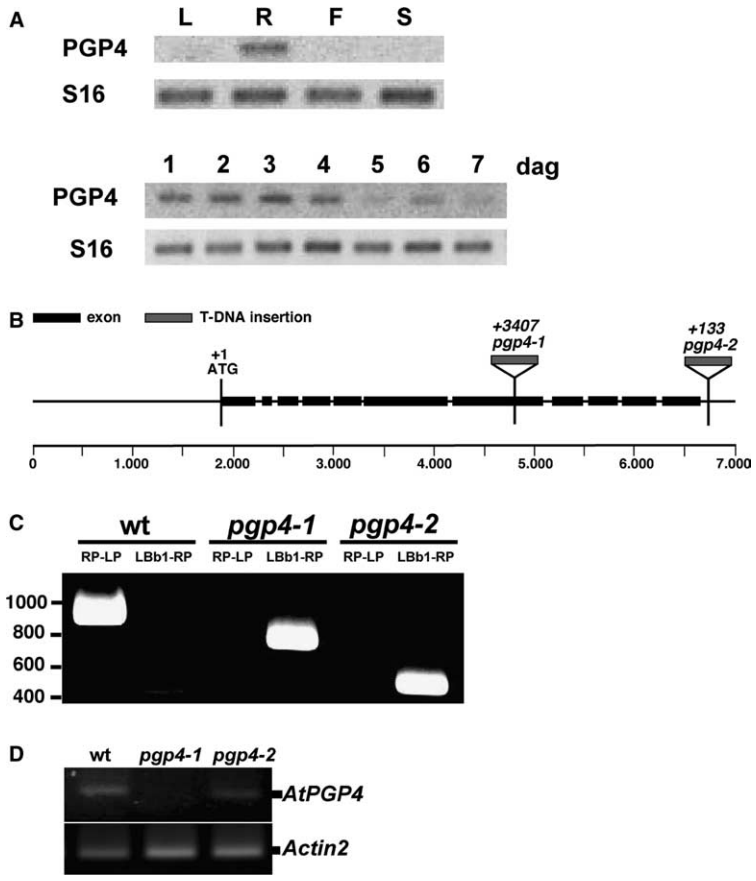


Fig. 1. Expression and mutant analysis of *AtPGP4*. (A) Results of RT-PCR analysis suggest that AtPGP4 is expressed in low amounts in the roots (upper panel) and at early stages of seedling development (lower panel). cDNAs from different tissues or developmental stages were synthesized from total RNA and AtPGP4 and 40S ribosomal subunit S16 (internal control) specific transcripts were detected by PCR. (B) Genomic structure of *AtPGP4* and T-DNA insertion sites. Start of open reading frame (ORF) is indicated by ATG; T-DNA insertions positions of mutant lines SALK_010207 (atpgp4-1) and SALK_067557 (atpgp4-2) are marked as nucleotide distances from +1 (ATG) and from the stop codon, respectively (see Section 3). (C) PCR verification of T-DNA insertions using primer combinations as described in Section 2. (D) RT-PCR verifies the absence in the atpgp4-1, but a low amount of transcript in the atpgp4-2 mutant. cDNAs were synthesized from total RNA extracted from root of 5 dag seedlings, and AtPGP4- and actin2 (internal control) specific transcripts were detected by PCR.

insertion in exon 7 (at position 3407 bp) and *atpgp4-2* (SALK_067557) in the 3′ UTR (133 bp downstream of the stop codon, see Fig. 1B). While for *atpgp4-1* no transcript was detected by semi-quantitative RT-PCR (Fig. 1D), it was markedly reduced in *atpgp4-2*. This is further supported by the phenotypic analysis presented below, which shows no significant differences between the two mutant alleles. Both mutants contained only single T-DNA insertions as was verified by Southern blot analysis (results not shown).

While pot-grown *atpgp4* mutant plants performed no obvious growth phenotype (results not shown), the strong expression in radical parts of the plant prompted us to investigate root growth in more detail. When grown on agar plates both mutant alleles of AtPGP4 showed slightly enhanced primary root growth at five days after germination (dag) compared to the wild-type while significant differences were only found for *atpgp4-1* (Suppl. Fig. 2).

