**Title of article:** Functional characterization of *Pa*LAX1, a putative auxin permease, in heterologous plant systems

**Author's names and institution addresses:**

Klára Hoyerová[2], Lucie Perry[2*], Paul Hand, Martina Laňková, Tomáš Kocábek, Sean May[3], Jana Kottová, Jan Pačes, Richard Napier, Eva Zažímalová

Institute of Experimental Botany, the Academy of Sciences of the Czech Republic, Rozvojová 263, CZ-165 02 Prague 6, Czech Republic (K.H., L.P., M.L., J.K., E.Z.)
HRI/ Univ. of Warwick, Wellesbourne, Warwick CV35 9EF, UK (P.H., S.M., R.N.)
Biological Centre of the Academy of Sciences of the Czech Republic, v.v.i., Institute of Plant Molecular Biology, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic (T.K.)
Department of Biochemistry, Faculty of Science, Charles University, Hlavova 2030, CZ-128 00 Prague 2, Czech Republic (M.L.)
Institute of Molecular Genetics, the Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-14220, Prague 4, Czech Republic (J.P.)
Department of Plant Physiology, Faculty of Science, Charles University, Viničná 5, CZ-128 44 Prague 2, Czech Republic (E.Z.)

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[2]These authors contributed equally to the paper.

[3]Present address: Nottingham Arabidopsis Stock Centre, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, UK

[*]Corresponding author; e-mail: perry@ueb.cas.cz; FAX: +420225106446.

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**Abstract**

We have isolated the cDNA of the gene Pa*LAX1* from a wild cherry tree (*Prunus avium*). The gene and its product are highly similar in sequences to both the cDNAs and the corresponding protein products of *AUX/LAX*-type genes, coding for putative auxin influx carriers. We have prepared and characterized transformed tobacco (*Nicotiana tabacum*) and Arabidopsis (*Arabidopsis thaliana*) plants carrying the gene Pa*LAX1*.

We have proved that constitutive overexpression of Pa*LAX1* is accompanied by changes in the content and distribution of free indole-3-acetic acid, the major endogenous auxin. The increase in free indole-3-acetic acid content in transgenic plants resulted in various phenotype changes, typical for the 'auxin-overproducing' plants. The uptake of synthetic auxin, 2,4-dichlorophenoxyacetic acid, was three times higher in transgenic lines compared to the wild type lines and the treatment with the auxin uptake inhibitor, 1-naphthoxyacetic acid, reverted the changes caused by the expression of *Pa*LAX1. Moreover, the agravitropic response could be restored by expression of *Pa*LAX1 in the mutant *aux1* plants, which are deficient in auxin influx carrier activity.

Based on our data, we have concluded that the product of the gene Pa*LAX1* promotes the uptake of auxin into cells and, as a putative auxin influx carrier, it affects the content and distribution of free endogenous auxin in transgenic plants.

## Introduction

Auxins, in co-action with cytokinins, play a crucial role in the regulation of plant growth and development. They are required for cell division, cell enlargement and differentiation, and they function as internal endogenous signals between cells, tissues and organs. Together with auxin metabolism, transport of auxins within a plant is involved in the regulation of intracellular auxin levels and in formation of auxin gradients. Typically, the cell-to-cell auxin transport is polar. Auxin influx and efflux carriers appear to be the central effectors of the polar auxin transport machinery; their activity and subcellular localization direct the auxin flow and underlie formation of auxin gradients (reviewed in Morris et al., 2004; Kramer and Bennett, 2006; Tanaka et al., 2006; Kerr and Bennett, 2007). There are mathematical models of polar auxin transport, which follow quantitative relationships between auxin flow into and out of the cells. These models provide the testable hypothesis of the direction and quantity of intercellular auxin movement and they emphasize the importance of both auxin efflux and influx carriers in intercellular polar auxin flow (Kramer, 2004; Heisler and Jönsson, 2006).

The auxin influx into cells in Arabidopsis (*Arabidopsis thaliana*) seems to be driven by proteins from AUX/LAX family of plasma membrane permeases, products of *AUX/LAX* genes (reviewed in Parry et al. 2001a; Kerr and Bennett, 2007). Other carriers/facilitators may also take part in auxin influx: ANT1 from Arabidopsis was shown to transport auxins when expressed in yeast (Chen et al., 2001). PGP4 from Arabidopsis, one of the MDR (multidrug-resistance)-type proteins, is a candidate for an energy-dependent auxin uptake transporter, even though its contribution to auxin uptake in Arabidopsis roots appears to be less than that of AUX1 (Terasaka et al., 2005; Santelia et al., 2005). Interestingly, some other PGP proteins are thought to function exclusively in auxin efflux. Generally, two types of putative auxin efflux carriers have been proved to function in Arabidopsis: PIN-type and MDR/PGP (multidrug-resistance/P-glycoprotein)-type transporters (reviewed by Morris et al. 2004; Benjamins et al., 2005; Blakeslee et al., 2005; Paponov et al., 2005; Geisler and Murphy, 2006; Zažímalová et al., 2007). The widely accepted model of mechanisms controlling the uptake and efflux of auxins indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-1-acetic acid (1-NAA) was proposed by Delbarre et al. (1996).

When considering the mechanism of auxin flow through cells, so far, the only mutant extensively characterized and shown to be closely related to the auxin influx was the root agravitropic and 2,4-D-resistant mutant *aux1* of Arabidopsis, which is lacking the functional gene *AUX1* (Marchant et al., 1999). *AUX1* gene was isolated by Bennett et al. (1996), who

showed that its product, AUX1, similar to plant amino acid permeases, presumably acted as a component and/or regulator of the auxin influx carrier. Data obtained in experiments with *aux1* mutants supported this presumption: Firstly, phenotype characterization of *aux1*, the loss-of-function mutant (Maher and Martindale, 1980; Bennett et al., 1996), clearly confirmed the presence of agravitropic roots which together with 2,4-D-resistant phenotype pointed to disruption in auxin influx. Secondly, the mutant agravitropic phenotype could be rescued by 1-NAA, an easily membrane-permeable synthetic auxin which does not require an active transport into cells. On the other hand, 2,4-D, a synthetic auxin which is transported into plant cells predominantly by a carrier (Delbarre et al., 1996), did not restore the wild type phenotype (Yamamoto and Yamamoto 1998, Marchant et al. 1999). The mutant agravitropic phenotype was also rescued by tissue-specific expression of *AUX1*. This renewed root gravitropic response depended on expression of the transgene in lateral root cap and expanding epidermis (Swarup et al., 2005; Dharmasiri et al., 2006). Thirdly, the roots of *aux1* accumulated significantly less 2,4-D than wild-type roots while no such difference was observed in accumulation of 1-NAA or IAA-like tryptophan, which, again, suggested deficiency in auxin uptake (Marchant et al. 1999). Lastly, *aux1* phenotype could be mimicked in wild type seedlings grown on media containing the inhibitors of auxin influx (Parry et al. 2001b).

Other data confirming the action of AUX1 have been reported on the level of protein: Its localization was explored by immunostaining, using a known epitope, the coding sequence of which was fused to both terminal regions of the *AUX1* gene. In Arabidopsis, the epitope of the fusion protein was localized in the root tips, in a subset of protophloem, columella, epidermal cells and lateral root cap (Swarup et al., 2001). The localization of the AUX1 at the plasma membrane of root cells, consistent with its proposed transport function, was proved and it was shown that alterations of the central domain of this permease led to its partial or complete loss of function (Swarup et al., 2004). Recent studies showed that subcellular localization and polar trafficking of AUX1 uses pathway(s) distinct from that of PIN1 efflux carrier (Dharmasiri et al., 2006; Kleine-Vehn et al., 2006; Zhuang et. al., 2006).

The AUX1 physiological function was studied in context with root gravitropic curvature (Swarup et al., 2001; Swarup et al., 2005), establishment and maintenance of apical-basal polarity in root hair cells (Grebe et al., 2002), the promotion of lateral root initiation (Marchant et al., 2002), formation of auxin gradients in the shoot apical meristem (Stieger et al., 2002), positioning of new leaf primordia (Reinhardt et al. 2003) and root hair positioning (Fischer et al., 2006). The most direct biochemical evidence for the auxin influx function of

AUX1 protein has been provided by Yang et al. (2006) who expressed the *AUX1* in *Xenopus* oocytes and proved its biochemical function as an auxin influx carrier. Only very recently, evidence has been provided for the expression of a sequentially similar gene (cDNA), *CgAUX1* from *Casuarina glauca* together with data supporting the role of the gene product as a functional homologue of AUX1 (Peret et al., 2007).

It is known that the *AUX/LAX* sequences are highly similar among plant species (for review see Parry et al., 2001a). cDNA sequences with high amino acid similarity to AUX1 have been cloned from various species (*Populus tremulus×tremuloides*, Schrader et al., 2003; *Zea mays*, Hochholdinger et al., 2000; *Medicago truncatula*, de Billy et al., 2001; *Cucumis sativa*, Kamada et al., 2003; *Pisum sativum*, Hoshino et al., 2005; Peret et al., 2007) and the expression of corresponding mRNAs in respective plants was confirmed.

In this paper, we describe the cloning of Pa*LAX1* gene from the cDNA library of wild cherry tree (*Prunus avium*), a commercially and ecologically important perennial woody plant, clonal propagation of which is complicated by poor rooting of the cuttings (Durkovic, 2006). Since the root development is dramatically affected by auxin distribution, genes relevant to this process are of high interest. We first describe a heterologous expression of such a gene, Pa*LAX1*, coding for a putative auxin transporter from *Prunus avium*, in transgenic plants and its impact on endogenous auxin level, the phenotype of transgenic tobacco (*Nicotiana tabacum*) and Arabidopsis plants, and the impact of the transgene expression on the auxin uptake. Also, we show that the agravitropic phenotype of *aux1* mutant lacking the function of auxin influx carrier is complemented by Pa*LAX1* expression and this complementation can be reverted back to *aux1* phenotype by the inhibitor of auxin uptake. Our results strongly support the role of *Pa*LAX1 as an auxin influx carrier/facilitator.

