Functional Characterization of PaLAX1, a Putative Auxin Permease, in Heterologous Plant Systems¹[W][OA]

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We have isolated the cDNA of the gene PaLAX1 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 Nicotiana tabacum and Arabidopsis thaliana plants carrying the gene PaLAX1. We have proved that constitutive overexpression of PaLAX1 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 3 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 PaLAX1. Moreover, the gravitropic response could be restored by expression of PaLAX1 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 PaLAX1 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.

Auxins, in coaction 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 (for review, see 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 the AUX/LAX family of plasma membrane permeases, products of AUX/LAX genes (for review, see Parry et al., 2001b; 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 (Saccharomyces cerevisiae; Chen et al., 2001). PGP4 from Arabidopsis, one of the multidrug resistance (MDR)-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 (Santelia et al., 2005; Terasaka et al., 2005). Interestingly, some other PGP (P-glycoprotein) 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-type transporters (for review,
The AUX1 physiological function was studied in context with root gravitropic curvature (Swarup et al., 2001, 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 homolog of AUX1 (Péret et al., 2007).

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

In this article, we describe the cloning of the PaLAX1 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). Because the root development is dramatically affected by auxin distribution, genes relevant to this process are of high interest. We first describe heterologous expression of such a gene, PaLAX1, coding for a putative auxin transporter from wild cherry tree, in transgenic plants and its impact on endogenous auxin level, the phenotype of transgenic tobacco (Nicotiana tabacum) and Arabidopsis plants, and the impact of transgene expression on auxin uptake. Also, we show that the agravitropic phenotype of the aux1 mutant lacking the function of auxin influx carrier is complemented by PaLAX1 expression and this complementation can be reverted back to the aux1 phenotype by the inhibitor of auxin uptake. Our results strongly support the role of PaLAX1 as an auxin influx carrier/facilitator.

RESULTS
Isolation of PaLAX1 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 wild cherry tree,
PaLAX1. 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 2,004 bp, although 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 and 44 bp. It was not clear whether these represented cloning artifacts or the evidence of regulation of mRNA translation as suggested by de Billy et al. (2001). Similarity between PaLAX1 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 PaLAX1 and PthLAX1 from hybrid aspen (Schrader et al., 2003). Identity of PaLAX1 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., 2001b). Phylogenetic analysis based on multiple alignment shows clearly that there are two distinct subfamilies of AUX/LAX proteins that differ in their amino acid sequences mainly in intracellular-oriented hydrophilic loops (Fig. 1, A and B; Supplemental Fig. S1).

A probe from the 5’ untranslated region of PaLAX1, hybridizing solely with the unique wild cherry tree sequence when used for Southern blot, was used for northern-blot analysis against total RNA extracted from different wild cherry tree tissues. PaLAX1 was expressed in roots, root tips, shoot apices, stems, and leaves (data not shown).

To confirm similar functions of products of genes PaLAX1 and AUX1, we constructed plant transformation vectors that were further used for tobacco and Arabidopsis transformation. We designed a set of primers that 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 PaLAX1 transgene/mRNA in both Arabidopsis and tobacco, whereas no amplification product was detected in either PCR or reverse transcription (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 PaLAX1 Tobacco Plants and Their Regeneration from Stem Internodal Segments

We obtained six independent transgenic tobacco lines that carried the PaLAX1 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 medium 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 Murashige and Skoog medium compared with controls (Fig. 2B). Moreover, after 3 weeks of cultivation on regeneration medium (containing 2,4-D, the auxin that is a good substrate for an active auxin uptake; Delbarre et al., 1996; Yamamoto and Yamamoto, 1998), the transgenic lines developed about four adventitious buds, whereas the control plants developed only zero to two adventitious buds (data not shown). To elucidate whether the observed rapidity of regeneration can be directly attributed to the expression of the PaLAX1 transgene and, concomitantly, to the increased auxin uptake, we placed tobacco stem segments on standard 2,4-D-containing Murashige and Skoog medium supplemented with auxin influx carrier inhibitor 1-naphthoxyacetic acid (1-NOA, 5 μM; Imhoff et al., 2000; Parry et al., 2001a) 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 a 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 medium containing 1-NOA improved regeneration of all explants even though not to the level achieved on standard medium without the inhibitor. The addition of 2,4-D to medium with inhibitor did not improve regeneration capacity of wild-type explants, whereas the stems of transgenic plants regenerated even more rapidly than on medium with 1-NAA and, in comparison with the controls grown on medium without
inhibitor, displayed only slightly delayed bud formation.