We quantified lateral root initiation, and found that atpgp4 seedlings after 5 dag developed significantly more LR primordia (137%) compared to the wt (Fig. 2A, Table 1). This difference diminishes after 7 and 9 days, which is inline with the expression profile for *AtPGP4*. LR formation is apparently induced by an endogenous and temporary shoot derived auxin burst [16,17,25]. In order to investigate if these differences in LR formation were directly a consequence of altered auxin levels, we tried to manipulate plant endogenous auxin levels by addition of low concentrations of IAA and the auxin transport inhibitor NPA. Exogenous IAA is known to inhibit root elongation but stimulates LR formation [16]. In agreement, growth at low concentration of exogenous IAA (10 nM) enhanced LR formation in wt at 5 dag thus mimicking the developmental defect of the atpgp4 mutants (Table 1). LR induction by IAA at 5 dag is significantly weaker in both atpgp4 mutant alleles (126%) compared to the wt (137%) while at later stages on IAA leads to a reduced induction both in wt and *atpgp4* (Table 1, Fig. 2B) until relative LR numbers wt and atpgp4 are indistinguishable after 9 dag.

The auxin transport inhibitor NPA has become an important pharmacological tool in auxin research as is known to block polar auxin transport, to bind to putative auxin transport complexes [12] and to mimic the pin-shaped mutant phenotype of putative auxin efflux facilitator PIN1 [2,4]. Recently, it was shown that NPA binds to Arabidopsis PGPs [9,12] and that NPA efficiently inhibits AtPGP1-mediated auxin transport [11]. Here we show that NPA reduces LR formation in the wt – verifying previous data [16,25] – but that inhibition