**Results**

*The isolation of Pa*LAX1 *cDNA, phylogenetic analysis of the predicted protein, the endogene and transgene expression*

We isolated cDNA of an AUX/LAX-like putative auxin influx carrier protein from *Prunus avium*, Pa*LAX1*. Several independent clones containing the complete amino acid sequences of AUX/LAX-like proteins were obtained. The coding sequences of the clones were identical, giving a protein of 483 amino acid residues. The majority of clones contained a cDNA of 2004 bp, though one clone contained an additional 142 bp at the 5' end, upstream of the translation start codon and another clone contained two small 5' deletions of 9 bp and 44 bp. It was not clear if these represented cloning artifacts or the evidence of regulation of mRNA translation as suggested by de Billy et al. (2001). Similarity between Pa*LAX1* and other plant *AUX/LAX*-like sequences was high at both the nucleotide and the amino acid levels. The greatest identity found was 89% between Pa*LAX1* and Ptt*LAX1* from hybrid aspen (Schrader et al. 2003). Identity of Pa*LAX1* and *AUX1* from Arabidopsis was 85%. We compared all the full-size known *AUX/LAX* cDNAs and their predicted protein products. The major differences between the predicted protein sequences were at both the amino and the carboxyl termini, as reported previously for the other AUX/LAX proteins (Parry et al., 2001a). Phylogenetic analysis based on multiple alignment shows clearly that there are two distinct subfamilies of AUX/LAX proteins which differ in their amino acid sequences in, mainly, intracellular-oriented hydrophilic loops (Figs. 1A, B, Supplemental Fig. S1).

A probe from the 5' untranslated region of Pa*LAX1*, hybridizing solely with the unique *Prunus avium* sequence when used for Southern blot, was used for Northern blot analysis against total RNA extracted from different *Prunus avium* tissues. Pa*LAX1* was expressed in roots, root tips, shoot apices, stems and leaves (data not shown).

In order to confirm similar functions of products of genes Pa*LAX1* and *AUX1*, we constructed plant transformation vectors which were further used for tobacco and Arabidopsis transformation. We designed a set of primers which specifically amplified only the transgene and not a putative endogene of *AUX/LAX* family; this set of primers gave a clear single product corresponding to Pa*LAX1* transgene/mRNA in both Arabidopsis and tobacco while no amplification product was detected in either PCR or RT-PCR reactions performed on wild-type control plants. In all transgenic lines obtained, we confirmed the stable presence of the transgene as well as its expression on the mRNA level.

*Phenotype of Pa*LAX1 *tobacco plants and their regeneration from stem internodal segments*

We obtained 6 independent transgenic tobacco lines that carried the Pa*LAX1* cDNA under transcriptional control of the *rolC* promoter (Schmülling et al., 1989), which allowed vascular-tissue-specific expression of the gene in leaves and roots. Based on distinct phenotypic changes, we chose two transgenic lines, *NtPaLAX1-5* and *NtPaLAX1-8*, showing the typical phenotype, for further experiments.

Compared to the wild-type control plants, the transgenic plants were generally smaller and in both transgenic lines the root gravitropism and/or their ability to penetrate growth media was significantly affected. Internodes of both transgenic lines were reduced in lengths and the leaves formed rosettes. Mature leaves were chlorotic and showed accelerated senescence. Also, the progression of leaf senescence was different in the leaves of transgenic plants, where the intercostal regions were chlorotic and the regions along the main veins retained chlorophyll. In the control wild-type plants, the pigmentation within the leaf area was not affected (Fig. 2A).

The most noticeable differences between the transgenic and wild-type plants were the petiole length, the stem length and the leaf surface area. Petioles and stems of transgenic plants were shorter than those of control wild-type plants. Even though the length and width of the leaf (leaf surface area) were reduced in transgenic plants, the calculated factors of circularity and elongation (see Materials and Methods) were not affected, which means that the leaf shape remained almost unchanged (Table I).

Stem internodal segments of transgenic lines *NtPaLAX1-5* and *NtPaLAX1-8* regenerated rapidly on standard MS media compared with controls (Fig. 2B). Moreover, after 3 weeks of cultivation on regeneration media (containing 2,4-D, the auxin which is a good substrate for an active auxin uptake; Delbarre et al., 1996; Yamamoto and Yamamoto, 1998), the transgenic lines developed about 4 adventitious buds while the control plants developed only 0-2 adventitious buds (details not shown). To elucidate whether the observed rapidity of regeneration can be directly attributed to the expression of Pa*LAX1* transgene and, concomitantly, to the increased auxin uptake, we placed tobacco stem segments on standard 2,4-D-containing MS media supplemented with auxin influx carrier inhibitor, 1-naphthoxyacetic acid (1-NOA, 5µM; Imhoff et al., 2000; Parry et al., 2001b) and observed their ability to regenerate.

Regeneration of wild-type explants (Fig. 2B) was completely inhibited by 1-NOA, which also remarkably suppressed regeneration of transgenic explants. We also tested the effect of higher concentration of 1-NOA (10 μM) and, in this case, the regeneration of both wild-type and transgenic explants was inhibited completely (data not shown). Addition of 1-NAA (the auxin entering the plant cell without the need for auxin transporter; Delbarre et al, 1996; Yamamoto and Yamamoto, 1998; Marchant et al., 1999), to media containing 1-NOA improved regeneration of all explants even though not to the level achieved on standard media without the inhibitor. The addition of 2,4-D to media with inhibitor did not improve regeneration capacity of wild-type explants while the stems of transgenic plants regenerated even more rapidly than on media with 1-NAA and, in comparison with the controls grown on media without inhibitor, displayed only slightly delayed bud formation.

*Phenotype of Pa*LAX1 *Arabidopsis plants and complementation of the* aux1 *mutant phenotype by strong expression of Pa*LAX1

After transformation, seventeen independent homozygous transgenic lines carrying the Pa*LAX1* cDNA under the strong constitutive CaMV35S promoter were obtained, twelve of them exhibiting the ’auxin phenotype‘(as previously described by Romano et al., 1995 and Zhao et al., 2001). Of these twelve transgenic lines, two lines (*AtPaLAX1-5* and *AtPaLAX1-15*), displaying the typical phenotype and growth characteristics, were chosen for further experiments (Fig. 3). Hypocotyls of transgenic lines *AtPaLAX1-5*, *AtPaLAX1-15* were longer than those of control seedlings as well as being slightly thicker in diameter (Table II). Compared to the control plants, the petioles in both transgenic lines were longer and their leaves were elongated (Fig. 3). The apical dominance of the main inflorescence was suppressed and the transformed plants developed multiple inflorescences (data not shown). The root hairs of transgenic plants developed more readily but not in greater abundance (Figs. 4A, B). To quantify this difference in development, we measured the average length of visible root hairs of four-day-old plants (Table II). The length of epidermal cells along the root remained unchanged. While roots of control plants exhibited positive gravitropic growth, the *AtPaLAX1-5* and *AtPaLAX1-15* lines showed disrupted gravitropism and occasional root coiling. However, the roots of transgenic lines were not completely agravitropic as those of *aux1* mutant plants (Fig. 4C). We quantified the gravitropism by the “gravitropic factor”, GF = ratio of root tip distance from the base of rosette measured before/after 90° reorientation of

the *in vitro* grown plants (calculation method based on Vicente-Agullo et al., 2004). This factor represents the curving of the root after being exposed to a changed gravity and the value GF corresponds to the ability of a given plant to react to such a change. The lower the value is, the more gravitropism the plant exhibits. The values of the GF factor were 0.6 ± 0.1 in control wild-type plants and 0.92 ± 0.1 in *AtPaLAX1-15* plants, which corresponds to the rooting differences we observed visually (Fig. 4C).

In order to prove the functional similarity of AUX1 and *Pa*LAX1, we transformed *aux1* mutant plants with a vector carrying Pa*LAX1* under control of the strong CaMV35S promoter. The expression of Pa*LAX1* transgene was confirmed by RT-PCR. In T1 population of homozygotes and heterozygotes, the root gravitropism was completely restored only in some plants (Fig. 4D), which are statistically likely to represent homozygotes. In the stable homozygous lines, the gravitropic phenotype was completely restored (Fig. 4E). When the transgenic plants were placed on media with addition of 1-NOA, complementation of the *aux1* phenotype was not achieved (data not shown).

*Endogenous IAA content in tobacco and Arabidopsis transgenic lines, distribution of auxin in transgenic Arabidopsis plants*

In tobacco plants, the content of free IAA was measured in mature leaves (leaves 3 to 6, leaf 6 being the oldest) of *NtPaLAX1-5* and *NtPaLAX1-8* and wild-type control plants regenerated from apical cuts (three weeks after the transfer of the apical cuts to the fresh media). The amount of IAA in *NtPaLAX1-5* and *NtPaLAX1-8* lines was almost four times higher than that in control leaves of wild-type plants (Fig. 5A).

In eight-day old Arabidopsis plants, the auxin content was measured separately in both leaves and whole roots. In the leaves of transgenic plants, the content of IAA was generally higher in comparison with wild-type plants and, moreover, the distribution pattern of IAA between leaves and roots was pronouncedly different from the control (Fig. 5B).

The distribution of auxin was also monitored in eight-day old Arabidopsis plants by reporter gene *GUS* placed under control of the auxin-inducible promoter *IAA2*. Generally, the higher auxin levels were noted in leaf bud primordia, primary leaf veins and future leaf-tip hydathodes in both wild-type and transgenic plants. The major difference in auxin distribution was noted at the root tips of transgenics, which were stained in a manner different from wild-

type control root tips. The reporter gene expression noted in stele, columella and lateral root cap of wild-type plants, extended to all cell layers of the root tips of transgenic plants (Fig. 6).

*Net 2,4-D uptake in midribs of tobacco leaves and in inflorescence stems of* A. thaliana

The synthetic auxin 2,4-D was previously reported to be a good substrate for the active auxin uptake in plant cells (Delbarre et al. 1996) which is why we used it for the measurements of the net auxin uptake in our experiments.

The very short internodes of the transgenic tobacco plants did not enable us to measure the net auxin uptake in the stem tissue. The accumulation of radiolabeled 2,4-D was, therefore, measured in segments of tobacco leaf midribs. The accumulation of radiolabeled 2,4-D was two to three times higher in transgenic lines than in a control wild-type line (Fig. 7A). We have also proved the effect of the auxin influx inhibitor, 10 μM 1-NOA on the accumulation of 2,4-D. In both wild-type and transgenic lines the accumulation was reduced after 1-NOA pre-treatment to approximately half compared to the controls without 1-NOA pre-tretment (Fig. 7B). The accumulation of permeable auxin 1-NAA remained unchanged in transgenic lines compared to wild-type line (Fig. 7C).

When segments of Arabidopsis inflorescence stems were used for the net 2,4-D uptake assay, transformed lines accumulated, similarly to tobacco plants, approximately 2.5 times more radiolabeled 2,4-D than the control line after 30 min of the treatment (Fig. 7D). The effect of 1-NOA application was also similar to that in tobacco and both the wild-type line and the transformants accumulated 1.5 to 2 times less 2,4-D when pre-treated with 10 μM 1-NOA in comparison with non-treated controls (Fig. 7E). 1-NAA accumulation was not different in transgenic lines when compared to wild-type (Fig. 7F).

## Discussion

*The evolution of AUX/LAX protein family based on analysis of the predicted protein sequences and placement of* Pa*LAX1 permease into subfamily AUX*

Based on expected nucleotide sequence similarity to the previously isolated and cloned gene *AUX1* from Arabidopsis (Maher and Martindale 1980, Bennett et al. 1996), a member of amino acid/auxin permease family (Young et al., 1999), cDNA of a new gene Pa*LAX1* has been cloned from *Prunus avium* cDNA library. In similar experiments, the *AUX1*-like cDNAs have been isolated from other plant species (Hochholdinger et al., 2000; de Billy et al., 2001; Kamada et al., 2003; Schrader et al., 2003; Hoshino et al., 2005; Peret et al., 2007; also reviewed by Parry et al., 2001a). According to data published and this study, the *AUX1*-type genes seem to be highly conserved in plant species. However, in spite of the high degree of conservation, the multiple alignment and subsequent phylogenetic analyses performed by three independent programs (PROML (Phylip Package), phyloML, CLUSTAL W) revealed two subfamilies (Fig. 1A). Within these two subfamilies, the members differ only in minor branching parameters (cf. also Peret et al., 2007). We suggest to name those two emerging subfamilies AUX (consisting of AtAUX1, AtLAX1, CsAUX1, MtLAX1, MtLAX2, PaLAX1, PsAUX1, PtAUX1, PttLAX1 and PttLAX2) and LAX (consisting of AtLAX2, AtLAX3, MtLAX3, OsLAX, PttLAX3 and ZmAUX1).

Representatives of both protein subfamilies are present in *Arabidopsis thaliana*, *Medicago truncatula* and *Populus tremula* x *tremuloides*. In other plants, at present we were able to identify members of only one of the respective subfamilies. However, complete genome sequences are not available for those plant species and thus we cannot state conclusively whether members of the other subfamily are present in the respective genomes or not.

Subfamily-specific amino acid differences occur only in the intracellular hydrophilic loops, as described previously in a structure model (Swarup et al. 2004), and the changes on specific positions (Fig 1B) indicate possible alteration(s) of biochemical properties determined by the particular amino acid residues. This finding points to potentially significant differences between the two subfamilies of the AUX/LAX proteins. We speculate that the members of the two described subfamilies have the same substrate specificity and affinity because of conserved secondary structure of the extracellularly-oriented hydrophilic loops. On the other hand, the amino acid changes in the less conserved intracellularly-oriented hydrophilic loops could result in interactions with different intracellular proteins and, thus, in modifications of

intracellular transport machinery and/or signaling. Together, these data can help the finding and distinguishing other members of AUX/LAX family in various species.

*PaLAX1 expressing transgenic plants have distinct phenotypes and corresponding higher levels of free IAA*

We have observed marked changes in phenotypes of plants carrying the Pa*LAX1* gene under control of strong constitutive promoters. While the viral promoter CaMV35S promotes strong gene expression in all cell types, bacterial promoter rolC has been reported to direct the expression preferentially to vascular tissues (Schmülling et al., 1989). We assumed that vascular-tissue-driven expression of auxin transport related gene Pa*LAX1* might have a more pronounced effect on the phenotypical parameters which we were studying. However, we have not noticed any major differences which would reflect this difference. Generally, the phenotypical changes (Figs. 2, 3 and 4) corresponded to those previously described in such plants where high internal levels of endogenous auxins were caused by modification of auxin metabolism (Romano et al., 1995; Gray et al., 1998; Buchanan et al. 2000; Zhao et al., 2001; van der Graaff et al., 2003). The elevated levels of free endogenous auxin in the transformed lines, resulting in changed auxin:cytokinin ratio, could be also responsible for chlorotic appearance and accelerated senescence, as observed in our transgenic lines.

Not surprisingly, higher levels of auxin were measured in both tobacco and Arabidopsis plants expressing Pa*LAX1*. Interestingly, the distribution of free IAA between leaves and whole roots is remarkably changed in transgenic Arabidopsis plants, which contain much higher proportion of free IAA in leaves than in roots (Figs 5A, B).

Ljung et al. (2001) showed that free IAA levels could undergo enormous changes related to the stage of development of organs and tissues in *A. thaliana* and tobacco. Our data on free auxin content are well consistent with previous measurements reported for tobacco leaves and Arabidopsis leaves and roots (Romano et al., 1995; Gray et al., 1998; Casimiro et al., 2001; van der Graaff et al., 2003; Nordström et al., 2004; Jones et al. 2004). Even though the IAA levels were significantly increased in all transformed plants (40 – 300 pmol $g^{-1}$ FW for wild type and 3 – 1000 pmol $g^{-1}$ FW for transgenics), they were still within the range of 'physiological' auxin levels similar to those reported for other plants with changed auxin levels.

Changes in the overall free auxin content and the free auxin distribution are difficult to explain without further data. The increased content of free auxin in some organs of transgenics can be attributed to metabolic changes and/or just to the auxin distribution being different from control plants. These metabolic changes, generally, may include a) higher rate of IAA biosynthesis and/or release from conjugates; b) lower rate of IAA degradation and/or conjugation. The total pool of IAA in Arabidopsis seedlings consists of as little as 1% of free IAA and the 99% of its conjugated forms (Tam et al., 2000); therefore, even a little shift in the rate of IAA conjugation might have a major effect on free IAA level. On the other hand, beside the metabolic changes mentioned above, the difference in leaf-root distribution of free IAA can be also a result of increased auxin degradation in roots and/or free auxin molecules having remained trapped in the "source" pool in leaves. The latter case could reflect a possible imbalance between the actions of auxin influx and efflux carriers, even though such an explanation does not entirely correspond to the reported feedback on expression of some auxin efflux carriers by auxin itself (Vieten et al., 2005).

Below, we discuss the higher auxin uptake by cells of transgenic Pa*LAX* plants but it should be noted here that the free IAA measurements cannot confirm the direct and exclusive relation between changes in auxin uptake and its endogenous content. The higher auxin content, presumably responsible for the changes of the phenotypes, might have arisen in response to the overall changes in hormonal homeostasis, related also to a stress reaction resulting from the overexpression of the Pa*LAX1* transgene.

*Functional analysis of PaLAX1 supports its role as an auxin uptake carrier*

The increased ability of transgenic tobacco stem segments to regenerate into whole plants (Fig. 2) corresponded to expected high levels/activity of auxin transporter(s). The 'intensified' development might well result from the abundance of the presumptive influx carrier *Pa*LAX1 in outer layers of stems, which would allow more pronounced auxin gradient formation. Co-operation of auxin influx and efflux carriers in leaf primordia initiation and formation has been reported (Stieger et al. 2002; Reinhardt et al., 2003), and the model of "reversed fountain" has been suggested for formation of aerial organs with the major role of PIN1 proteins in auxin distribution (Benkova et al., 2003). We expect that in the Pa*LAX1*-transgenic plants the function of the endogenous auxin efflux carriers is either not affected or it can be even stimulated by higher auxin levels (in concert with the data by Vieten et al., 2005).

Assuming that higher auxin influx rate can be compensated by auxin efflux carrier(s), the more intense organ formation in transgenics might result from more intense auxin flow and resulting auxin accumulation in some organs, rather than from the higher overall 'statical' auxin content.

The differential sensitivity of regeneration of control and transgenic plants to auxin influx inhibitor also points to the changes in auxin uptake. The transgenic and wild-type control stem segments placed on auxin uptake inhibitor, 1-NOA, did not regenerate into shoots. Placed on media containing both 1-NOA and 1-NAA, the auxin entering the cell readily even in the absence of an auxin transporter, both the control and transgenic samples showed an improved regeneration capacity even if it was not as prominent as that on standard regeneration media. Remarkable difference was achieved when the stem segments were placed on media with the uptake inhibitor and 2,4-D, the auxin preferentially delivered into cells by a carrier (Delbarre et al., 1996). While control wild-type segments did not react to the presence of this synthetic auxin and their regeneration remained completely inhibited, the transgenic segments expressing Pa*LAX1* readily regenerated.

Reporter assays in Pa*LAX1*-transgenic plants revealed higher levels of auxin signal in all cell layers of the root tip. This is in contrast to the wild-type roots, where the auxin signal was limited to the vasculature, columella and root cap (in concert with data published previously by Swarup et al., 2005, Casimiro et al., 2001).

Our results revealed that, under some circumstances, Pa*LAX1* expression (see below), could also complement the agravitropic phenotype of Arabidopsis loss-of-function *aux1* mutant. Moreover, this phenotypical complementation of AUX1 function could be reverted back to agravitropic phenotype by the auxin uptake inhibitor 1-NOA. Interestingly, only transgenic lines homozygous in Pa*LAX1* showed fully restored gravitropism (in all lines). Whether (and why) the gravitropism restoration relates strictly to homologous phenotype and what could be the possible causes of this phenomenon has yet to be explored (e.g. stronger expression from more copies of the gene is needed for the phenotype restoration, some other phenomena related to tissue-specific expression or development-specific expression are involved, etc.,).

In addition to the above mentioned phenotypical changes, which point to the role of *Pa*LAX1 as an auxin influx carrier, we have proved that strong expression of Pa*LAX1* is accompanied by an increase in the net auxin uptake in both tobacco and Arabidopsis plants. For comparisons of the auxin uptake in transgenic and wild type plants, radiolabeled 2,4-D was used. This synthetic auxin was reported to be a good substrate for auxin influx carriers, but not for efflux carriers and, probably due to the polarity of its molecule, it is not taken up into

cells, at significant rate, by passive diffusion (Delbarre et al. 1996). In concert with this, the agravitropic phenotype of the *aux1* mutant could not be rescued by 2,4-D while it was rescued by the membrane permeable (Yamamoto and Yamamoto, 1998; Marchant et al., 1999) auxin 1-NAA. On the other hand, IAA was previously reported (in accordance with the fact that it is a native auxin) to be a good substrate for both influx and efflux carriers and it could also enter cells by passive diffusion (Delbarre et al., 1996). Thus, using radiolabeled IAA, its accumulation reflects the "balance" between the rates of both passive and carrier-driven influx, as well as carrier-driven efflux. In contrast to this, using labeled 2,4-D for measurements of the net auxin uptake in plant segments made our assays 'specific' for monitoring the activity of the auxin uptake carriers.

The transformed tobacco plants had very short stems; thus, in this case, we could not use tobacco stem segments for a standard auxin uptake assay. Instead, we designed a method using tobacco leaf midribs for comparison of "capacity" for the net auxin accumulation between transformed and control tobacco plants. In Arabidopsis, the inflorescence stems were used for the standard labeled 2,4-D uptake assay (Parry et al. 2001b, Morris and Robinson, 1998). Given the importance of the net uptake (more precisely "accumulation") assays and the comparisons between transformants and controls, the question of how to express the uptake data is crucial. To exclude the impact of differences in 'robustness' between transformants and controls we have compared the data calculated as relative to fresh weight and the plants of approximately equal size were always used.