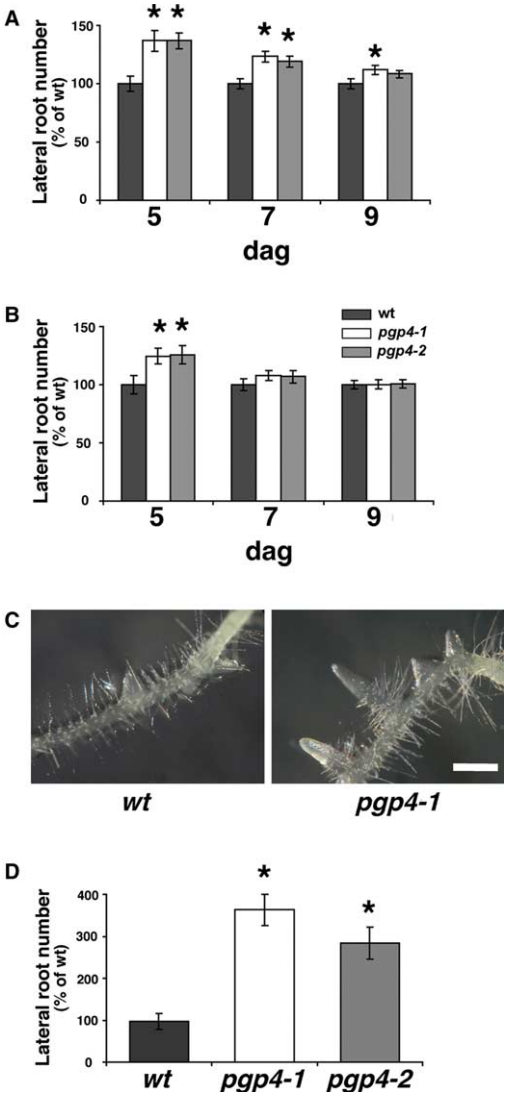


Fig. 2. atpgp4 mutants show enhanced lateral root formation and reduced sensitivity to the auxin transport inhibitor, NPA. (A) Lateral root formation is significantly enhanced in atpgp4 mutant alleles at 5 dag but decreases at later stages. (B) Lateral root induction by IAA at 5 dag is significantly weaker in both atpgp4 mutant alleles compared to the wt, while at later stages, IAA leads to a reduced induction both in wt and atpgp4 (see Table 1 for absolute values). (C) Lateral root phenotype of atpgp4-1 grown in the presence of 5 μM NPA for 11 days. Atpgp4 mutants are less sensitive to the inhibitory effect of NPA. Scale bar = 500 μm. (D) Lateral root formation in atpgp4 mutants is 3–4 times enhanced compared to the wt. Lateral root number of vertically grown plants (40 seedlings each) in the presence or absence 10 nM IAA (determined at dag 5, 7 and 9) or 5 μM NPA (11 dag). Shown are mean numbers ± S.E., significant differences to wt growth (*P* values analyzed by Student's *t* test) are marked with an asterisk. Absolute values are presented in Table 1.

is lower in the atpgp4 mutants (Fig. 2C, Table 1) leading to a drastic (3–4-fold) enhanced relative LR number (Fig. 2D). We also found that LR shape is distorted (Fig. 2C) and that LR emergence site is shifted nearby the root apex (data not shown). Our data indicate that external IAA reduces enhanced LR formation in atpgp4 while NPA increases this difference pointing to an involvement of AtPGP4 in auxin homeostasis of the root.

Table 1
atpgp4 mutants show enhanced lateral root formation and reduced sensitivity to the auxin transport inhibitor, NPA

| Treatment | Dag | Wt | pgp4-1 | pgp4-2 |
|---|---|---|---|---|
| control | 5 | 3.33 ± 0.22 | 4.55 ± 0.29 | 4.55 ± 0.23 |
| | 7 | 12.12 ± 0.54 | 14.95 ± 0.58 | 14.42 ± 0.58 |
| | 9 | 30.35 ± 1.29 | 34.025 ± 1.18 | 32.9 ± 1.02 |
| IAA | 5 | 4.57 ± 0.34 | 5.7 ± 0.31 | 5.75 ± 0.35 |
| | 7 | 12.87 ± 0.69 | 13.85 ± 0.54 | 13.75 ± 0.66 |
| | 9 | 29.88 ± 1.03 | 29.98 ± 1.08 | 30.00 ± 1.07 |
| NPA | 11 | 1.63 ± 0.32 | 6.08 ± 0.6 | 4.75 ± 0.63 |

Lateral root number of vertically grown plants (40 seedlings each) in the presence or absence of 10 nM IAA or 5 μM NPA. Shown are mean numbers ± S.E. Relative values are presented in Fig. 2.

### 3.3. Root hairs of atpgp4 mutants are longer and more variable in length

Like for LRs, RH initiation and elongation is well known to be regulated by auxin [22]. The growth defect of LRs in the atpgp4 mutants thus prompted us to investigate RH number and lengths in atpgp4. While both *atpgp4* alleles have similar RH numbers compared to the wt (Fig. 3A), RHs of the mutant were longer (136%, Fig. 3D) and more variable in lengths (Fig. 3B) under control conditions.

It has been shown that growth in the presence of either auxin or auxin transport inhibitors results in enhanced root hair length [21,22]. Here we report that exogenous IAA stimulated RH elongation in wt and atpgp4 seedlings (Fig. 3B). However, like for the LRs, enhanced RH elongation was reduced in the mutant (118%) when compared to the wt (133%). In contrast, growth on NPA leads to enhanced RH length similarly in wt and atpgp4 (Fig. 3D).

Seedlings grown on NPA plates showed constant differences in RH length between wt and atpgp4 on control and NPA plates (between 135% and 141%), while differences on IAA plates were lower (around 118%). Interestingly, mutant RH on NPA displayed a far flatter and non-Gaussian distribution in length comparing to the wt (Fig. 3B).