In all experiments performed, the transgenic explants accumulated about three times more radiolabeled 2,4-D compared to the control wild-type explants. The accumulation rate of transformed segments, when pre-treated with the auxin uptake inhibitor, 1-NOA, corresponded to that of wild-type. As expected, the untreated wild-type control showed a higher rate of auxin accumulation compared to wild-type samples treated with 1-NOA. The comparative accumulation of 1-NAA, the membrane-permeable auxin, remained unaffected (Fig. 7). Thus, the significantly higher ability of transgenic plants to accumulate 2,4-D, the auxin transported preferentially via active uptake, strongly supported the direct involvement of *Pa*LAX1 in the uptake of auxin molecules into cells, probably as the auxin influx carrier itself.

*Conclusion*

Even though it is very difficult to prove the molecular function of *Pa*LAX1 by the means of the biochemical methods available, our 2,4-D-accumulation results, showing increased auxin uptake in Pa*LAX1*-transgenic plants (which can be specifically inhibited by the specific auxin uptake inhibitor), together with the phenotypical characterization of transgenic plants and the protein sequence similarity of *Pa*LAX1 to the single-component amino acid permeases (Young et al., 1999; Swarup et. al 2004) provide quite solid background for marking *Pa*LAX1 protein, together with other permeases of AUX and LAX subfamilies, as likely candidates for auxin influx carriers.

## Materials and Methods

### *Chemicals*

Unless stated otherwise, all the commonly used chemicals were supplied by Sigma Aldrich and the kits for gene cloning and detection of expression were obtained from Qiagen. [$^3$H]IAA and [$^3$H]2,4-D (both of specific radioactivity 20 Ci mmol$^{-1}$) were produced by American Radiolabeled Chemicals, Inc.

### *Sequence analysis*

Identification of *At*AUX1 homologues was carried out by a BLAST search at the National Center for Biotechnology Information (Altschul et al., 1990). Similarity of the amino acid sequences retrieved from the BLAST search was analyzed by multiple-sequence alignment using the MUSCLE program (Edgar, 2004), and PROML from the Phylip package, which estimates phylogenies from protein amino acid sequence alignment by maximum likelihood (Felsenstein, 1989). For the amino acid multiple alignment we used the highly conserved region, which, generally, represents the central part of the predicted protein (Supplemental Figure 1).

### *Isolation of Pa*LAX1 *cDNA, construction of transformation vectors, PCR and RT-PCR*

For the isolation of *AUX1*-related clones from *Prunus avium*, the cDNA library was prepared from shoot tips collected from mature trees in the spring. Tips consisted of vegetative shoot apices and surrounding leaf primordial and expanding leaves. The library was constructed in the lambda Zap II vector (Stratagene). An aliquot of the library was first screened by PCR using primers for conserved regions of the *Arabidopsis thaliana AUX1* gene (the gene was kindly provided by Prof. M. Bennett, Univ. Nottingham, UK). The sequence of *AUX1*-like amplification products was used to design *Prunus-avium*-specific primers. A combination of a 5' SK vector sequence primer (CGCTCTAGAACTAGTGGATC) and a 3' PauxR3 *Prunus-avium*-specific primer (GTTGAGAAGCTCATCACCAAA) was found to amplify approx. 1 kb of 5' region of *AUX1*-like cDNA. This primer pair was used to screen row and column pools of aliquots of the cDNA library arrayed into 96-well plates. Positive aliquots were diluted and re-arrayed and further rounds of screening were carried out. Finally, aliquots were

plated in agar at low density, plaque purified and then positive single plaques were converted to plasmid clones in the vector pBluescript SK - using the manufacturers' protocol. Clones were classified by restriction digestion and those containing the longest inserts were sequenced on both strands with an ABI 373A automated sequencer (Applied Biosystems, Warrington, UK). Sequence data was analyzed using GCG software (Genetics Computer Group, Madison, USA). Nucleotide and protein alignments were made using ClustalW software (Thompson et al., 1994). The nucleotide sequence data were deposited in the EMBL nucleotide sequence database under accession no. AJ862887.

A full length Pa*LAX1* cDNA clone was used for the construction of transformation vectors pROKPaLAX1 and pCPaLAX1 (Fig. 1C). Both these vectors are derivatives of pBIN19 (Bevan, 1984) and they carry the plant selectable marker neomycin phosphotransferase (*NPTII*). In *pROKPaLAX1* (Pa*LAX1 cDNA* placed under control of CaMV35S promoter), BglII/SacI fragment of pBSchaxfull (Pa*LAX1* cDNA) was ligated into pROK2 vector (Baulcombe et al., 1986) digested with BamHI/SacI. This vector was used for transformation of *Arabidopsis thaliana* plants. In *pCPaLAX1* (Pa*LAX1 cDNA* placed under control of rolC promoter), the initial vector pBIpoc (Lucie Perry, unpublished) was constructed by replacement of the HindIII/SmaI CaMV35S promoter region of pBI121 (Clontech) by a HindIII/EcoRV polylinker of pUC57 (Fermentas). Agrobacterial *rolC* gene promoter from the pRiA4 plasmid of *Agrobacterium rhizogenes* was amplified. The SalI/BglII fragment of the PCR product together with SacI/BglII digested Pa*LAX1* cDNA were ligated into pBIpoc digested with SalI/SacI. This vector was used for transformation of *Nicotiana tabacum* plants.

Detection of transgene and its expression was performed by PCR and RT-PCR, respectively (CHAXf 5'-TACACAGCCTGGTACTTG-3'; CHAXr 5'-ATCACAACTGGAAGCCTA-3' Pa*LAX1*-specific primers; Ta=52°C). Amplification from specific primers did not result in visible product when performed on control non-transgenic material. The DNA techniques were performed according to the usual laboratory protocols described in Ausubel (1995) with modifications (Krizkova and Hrouda 1998).

*Transformation,* in vitro *cultivation and regeneration of tobacco plants*

Tobacco plants (*Nicotiana tabacum* L., cv. Maryland Mammoth) were used for the leaf disc transformation *via Agrobacterium tumefaciens* as described in Clark (1997). All cultivations were performed at 20ºC/23ºC, 16 h light/8 h dark photoperiod cycles. Regenerated transformants were grown on modified solid MS media (4.3 g $l^{-1}$ MS basal salt mixture, 104

mg $l^{-1}$ MS vitamins, 4% sucrose, 1 g $l^{-1}$ casein-hydrolysate, 100 mg $l^{-1}$ inositol, 0.8 % Agar-Agar, pH 5,6; supplemented with 100 µg $ml^{-1}$ kanamycin (kanamycin sulphate; Sigma) and 1 mg $ml^{-1}$ cefotaxim (Claforan; Roussel). The apical cuts were transferred to fresh media every 3-4 weeks and regenerated to new plants.

For regeneration experiments, the stems of six weeks old transformed and control plants (i.e. six weeks after the transfer of the apical cuts to the fresh media) were cut into 5 mm internodal segments. The segments were placed on regeneration MS media (six segments per Magenta box): 4.3 g $l^{-1}$ MS basal salt mixture, 104 mg $l^{-1}$ MS vitamins, 5% glucose, 100 mg $l^{-1}$ inositol, 0.8 % Agar-Agar, 0.2 mg $l^{-1}$ IAA, 0.2 mg $l^{-1}$ kinetin, pH 5.6 supplemented with 100 mg $l^{-1}$ kanamycin (kanamycin sulphate; Sigma) and cultivated for 3 weeks. In inhibition experiments 5 µM 1-NOA, 5 µM 1-NOA together with 0,1 µM 2,4-D or 5 µM 1-NOA together with 1 µM 1-NAA were added to regeneration media, respectively. For the leaf shape description the circularity $(4\pi*\text{area})/\text{perimeter}^2$, max. circularity = 1) and elongation (max. Feret/min. Feret, $\alpha \in (0, 180)$; LUCIA $\alpha$ = 0, 10, 20...180) factors were calculated (image analysis software LUCIA G v. 4.71, designed by Laboratory Imaging, Prague, Czech Republic).

*Transformation of Arabidopsis plants, derivation of homozygous lines and genetic crosses*

Arabidopsis plants (*Arabidopsis thaliana*, (L.) Heynh., Columbia ecotype) were grown in a greenhouse for 4 to 5 weeks. All cultivations were performed at 20ºC/23ºC, 16 h light/8 h dark photoperiod cycles. The terminal stem was cut off to induce formation of secondary stems. Well developed plants with plenty of buds were transformed *via Agrobacterium tumefaciens* by floral dip method (Clough and Bent, 1998).

Collected T1 seeds were surface-sterilized using 70 % ethanol for 2 min. Selection of transformed plants was performed on modified solid MS media (MS half dose, 1 % sucrose) supplemented with 100 µg $ml^{-1}$ kanamycin (kanamycin sulphate; Sigma) and 1 mg $ml^{-1}$ cefotaxim (Claforan, Roussel). Selected plants were transferred to non-sterile conditions. The segregation ratio of kanamycin resistant and kanamycin sensitive plants was scored on three week old T2 and T3 seedlings. Lines homozygous for a T-DNA insert were grown in soil and further cultivated in a controlled climate cultivation chamber for 5 to 6 weeks to obtain well grown stems for further analyses.

For genetic crossing, auxin-inducible reporter *IAA2::GUS* lines (Swarup et al., 2001) were used (seeds kindly provided by Prof. M. Bennett, Univ. Nottingham, UK). For complementation experiments (transformation with pROKPaLAX1), the mutant *aux1* line (Maher and Martindale, 1980) was used (seeds of line N3074 obtained from NASC, Univ. Nottingham, UK). For GUS qualitative assay, transgenic seedlings were infiltrated for 15 min with GUS staining solution (100 mM $Na_2HPO_4$, 10 mM EDTA, 1mg $ml^{-1}$ X-Gluc), incubated at 37°C for 16 h and cleared with 70% ethanol overnight.

*Measurement of the net auxin uptake (auxin accumulation) in leaf midribs of tobacco and in inflorescence stems of Arabidopsis*

For studies of the net auxin uptake (auxin accumulation)), 3-mm-segments were cut from isolated tobacco leaf midribs (taken from 8 week-old plants) or from Arabidopsis inflorescence stems (taken from 5-week-old plants). For measurements, segments of approximately equal weight, length and diameter were used for both the transgenic and the wild type control plants. Since the stems of transgenic tobacco were too short to provide a suitable material for the measurements of the net auxin uptake (auxin accumulation), we have developed an adequate method of measuring the auxin uptake in leaf midribs. This method has proved to be statistically plausible for the detection of differences in the auxin uptake between the transgenic plants and the wild type controls. For accumulation assays in Arabidopsis, standard material (inflorescence stem segments) was used (Parry et al., 2001b).
The segments were placed into the ice-cold uptake buffer (1.5% sucrose, 23 mM MES, pH 5.5 KOH) for 15 min and washed two times in the fresh uptake buffer for 15 min. Segments were surface-dried on filter paper. 'Dry' segments were weighed.
100 mg of segments were dropped into 5 ml 2nM [$^3$H]2,4-D or 2nM [$^3$H]1-NAA for 0, 5, 10 and 15 min, respectively, in 3 repeats per each sample. Accumulation of the labeled 2,4-D was terminated by rapid filtration under reduced pressure on 22-mm-diameter cellulose filters. The label was extracted in 1ml of 96 % ethanol for 30 min and afterwards 5 ml of scintillation solution (EcoLite; ICN, USA) were added and the sample was incubated overnight (Morris and Robinson, 1998). Radioactivity was then measured using the liquid scintillation analyzer Packard tri-Carb 2900TR (Packard Instruments Co., Meriden, CT). When the effect of 1-NOA was examined, the 20 min pre-treatments with 10μM 1-NOA preceded the incubation with [$^3$H]-2,4-D.

*Determination of internal levels of endogenous auxin in leaves of tobacco and Arabidopsis transformed lines*

Free IAA was extracted by methanol/formic acid/water (15/1/4, v/v/v) from mature leaves 3-6 (leaf 6 being the oldest one) (tobacco) or the whole rosettes (Arabidopsis) homogenized in liquid nitrogen. The extract was purified using dual-mode solid phase extraction method as described in Dobrev and Kaminek (2002). The IAA determination itself was performed using two-dimensional HPLC. IAA was purified on two different HPLC columns and determined using on-line fluorescence detector with excitation and emission wavelengths adequate for IAA (Dobrev et al., 2005). The identity of IAA peak was verified using GC-MS with deuterated IAA standard. Internal standard of [$^{3}$H]IAA was used for checking the recovery of IAA during extraction.

*Statistics*

Each experiment was done three times, if not stated otherwise. Free IAA content was measured in three samples per plant in three plants of each, control or transgenic, line. In auxin accumulation assay, the number of repeats in one experiment was three using either stem segments or main veins per one plant, and three plants of each, control and transgenic, line were used for one experiment. Measured data were analyzed simultaneously by calculating arithmetical means of repeats, and their standard deviations and variations. Image analysis software LUCIA G v. 4.71 (Laboratory Imaging, Prague, Czech Republic) was used for phenotype data evaluation.

**Supplemental Material**

Supplemental Material includes multiple alignment of AUX/LAX family.

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**Literature Cited**

**Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ** (1990) Basic local alignment search tool. J. Mol. Biol. **215**: 403–410

**Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K** (1995) Current Protocols in Molecular Biology. John Wiley and Sons, New York, NY

**Baulcombe DC, Saunders GR, Bevan MW, Mayo MA, Harrison BD** (1986) Expression of biologically-active viral satellite RNA from the nuclear genome of transformed plants. Nature **321**: 446-449

**Benjamins R, Malenica N, Luschnig C** (2005) Regulating the regulator: the control of auxin transport. Bioessays **27**: 1246-1255

**Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertová D, Jürgens G, Friml J** (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell **115**: 591-602

**Bennett MJ, Marchant A, Green HG, May ST, Ward SP, Millner PA, Walker AR, Schultz B, Feldmann KA** (1996) Arabidopsis AUX1 gene: a permease-like regulator of root gravitropism. Science **273**: 948-950

**Bennett MJ, Marchant A, May ST, Swarup R** (1998) Going the distance with auxin: unrevealling the molecular basis of auxin transport. Phil. Trans. R. Soc. Lond. B **353**: 1511-1515

**Bevan MW** (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. **12**: 8711-8721

**de Billy F, Grosjean C, May S, Bennett M, Cullimore JV** (2001) Expression studies on AUX1-like genes in Medicago truncatula suggest that auxin is required at two steps in early nodule development. Mol. Plant-Microbe Interac. **14**: 267-277

**Blakeslee J, Peer WA, Murphy AS** (2005) Auxin transport. Curr. Opin. Plant Biol. **8**: 494-500

**Buchanan BB, Gruissem W, Jones RL** (2000) Biochemistry and Molecular Biology of Plants. (American Society of Plant Physiologists, Rockville, Maryland), Fig. 17.51, p. 895.

**Casimiro I, Marchant A, Bhalerao RP, Beeckman T, Dhooge S, Swarup R, Graham N, Inze D, Sandberg G, Casero PJ, Bennett M** (2001) Auxin transport promotes Arabidopsis lateral root initiation. Plant Cell **13**: 843-852

**Chen L, Ortiz-Lopez A, Jung A, Bush DR** (2001) ANT1, an aromatic and neutral amino acid transporter in Arabidopsis. Plant Physiol. **125**: 1813-1820

**Clark MS** (1997) Plant Molecular Biology. A laboratory manual. Springer-Verlag, Berlin, Heidelberg

**Clough SJ, Bent AF** (1998) Floral dip: simplified method for *Agrobacterium* – mediated transformation of *Arabidopsis thaliana.* Plant J. **16**: 735-743

**Delbarre A, Muller P, Imhoff V, Guern J** (1996) Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. Planta **198**: 532-541

**Dharmasiri S, Swarup R, Mockaitis K, Dharmasiri N, Singh SK, Kowalchyk M, Marchant A, Mills S, Sandberg G, Bennett MJ, Estelle M** (2006) AXR4 is required for localization of the auxin influx facilitator AUX1. Science **312**: 1218-1220

**Dobrev P, Kamínek M** (2002) Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. J. Chromatogr. A **950**: 21-29

**Dobrev P, Havlíček M, Vágner J, Malbeck J, Kamínek M** (2005) Purification and determination of plant hormones auxin and abscisic acid using solid phase extraction and two-dimensional high performance liquid chromatography. J. Chromatogr. A **1075**: 159-166

**Durkovic J** (2006) Rapid micropropagation of mature wild cherry. Biol. Plant. **50**: 733-736

**Edgar RC** (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput, Nucleic Acids Research **32** (5): 1792-97

**Felsenstein** J (1989) PHYLIP-Phylogeny Inference Package (Version 3.2), Cladistics **5**: 164-166

**Fischer U, Ikeda Y, Ljung K, Serralbo O, Singh M, Heidstra R, Palme K, Scheres B, Grebe M** (2006) Vectorial information for Arabidopsis planar polarity is mediated by combined AUX1, EIN2, and GNOM activity. Curr. Biol. **16**: 2143-2149

**Geisler M, Murphy AS** (2006) The ABC of auxin transport: The role of p-glycoproteins in plant development. Febs Lett. **580**: 1094-1102

**van der Graaff E, Boot K, Granbom R, Sandberg G, Hooykaas PJJ** (2003) Increased endogenous auxin production in Arabidopsis thaliana causes both earlier described and novel auxin-related phenotypes. J. Plant Growth Reg. **22**: 240-252

**Gray WM, Ostin A, Sandberg G, Romano CP, Estelle M** (1998) High temperature promotes auxin-mediated hypocotyl elongation in Arabidopsis. Proc. Natl. Acad. Sci. USA **95**: 7197–7202

**Grebe M, Friml J, Swarup R, Ljung K, Sandberg G, Terlou M, Palme K, Bennett MJ, Scheres B** (2002) Cell polarity signaling in Arabidopsis involves a BFA-sensitive auxin influx pathway. Curr. Biol. **12**: 329-334

**Heisler MG, Jonsson H** (2006) Modeling auxin transport and plant development. J. Plant Growth Reg. **25** : 302-312

**Hochholdinger F, Wulff D, Reuter K, Park WJ, Feix G** (2000) Tissue-specific expression of AUX1 in maize roots. J. Plant Physiol. **157**: 315-319

**Hoshino T, Hitotsubashi R, Miyamoto K, Tanimoto E, Ueda J** (2005) Isolation of PsPIN2 and PsAUX1 from etiolated pea epicotyls and their expression on a three-dimensional clinostat. Adv. Space Res. **36**: 1284-1291

**Imhoff V, Muller P, Guern J, Delbarre A** (2000) Inhibitors of the carrier-mediated influx of auxin in suspension-cultured tobacco cells. Planta **210**: 580-588

**Jones SE, Demeo JS, Davies NW, Noonan SE, Ross JJ** (2005) Stems of the Arabidopsis pin1-1 mutant are not deficient in free indole-3-acetic acid. Planta **222**: 530-534

**Kamada M, Yamasaki S, Fujii N, Higashitani A, Takahashi H** (2003) Gravity-induced modification of auxin transport and distribution for peg formation in cucumber seedlings: possible roles for CS-AUX1 and CS-PIN1. Planta **218**: 15-26

**Kerr ID, Bennett MJ** (2007) New insight into the biochemical mechanisms regulating auxin transport in plants. Biochem. J. **401**: 613-622

**Kleine-Vehn J, Dhonukshe P , Swarup R, Bennett M, Friml J** (2006) Subcellular trafficking of the Arabidopsis auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. Plant Cell **18**: 3171-3181

**Kramer ER** (2004) PIN and AUX/LAX proteins: their role in auxin accumulation Trends Plant Sci. **9**: 579-582

**Kramer EM, Bennett MJ** (2006) Auxin transport: a field in flux. Trends Plant Sci. **11**: 382-386

**Krizkova L, Hrouda M** (1998) Direct repeats of T-DNA integrated in tobacco chromosomes: characterization of junction regions. Plant J. **16**: 673-680

**Ljung K, Bhalerao RP, Sandberg G** (2001) Sites and homeostatic control of auxin biosynthesis in Arabidopsis during vegetative growth. Plant J. **28**: 465-474

**Maher EP, Martindale SJ** (1980) Mutants of *Arabidopsis thaliana* with altered responses to auxins and gravity. Biochem. Genet. **18**: 1041-1053

**Marchant A, Kargul J, May ST, Muller P, Delbarre A, Perrot-Rechenmann C, Bennett MJ** (1999) AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. EMBO J. **18**: 2066-2074

**Marchant A, Bhalerao R, Casimiro I, Eklöf J, Casero PJ, Bennett MJ, Sandberg G** (2002) AUX1 promotes lateral root formation by facilitating Indole-3-acetic acid distribution between sink and source tissues in the Arabidopsis seedling. Plant Cell **14**: 589-597

**Morris DA, Robinson JS** (1998) Targeting of auxin carriers to the plasma membrane: differential effects of brefeldin A on the traffic of auxin uptake and efflux carriers. Planta **205**: 606-612

**Morris DA, Friml J, Zažímalová E** (2004) in Plant Hormones: Biosynthesis, Signal Transduction, Action!, ed. Davies, P.J. (Kluwer Acad. Publ., Dordrecht), pp 437-470

**Nordstrom A, Tarkowski P, Tarkowska D, Norbaek R, Astot C, Dolezal K, Sandberg G** (2004) Auxin regulation of cytokinin biosynthesis in Arabidopsis thaliana: A factor of potential importance for auxin-cytokinin-regulated development. Proc. Natl. Acad. Sci. USA **101**: 8039-8044