Genetic and physiological evidences implicate that auxin cross talks with ethylene in promoting root hair elongation [21,22]. In order to dissect the nature of this effect, and in order to test an involvement of AtPGP4 in growth defects caused by an individual plant hormone we repeated root hair measurements in the presence either of ethylene precursor aminocyclopropane carboxylic acid (ACC) which induces ectopic root hair formation or ethylene biosynthesis inhibitor aminovinylglycine (AVG) which abolishes root hairs, respectively [21]. In our experiments we found indeed that 200 nM ACC dramatically enhances RH lengths (Fig. 3C) while 1 μM AVG blocks efficiently RH elongation (Fig. 3A and C). This was true for both wt and atpgp4; however, as found for experiments using exogenous manipulation of auxin levels, both treatments lead to a flatter and more unequal distribution (Fig. 3C). Further, our data indicate that atpgp4 plants are even under low endogenous ethylene able to develop a population of root hairs that are as long as under control conditions (Fig. 3A and C).

Interestingly, both exogenous up- and down-regulation of intracellular ethylene by ACC and AVG, respectively, reverts the mutant phenotype (enhanced RH length). Under control conditions relative root hair length of atpgp4 is enhanced to around 136% while on ACC (wt: 180%, *atpgp4*: 165%) and AVG (wt: 30%, *atpgp4*: 37%) differences are smaller. This is