**Paponov IA, Teale WD, Trebar M, Blilou K, Palme K** (2005) The PIN auxin efflux facilitators: evolutionary and functional perspectives. Trends Plant Sci. **10**: 170-177

**Parry G, Marchant A, May S, Swarup R, Swarup K, James N, Graham N, Allen T, Martucci T, Yemm A, Napier R, Manning K, King G, Bennett M** (2001a) Quick on the uptake: Characterization of a family of plant auxin influx carriers. J. Plant Growth Regul. **20**: 217-225

**Parry G, Delbarre A, Marchant A, Swarup R, Napier R, Perrot-Rechenmann C, Bennett MJ** (2001b) Novel auxin transport inhibitors phenocopy the auxin influx carrier mutation *aux1*. Plant J. **25**: 399-406

**Péret B, Swarup R, Jansen L, Devos G, Auguy F, Collin M, Santi C, Hocher V, Franche C, Bogusz D, Bennett M, Laplaze L** (2007) Auxin influx activity is associated with *Frankia* infection during actinorhizal nodule formation in *Casuarina glauca*. Plant Physiol. **144**: 1852-1862

**Reinhardt D, Pescue ER, Stieger P, Mandel T, Baltensperger K, Bennett M, Traas J, Friml J, Kuhlemeier C** (2003) Regulation of phyllotaxis by polar auxin transport. Nature **426**: 255-260

**Romano CP, Robson PR, Smith H, Estelle M, Klee H** (1995) Transgene-mediated auxin overproduction in Arabidopsis – hypocotyl elongation phenotype and interactions with the hy6-1 hypocotyl elongation and axr1 auxin-resistant mutants. Plant Mol. Biol. **27**: 1071-1083

**Santelia D, Vincenzetti V, Azzarello E, Bovet L, Fukao Y, Duchtig P, Mancuso S, Martinoia E, Geisler M** (2005) MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development. Febs Lett. **579**: 5399-5406

**Schmülling T, Schell J, Spena A** (1989) Promoters of the *rolA, B,* and *C* genes of *Agrobacterium rhizogenes* are differentially regulated in transgenic plants. Plant Cell **1**: 665-670

**Schrader J, Baba K, May ST, Palme K, Bennett M, Bhalerao RP, Sandberg G** (2003) Polar auxin transport in the wood-forming tissues of hybrid aspen is under simultaneous control of developmental and environmental signals. Proc. Natl. Acad. Sci. USA **100**: 10096-10101

**Stieger PA, Reinhardt D, Kuhlemeier C** (2002) The auxin influx carrier is essential for correct leaf positioning. Plant J. **32**: 509-517

**Swarup R, Friml J, Marchant A, Ljung K, Sandberg G, Palme K, Bennett M** (2001) Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the Arabidopsis root apex. Genes Dev. **15**: 2648-2653

**Swarup R, Kargul J, Marchant A, Zadik D, Rahman A, Mills R, Yemm A, May S, Williams L, Millner P, Tsurumi S, Moore I, Napier R, Kerr ID, Bennett MJ** (2004) Structure-function analysis of the presumptive Arabidopsis auxin permease AUX1. Plant Cell **16**: 3069-3083

**Swarup R, Kramer EM, Perry P, Knox K, Leyser HMO, Haseloff J, Beemster GTS, Bhalerao R, Bennett MJ** (2005) Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. Nat. Cell Biol. **7**: 1057-1065

**Tam YY, Epstein E, Normanly J** (2000) Characterization of auxin conjugates in arabidopsis. Low steady-state levels of indole-9-acetyl-aspartate indole-3-acetyl-glutamate, and indole-3-acetyl-glucose. Plant Physiol. **123**: 589-595

**Tanaka H, Dhonukshe P, Brewer PB, Friml J** (2006) Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. Cell. Mol. Life Sci. **63**: 2738-2754

**Terasaka K, Blakeslee JJ, Titapiwatanakun B, Peer WA, Bandyopadhyay A, Makam SN, Lee OR, Richards EL, Murphy AS, Sato F, Yazaki K** (2005) PGP4, an ATP binding cassette P-glycoprotein, catalyzes auxin transport in Arabidopsis thaliana roots. Plant Cell **17**: 2922-2939

**Thompson JD, Higgins DG, Gibson TJ** (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**: 4673-4680

**Vicente-Agullo F, Rigas S, Desbrosses G, Dolan L, Hatzopoulos P, Grabov A** (2004) Potassium carrier THR1 is required for auxin transport in Arabidopsis roots. Plant J. **40**: 523-535

**Vieten A, Vanneste S, Wisniewska J, Benkova E, Benjamins R, Beeckman T, Luschnig C, Friml J** (2005) Functional redundancy of PIN proteins is accompanied by auxindependent cross-regulation of PIN expression. Development **132**: 4521-4531

**Yamamoto M, Yamamoto KT** (1998) Differential effects of 1-naphthalene acetic acid, indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid on the gravitropic response of roots in an auxin resistant mutant of Arabidopsis, *Aux1*. Plant Cell Physiol. **39**: 660-664

**Yang YD, Hammes UZ, Taylor CG, Schachtman DP, Nielsen E** (2006) High-affinity auxin transport by the AUX1 influx carrier protein. Curr. Biol. **16**: 1123-1127

**Young GB, Jack DL, Smith DW, Saier MH** (1999) The amino acid/auxin : proton symport permease family. Bioch. Biophys. Acta-Biomembranes **1415**: 306-322

**Zažímalová E, Křeček P, Skůpa P, Hoyerová K, Petrášek J** (2007) Polar transport of the plant hormone auxin - the role of PIN-FORMED (PIN) proteins. Cell. Mol. Life Sci., **64**: 1621–1637

**Zhao Y, Christensen SK, Frankhauser C, Cashman JR, Cohen JD, Weigel D, Chory J** (2001) A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science **291**: 306-309

**Zhuang XL, Jiang JF, Li JH, Ma QB, Xu YY, Xue YB, Xu ZH, Chong K** (2006) Over-expression of OsAGAP, an ARF-GAP, interferes with auxin influx, vesicle trafficking and root development. Plant J. **48**: 581-591

**Figure legends**

Fig. 1

A Unrooted phylogenetic tree of AUX1-like proteins.

The results of two independent analyses are presented. Proteins were aligned using MUSCLE; a phylogenetic tree was constructed using the maximum-likelihood method (PROML in Phylip Package). The branch lengths are proportional to the sequence divergence. The number at each node indicates the percentage of 1000 bootstrap replicates for statistical support. Accession numbers are as follows: *Arabidopsis thaliana* (At): AUX1, X98772; LAX1, AJ249442; LAX2, AJ243221; LAX3, AC012193; *Cucumis sativa* (Cs): AUX1, AB085896; *Medicago truncatula* (Mt): LAX1, AJ299397; LAX2, AJ299398; LAX3, AJ299399; *Oryza sativa* (Os): LAX, NM194844; *Prunus avium* (Pa): LAX1, AJ862887; *Pisum sativum* (Ps): AUX1, AB107919; *Populus tomentosa* (Pt): AUX1, AY864733; *Populus tremula x tremuloides* (Ptt): LAX1, AF115543; LAX2, AF190880; LAX3, AF263100; *Zea mays* (Zm): AUX1, AJ011794.

B Topological predictions of secondary structures in AUX/LAX family and primary structures of family members.

Upper panel: AUX1 secondary structure and protein topology (drawing based on model by Swarup et al. 2004, with permission) with marked amino acid changes between two predicted AUX/LAX subfamilies. The letters (standard amino acid code) represent the actual amino acid exchanges. The marked positions (red) correspond to amino acid changes between AUX and LAX subfamilies shown bellow. Symbols encircled in bold are depicted for easier orientation, they represent every 10th amino acid.

Lower panel: Parts of amino acid sequences of proteins of both LAX (upper section) and AUX (lower section) subfamilies showing the differences in their primary structures. The amino acids are colored according to their similarities based on PAM250 protein weight matrix.

Fig. 2

A Phenotypes of *in vitro* tobacco plants.

In comparison with wild type plants, transgenic lines *NtPaLAX1-5* and *NtPaLAX1-8* have shorter internodes and they form rosettes of chlorotic leaves (see also Table I for quantitative data). The petiole length in detached leaves of seven-week-old plants is noticeably shortened in both transgenic lines compared to the control plants. Three weeks after placing on regeneration media, regeneration of internodal stem segments is more prominent in both transgenic lines compared to that in controls. Root gravitropism and/or the ability to penetrate growth media are significantly impaired in transgenic plants (four weeks after clonal propagation of the apical cuts). Scale bar segment (left) = 1 cm.

B Effect of the auxin uptake inhibitor (1-NOA) on regeneration of tobacco stem internodal segments.

Three weeks after placing on regeneration media with 1-NOA (5μM), regeneration is impaired in both transgenic lines (*NtPaLAX1-5* and *NtPaLAX1-8*) and completely inhibited in wild-type (wt) compared to regeneration on standard media (control). Addition of 1-NAA (1μM) to media with 1-NOA partially restores the regeneration of both the transgenic and control segments. Addition of 2,4-D (0.1 μM) to media with 1-NOA restores the regeneration of transgenic segments and does not affect controls.

Fig. 3

Phenotypes of *in vitro* cultivated Arabidopsis plants.
Comparison of the overall morphology and leaves of plants of Arabidopsis transgenic line *AtPaLAX1-15* and control line (wt). Photographs taken in the same magnification (upper panel). Scale bars = 1 cm (lower panel).

Fig. 4
Rooting of *in vitro* cultivated Arabidopsis seedlings.
A, B Root hairs in the four-day-old transgenic Arabidopsis plants (B) growmore readily compared to controls (wt, A). Scale bars = 2 mm.
C, D, E The root gravitropism of the transgenic plants (*AtPaLAX1-15*) is disrupted even though the roots of ten-day-old plants are not completely agravitropic as can be seen in *aux1* mutant plants (C). Transformation of *aux1* mutant plants with Pa*LAX1* does not affect gravitropism in all ten-day-old plants; it only restores gravitropism in plants presumably homozygous for Pa*LAX1*; indicated by arrows (D). Complementation of the agravitropic phenotype is stable in ten-day-old plants of homozygous *aux1*Pa*LAX1* lines (E). Scale bars = 1 cm.

Fig. 5
Free endogenous IAA content in tobacco and Arabidopsis leaves grown *in vitro*.
A Free IAA content in tobacco wild-type (wt, white column) and transgenic plants (*NtPaLAX1-5*, gray column; *NtPaLAX1-8*, black column) measured in mature leaves (leaves 3-6) eight weeks after clonal propagation of the apical cuts. Vertical bars indicate SD (n=3 per one plant, 3 plants measured).

B Free IAA content in Arabidopsis wild-type (wt) and transgenic plants (*AtPaLAX1-5* and *AtPaLAX1-15*) measured in leaf rosettes (white columns) and whole roots (black columns) of eight-week-old plants. Vertical bars indicate SD (n=3 per one plant, 3 plants measured).

Fig. 6
Auxin-responsive *IAA2::GUS* reporter expression in Arabidopsis.
Auxin-responsive *IAA2::GUS* expression in root apicesof wild type (wt) and *AtPaLAX1-15* lines. Scale bars = 0.5 mm.

Fig. 7
Net accumulation of [$^3$H]2,4-D and [$^3$H]1-NAA in tobacco (A, B, C) and Arabidopsis (D, E, F).
A Net accumulation of [$^3$H]2,4-D in midribs of tobacco leaves (mix of leaves at various growth stages, eight-week-old plants). Wild-type control (white column), transgenic plants *NtPaLAX1-5* and *NtPaLAX1-8* (gray and black columns, respectively) at the time 5 min after addition of radiolabelled auxin.
B Time course of the net accumulation of [$^3$H]2,4-D in midribs of tobacco leaves (eight-week-old plants) after pre-treatment (20 min) with 1-NOA (10 μM). Wild-type (squares), transgenic plants *NtPaLAX1-5* and *NtPaLAX1-8* (triangles and diamonds, respectively), wild-type control without 1-NOA pre-treatment (circles).
C Time course of the net accumulation of [$^3$H]1-NAA in midribs of tobacco leaves (eight-week-old plants). Wild-type (squares), transgenic plants *NtPaLAX1-5* and *NtPaLAX1-8* (triangles and diamonds, respectively).
D Time course of the net accumulation of [$^3$H]2,4-D in Arabidopsis inflorescence stem segments (five-week-old plants). Wild-type (squares), transgenic plants *AtPaLAX1-5* and *AtPaLAX1-15* (triangles and diamonds, respectively).

E Time course of the net accumulation of [$^{3}$H]2,4-D in Arabidopsis inflorescence stem segments (five-week-old plants) after pre-treatment (20 min) with 1-NOA (10 μM). Wild-type (squares), transgenic plants *AtPaLAX1-5* and *AtPaLAX1-15* (triangles and diamonds, respectively), wild-type control without 1-NOA pre-treatment (circles).
F Time course of the net accumulation of [$^{3}$H]1-NAA in Arabidopsis inflorescence stem segments (five-week-old plants). Wild-type (squares), transgenic plants *AtPaLAX1-5* and *AtPaLAX1-15* (triangles and diamonds, respectively). Vertical bars indicate SD (n=3 per one plant, 3 plants measured).
Where invisible, SDs were smaller than the symbol.

| | petiole length (mm; n=9) | stem length (mm; n=6) | leaf circularity (n=6) | leaf elongation (n=6) | leaf surface area ($mm^2$; n=6) |
|---|---|---|---|---|---|
| *wt* | 17.1 ± 1.2 | 56.2 ± 4.8 | 1.7 | 0.84 | 435 ± 62 |
| *NtPaLAX1-5* | 10.3 ± 1.1 | 11.7 ± 3.2 | 1.5 | 0.88 | 310 ± 48 |
| *NtPaLAX1-8* | 8.0 ± 0.9 | 16.5 ± 4.2 | 1.6 | 0.84 | 538 ± 84 |

Table I
Quantitative effects of *Pa*LAX1 ectopic expression in tobacco.
Circularity calculation: $4\pi$*area)/perimeter$^2$, max. circularity = 1; elongation calculation: max. Feret/min. Feret, $\alpha \in (0, 180)$; LUCIA $\alpha$ = 0, 10, 20...180 (Image analysis software LUCIA G v. 4.71, Laboratory Imaging, Prague; n = number of measurements per each of three seven-week-old plants).

| | hypocotyl diameter (µm) | hypocotyl length (mm) | root hair growth (length of hairs in µm) |
|---|---|---|---|
| *wt* | 170 ± 19 (n=22) | 2.84 ± 0.39 (n=55) | 650 ± 125 (n=400) |
| *AtPaLAX1-5* | n.d. | 6.32 ± 0.89 (n=35) | n.d. |
| *AtPaLAX1-15* | 222 ± 18 (n=22) | 6.74 ± 0.81 (n=44) | 867 ± 100 (n=400) |

Table II
Quantitative effects of *Pa*LAX1 ectopic expression in Arabidopsis. Hypocotyl diameter (five-day-old plants) and length (seven-day-old plants) (n = number of plants).
Root hair length in four-day-old plants (n = total number of hairs measured in sixty plants/samples); root segments measured were of identical length and distance from the root tip.