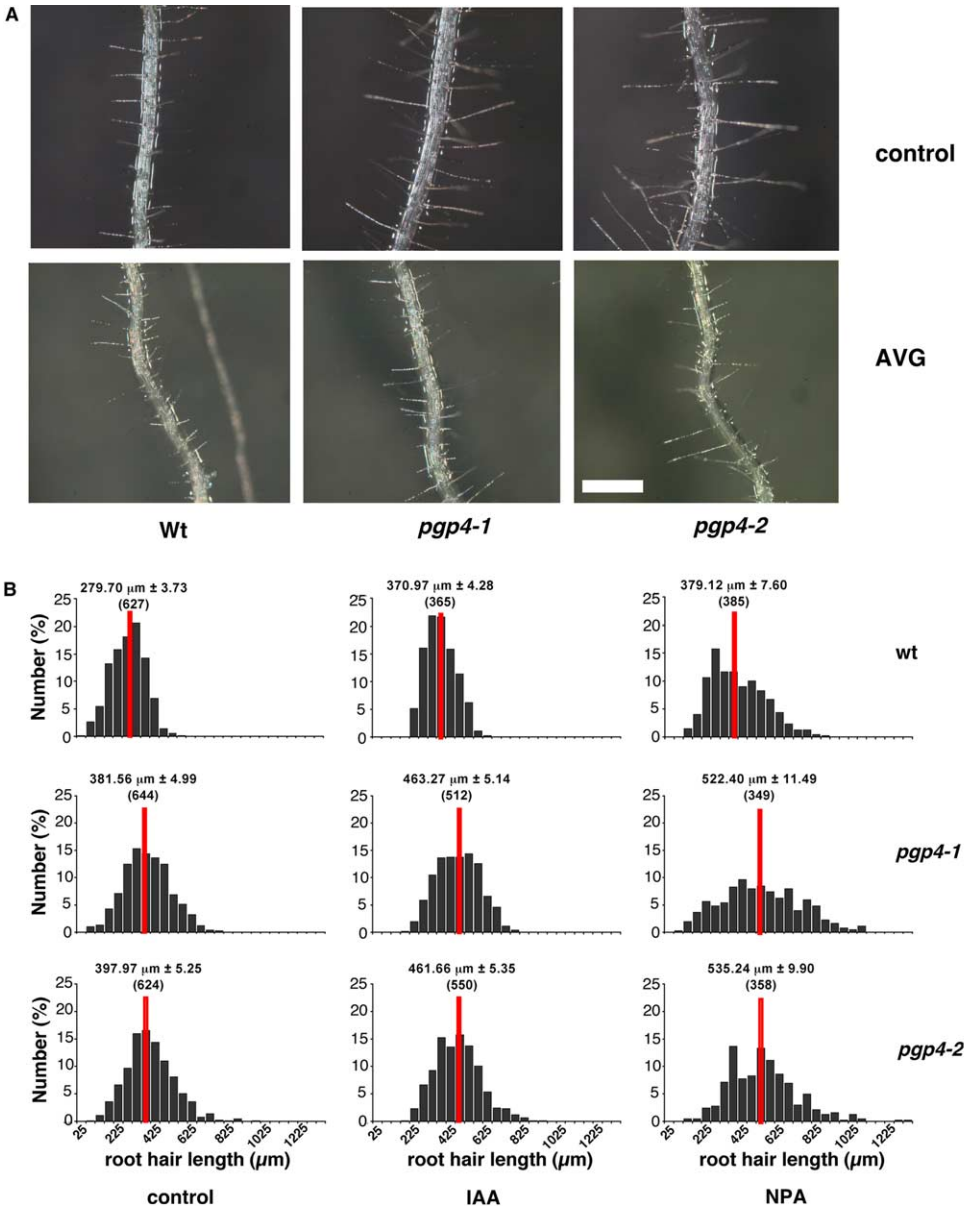


Fig. 3. Root hairs of atpgp4 mutants are longer and more variable in length. (A) Root hair (RH) phenotype of atpgp4 mutants grown on unsupplemented media (upper panel) or on 1 μM AVG (lower panel) for five days. Scale bar = 500 μm. (B) Atpgp4 mutants have longer RHs and their distribution is flatter compared to the wt. On IAA enhanced RH elongation was reduced in the mutant compared to the wt. (C) In wt and atpgp4 seedlings, ACC enhanced RH lengths, while AVG efficiently blocked RH elongation. (D) Comparison of relative root hair lengths between wt and atpgp4 mutants seedlings grown for 5 days in the presence of 10 nM IAA, 5 μM NPA, 1 μM AVG or 200 nM ACC. Distribution of root hair lengths in 5-day-old seedlings grown on unsupplemented media (control), 10 nM IAA, 5 μM NPA, 1 μM AVG or 200 nM ACC. Values are mean numbers ± S.E. (*P* values analyzed by Student's *t* test). In parenthesis are shown the numbers of root hair measured per each sample.

in contrast to the variation of auxin levels where the relative difference in RH lengths between wt and atpgp4 was only slightly affected. Keeping the fact in mind that auxin application itself does not cause ectopic RH development [21,24] our data on auxin and ethylene treatments suggest that AtPGP4 is more likely to be involved in auxin- than in ethylene-dependent RH development.

### 3.4. *atpgp4 mutants reveal elevated auxin levels and auxin transport deficiencies*

Auxin promotes LR initiation [16,25] and RH elongation [21,22] while enhanced LR formation and RH lengths in *atpgp4* plants can be mimicked by exogenous auxin. This prompted us to investigate free auxin levels in the mutant root. GC–MS analysis indeed showed elevated free IAA in the root of both mutant alleles of atpgp4 (224%, Fig. 4) while in the shoot differences were not significantly different (*atpgp4-1*) or only slightly different (*atpgp4-2*).

Auxin-related growth phenotypes and elevated auxin concentrations in the root suggested transport deficiencies in the root. Therefore, we measured in vivo $^{3}$H-IAA uptake into wt and atpgp4 roots. Surprisingly, both mutant alleles showed significantly reduced IAA uptake compared to the wt (Fig. 5A).

In order to verify these data, a novel self-referencing microelectrode [33] was used. In short, this electrode allows

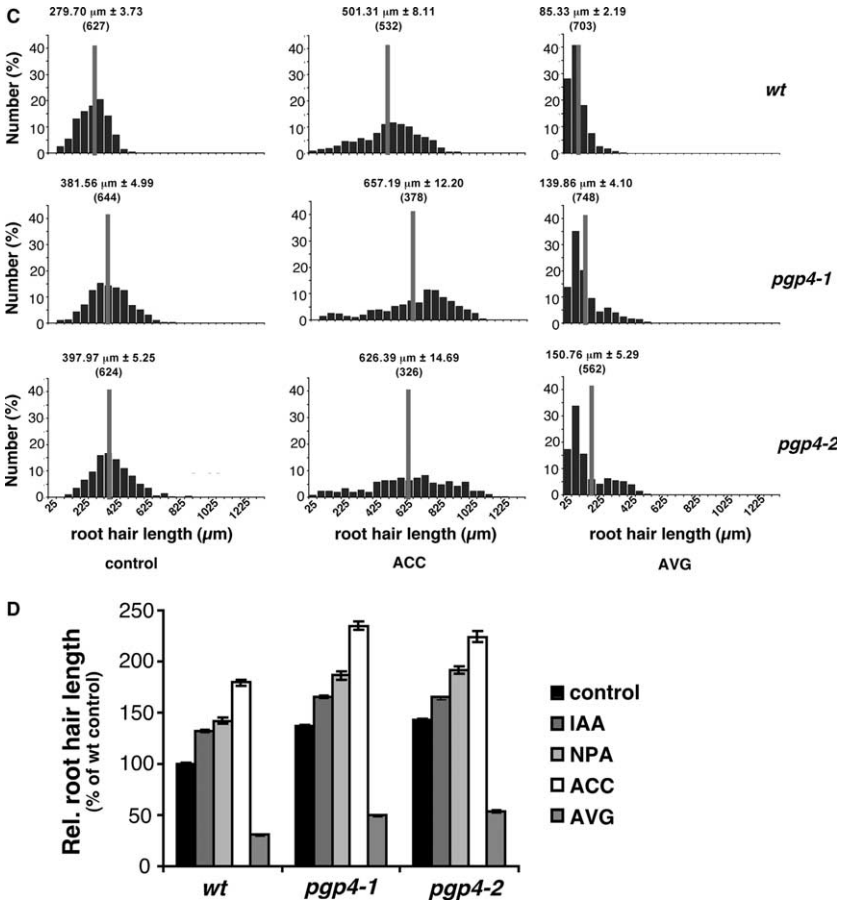


Fig. 3 (*continued*)

non-invasive and continuous recordings of auxin fluxes in intact root apices and has been shown to be highly selective for IAA. Characteristically, IAA influxes showed a distinct peak at 0.2–0.3 mm from the root apex in the so-called transition zone (TZ) or distal elongation zone of the root apex. In wt roots this transition zone peak averaged $193 \pm 16$ fmol $cm^{-2}$ $s^{-1}$, while in atpgp4-1 the maximum influx in the TZ was significantly reduced averaging $133 \pm 13$ fmol $cm^{-2}$ $s^{-1}$ (Fig. 5B). At more distal positions, corresponding to cells accomplishing their onset into rapid cell elongation (>0.5 mm), the IAA influx was smaller and remained unchanged at positions as far back as 1 mm from the root apex. AtPGP4 roots showed also a reduced response to the inhibitory effect of NPA on IAA transport. In wt roots, the IAA influxes measured in the TZ of the root apex after 2 h of NPA treatment (20 μM) were about $100 \pm 5$ fmol $cm^{-2}$ $s^{-1}$ (around 50% inhibition), whereas in atpgp4-1 roots the inhibitory effect of NPA was just 25% ($133 \pm 13$ fmol $cm^{-2}$ $s^{-1}$ against $98 \pm 10$ fmol $cm^{-2}$ $s^{-1}$). Interestingly, reduced sensitivity of IAA influx toward NPA in the atpgp4 TZ are in accordance with the reduced effect of NPA on LR and RH formation in atpgp4 mutants.

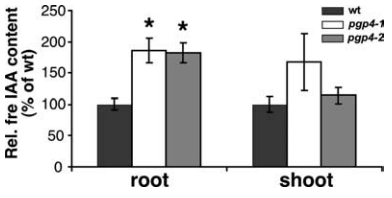


Fig. 4. Roots of atpgp4 plants show elevated levels of free IAA. Root and shoot segments were methanol extracted and free IAA analyzed by GC–MS. Shown are mean numbers of two independent experiments (each 30–50 seedlings) ± S.E., significant differences to wt growth (*P* values analyzed by Student's *t* test) are marked with an asterisk.