Fig.1A

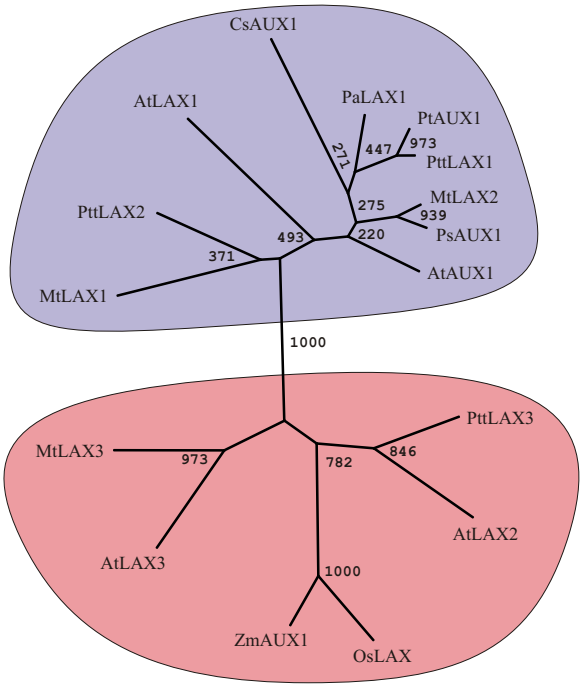



Fig.1B

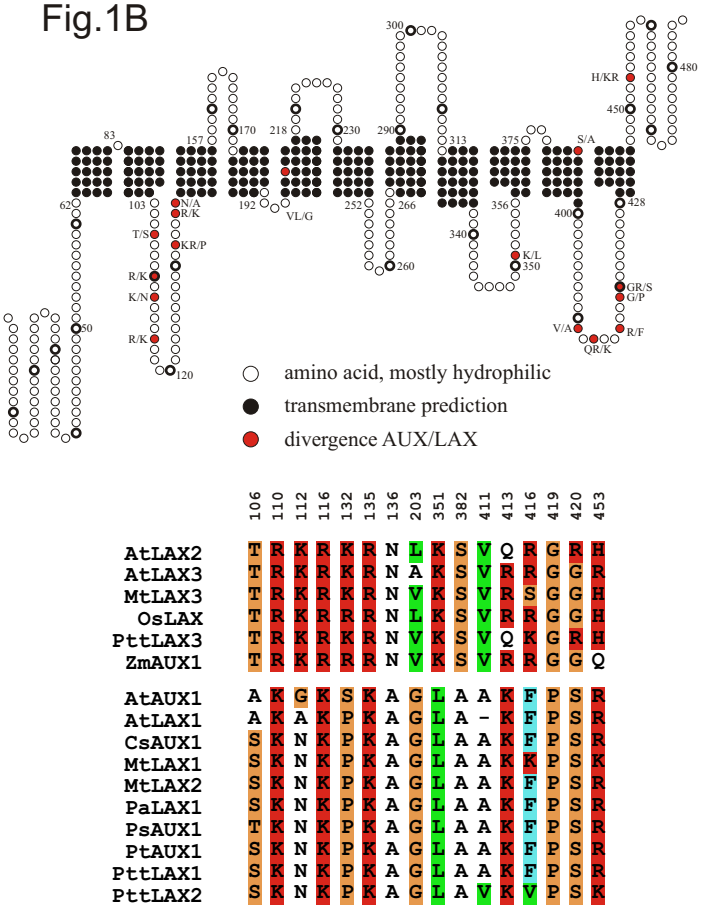



Fig. 2A

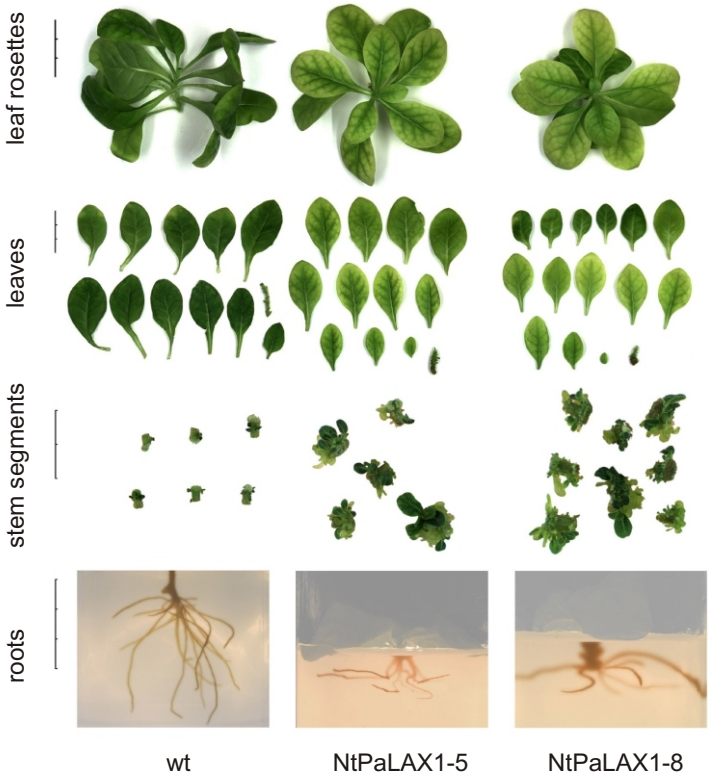



Fig. 2B

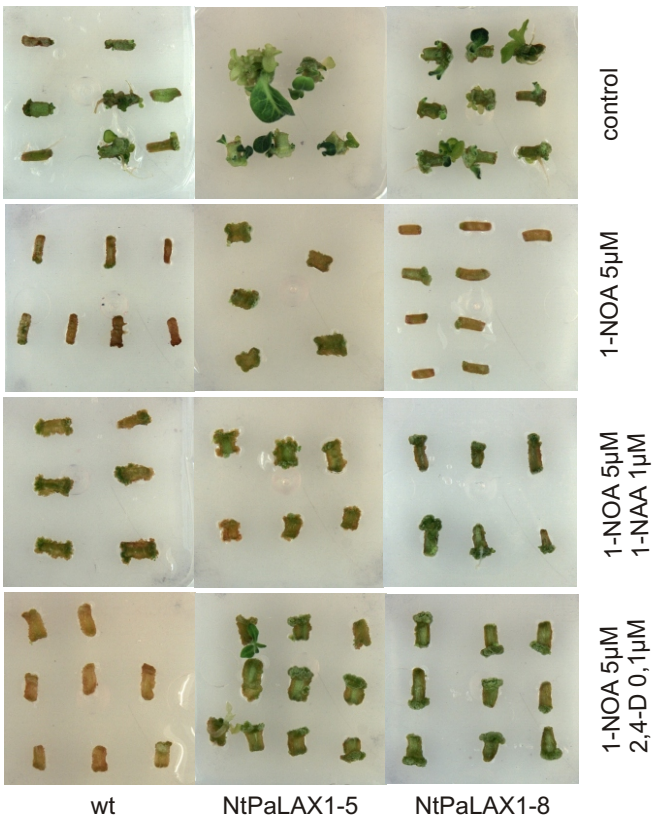


Fig.3

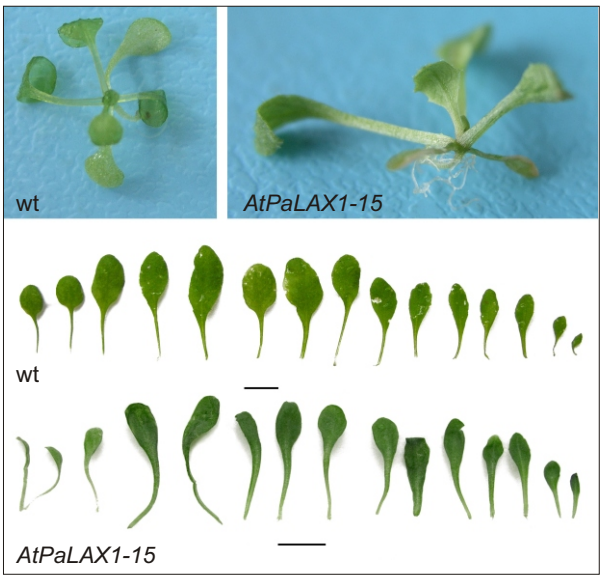



Fig.4

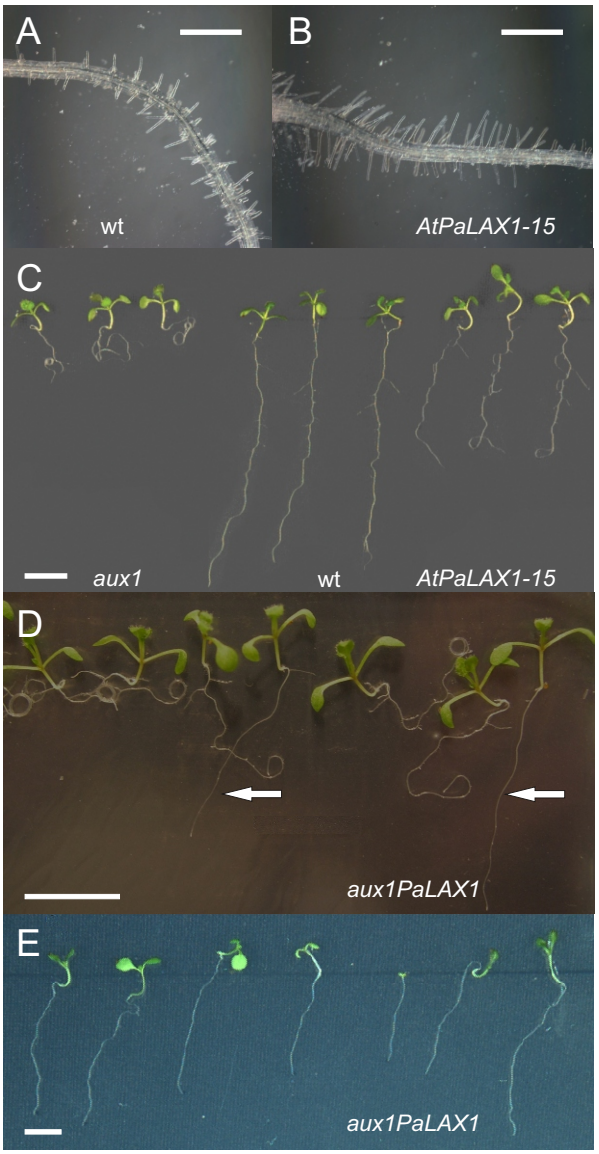



Fig.5A

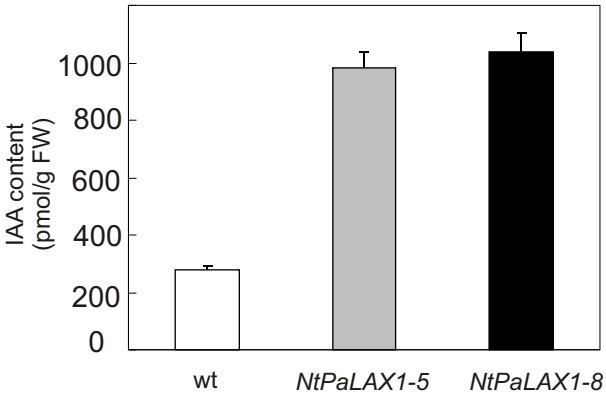



Fig.5B

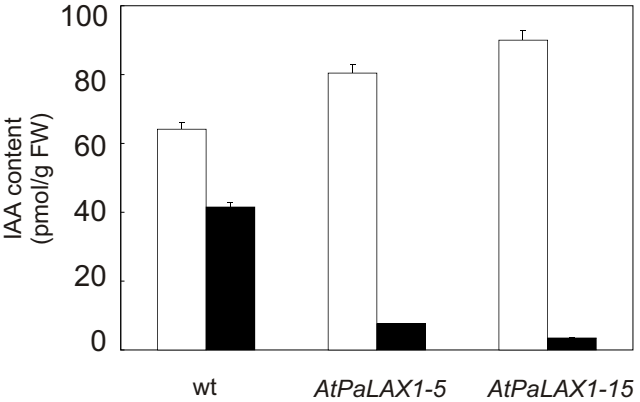
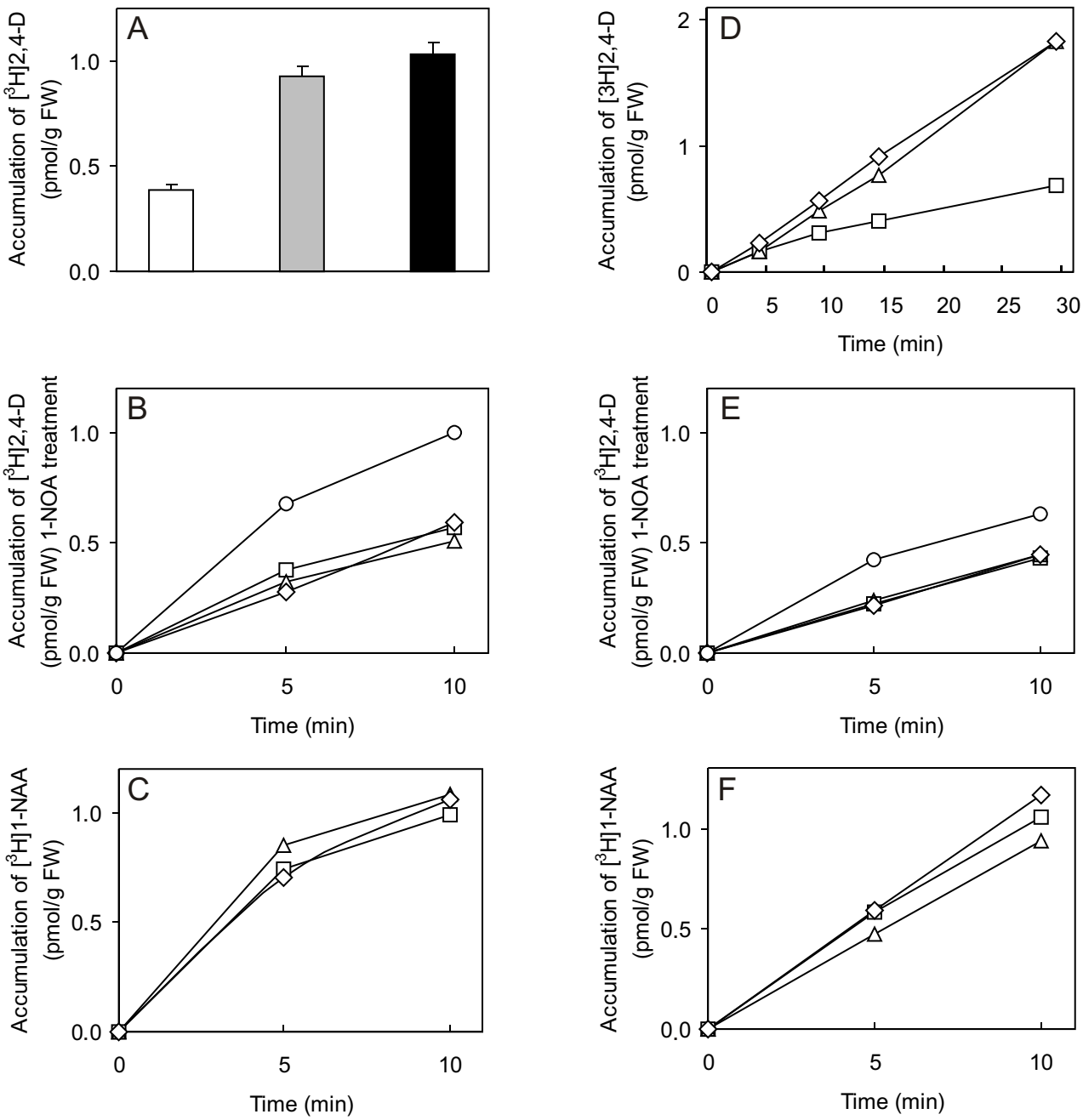


Fig.6


wt
AtPaLAX1-15

Fig.7


A
Accumulation of [3H]2,4-D (pmol/g FW)
0.0
0.5
1.0
D
Accumulation of [3H]2,4-D (pmol/g FW)
0
1
2
0
5
10
15
20
25
30
Time (min)
B
Accumulation of [3H]2,4-D (pmol/g FW) 1-NOA treatment
0.0
0.5
1.0
0
5
10
Time (min)
E
Accumulation of [3H]2,4-D (pmol/g FW) 1-NOA treatment
0.0
0.5
1.0
0
5
10
Time (min)
C
Accumulation of [3H]1-NAA (pmol/g FW)
0.0
0.5
1.0
0
5
10
Time (min)
F
Accumulation of [3H]1-NAA (pmol/g FW)
0.0
0.5
1.0
0
5
10
Time (min)