In order to investigate at the cellular level if AtPGP4 itself is the component responsible for IAA transport, we functionally expressed AtPGP4 in yeast. Fluorinated indolic derivates, toxic analogs of potential IAA precursors, are cytotoxic to yeast and have become a valuable tool used to investigate auxin transport [34]. Using yeast strain JK93da that lacks endogenous PGP-like proteins [7] we found that AtPGP4 provides hypersensitivity toward 5-fluro-indol compared to the vector control (Fig. 5C, left panel). In order to verify these data, we repeated growth analysis using the IAA-sensitive mutant yeast strain *yap-1-1*. In short, this yeast strain is sensitive toward low concentrations of IAA due to transcriptional upregulation of endogenous AUX1-like IAA uptake systems [31] and has therefore become an excellent system to demonstrate auxin transport [11]. Again, AtPGP4 confers slight but significant hypersensitivity to yap1-1 yeast (Fig. 5B, right panel). AtPGP4 expression is under control of a GAL promoter; the fact that hypersensitivity is dependent on galactose in the growth medium (results not shown) excludes secondary effects. Unlike AtPGP1, which specifically complemented growth deficiencies on 5-fluoro-indol and IAA thus demonstrating IAA export [11] both growth tests argue for an import direction. In summary, both root transport and yeast complementation data

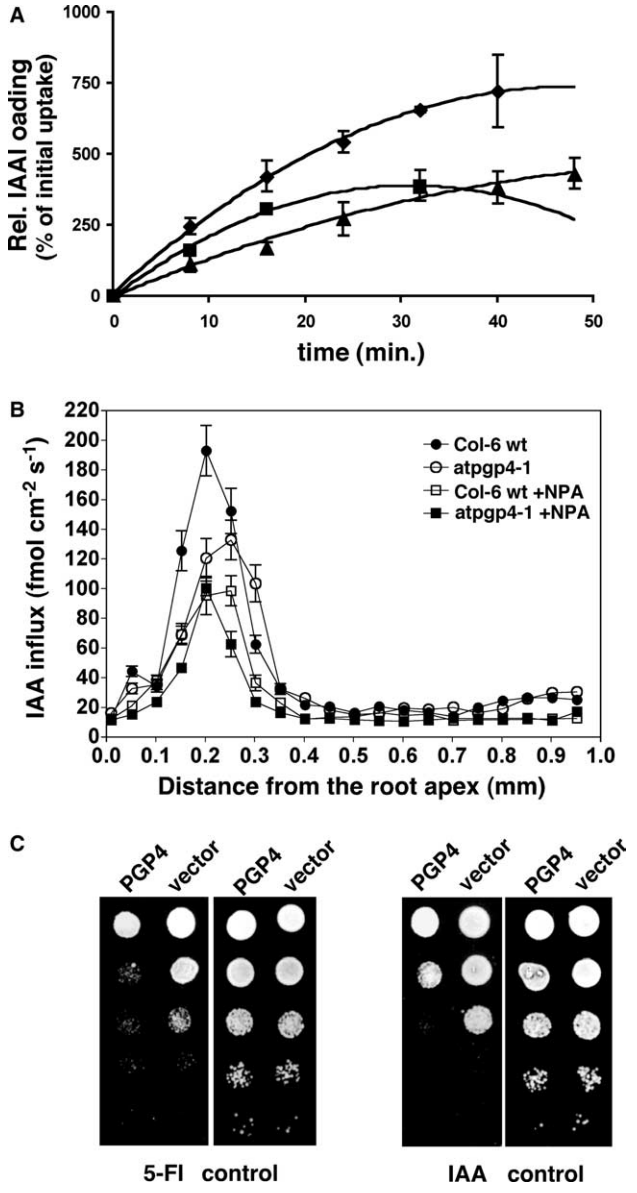


Fig. 5. Auxin transport is altered in *atpgp4* plants. (A) Roots of atpgp4-1 (filled square) and of atpgp4-2 (filled triangle) show reduced 3H-IAA uptake compared to the wild-type (filled circle). (B) Diagram illustrating the IAA influx profile along single roots of wt and atpgp4-1 plants and the effect of NPA (20 μM). Differential current from an IAA-selective microelectrode placed 2 μm from the root surface and used in a self-referencing mode. The sensor was vibrated between two positions 10 μm distant at a rate of 0.1 Hz. Positive fluxes represent a net IAA influx. Data shown were collected continuously over a 10 min period and are means of eight replicates. Error bars represent S.E. (C) AtPGP4 confers hypersensitivity to IAA sensitive *yap1-1* mutant strain. 10-fold dilutions of yeast cells transformed with AtPGP4 or the corresponding vector control were spotted on control plates or plates supplemented with 10 μM IAA.

point to an in vivo uptake of IAA over the plasma membrane. Uptake by an ABC MDR-like ABC transporter is an unusual event and has to our knowledge only been demonstrated once: CjMDR1 from *Coptis japonica* has been shown to catalyze the primary active uptake of the alkaloid berberine [27]. Interestingly, AtPGP4 shares (beside to its closest homologue AtPGP21) highest homology with CjMDR1 (65%) and in a phylogenetic tree AtPGP4/AtPGP21 and CjMDR1 cluster together on a subcluster that is clearly distinct from the one that is characterized by AtPGP1 and AtPGP19 (results not shown). This indicates that in analogy to CjMDR1, AtPGP4 might catalyze the cellular import of auxin.

## 4. Conclusions

Using reverse genetics we provide several lines of evidence suggesting that AtPGP4 is involved in auxin-mediated LR and RH development: (i) A survey of available microarray data and RT-PCR verification demonstrate expression in early root development. (ii) atpgp4 plants show enhanced LR initiation and RH elongation both known to be controlled by auxin. (iii) Exogenous IAA and auxin transport inhibitor, NPA, can mimic both mutant phenotypes when applied to the wt but wt and atpgp4 plants show different sensitivities toward IAA and NPA. (iv) AtPGP4 loss-of-function mutants reveal elevated root free auxin levels.

The reduced root IAA uptake capacities shown by transport studies and an IAA-selective electrode as well as the fact that AtPGP4 confers hypersensitivity to the IAA sensitive yap1-1 yeast mutant suggest an involvement of AtPGP4 in auxin transport like previously shown for AtPGP1 [11]. Surprisingly, all experimental setups suggest an uptake direction, which has been demonstrated only once for the closely related CjMDR1 [27]. Elevated root auxin levels might account for the described auxin-related growth phenotypes but seem on a first view to be contradictory with the proposed transport direction. The simplest model to explain growth phenotypes, elevated auxin concentration and a putative involvement in root auxin uptake would be to postulate an involvement of AtPGP4 in polar auxin transport. In this so far speculative model, AtPGP4 would co-function with AtPGP1 in basipetal auxin transport from the root to the shoot. AtPGP4 and AtPGP1 would catalyze active (ATP-dependent) auxin import and export, respectively, at opposite (apical and basal, respectively [36]) plasma membrane domains of the cell. Obviously, this would require a polar expression pattern of AtPGP4 but so far the precise location of AtPGP4 is unknown.

However, a putative polar expression and involvement in PAT, like found recently for AtPGP1 in the root elongation zone [11], is supported by two findings. First, LR formation in atpgp4 mutant plants is less affected than in wt plants by NPA, known to block basipetal IAA reflux from the root tip [16]; NPA inhibition [11] and binding [12] was shown for AtPGP1. Second, AtPGP4 has been identified in a proteomic inventory to reside on detergent-resistant plasma membranes (DRMs) or lipid rafts [28]. In plant cells, these microdomains are thought to play an important role in protein targeting and polar distribution of plasma membrane proteins, including putative auxin facilitator PIN1 [28,29].

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.08.061.

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