Phenotype of PaLAX1 Arabidopsis Plants and Complementation of the aux1 Mutant Phenotype by Strong Expression of PaLAX1

After transformation, 17 independent homozygous transgenic lines carrying the PaLAX1 cDNA under the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter were obtained, 12 of them exhibiting the auxin phenotype (as previously described by Romano et al. [1995] and Zhao et al. [2001]). Of these 12 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 and 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.

Figure 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 1,000 bootstrap replicates for statistical support. Accession numbers are as follows. Arabidopsis (At): AUX1, X98772; LAX1, AJ249442; LAX2, AJ43221; LAX3, ACO12193; cucumber (Cs): AUX1, AB085896; M. truncatula (Mt): LAX1, AJ299397; LAX2, AJ299398; LAX3, AJ299399; Oryza sativa (Os): LAX, NM194844; wild cherry tree (Pa): LAX1, AJ862887; pea (Ps): AUX1, ABI07919; Populus tomentosa (Pt): AUX1, AY864733; hybrid aspen (Pt): LAX1, AF115543; LAX2, AF190880; LAX3, AF263100; maize (Zm): AUX1, AY864733; hybrid aspen (Pt): LAX1, AF115543; LAX2, AF190880; LAX3, AF263100; maize (Zm): AUX1, AY864733; hybrid aspen (Pt): LAX1, AF115543; LAX2, AF190880; LAX3, AF263100;

B, Topological predictions of secondary structures in AUX/LAX family and primary structures of family members. Top, 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 below. Symbols encircled in bold are depicted for easier orientation; they represent every 10th amino acid. Bottom, Parts of amino acid sequences of proteins of both the LAX (top) and AUX (bottom) subfamilies showing the differences in their primary structures. The amino acids are colored according to their similarities based on PAM250 protein weight matrix.
To quantify this difference in development, we measured the average length of visible root hairs of 4-d-old plants (Table II). The length of epidermal cells along the root remained unchanged. Whereas roots of control plants exhibited positive gravitropic growth, the \textit{AtPaLAX1-5} and \textit{AtPaLAX1-15} lines showed disrupted gravitropism and occasional root coiling. However, the roots of transgenic lines were not completely agravitropic as those of \textit{aux1} mutant plants (Fig. 4C). We quantified the gravitropism by the gravitropic factor (GF): 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 were 0.6 ± 0.1 in control wild-type plants and 0.92 ± 0.1.

### Table I. Quantitative effects of \textit{PaLAX1} ectopic expression in tobacco

|                          | Petiole Length | Stem Length | Leaf Circularity | Leaf Elongation | Leaf Surface Area |
|--------------------------|----------------|-------------|------------------|-----------------|------------------|
|                          | (n = 9)        | (n = 6)     | (n = 6)          | (n = 6)         | (n = 6)          |
| Wild type                | 17.1 ± 1.2     | 56.2 ± 4.8  | 1.7              | 0.84            | 435 ± 62         |
| \textit{NtPaLAX1-5}      | 10.3 ± 1.1     | 11.7 ± 3.2  | 1.5              | 0.88            | 310 ± 48         |
| \textit{NtPaLAX1-8}      | 8.0 ± 0.9      | 16.5 ± 4.2  | 1.6              | 0.84            | 538 ± 84         |

(Fig. 4, A and B). To quantify this difference in development, we measured the average length of visible root hairs of 4-d-old plants (Table II). The length of epidermal cells along the root remained unchanged. Whereas roots of control plants exhibited positive gravitropic growth, the \textit{AtPaLAX1-5} and \textit{AtPaLAX1-15} lines showed disrupted gravitropism and occasional root coiling. However, the roots of transgenic lines were not completely agravitropic as those of \textit{aux1} mutant plants (Fig. 4C). We quantified the gravitropism by the gravitropic factor (GF): 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 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).

To prove the functional similarity of AUX1 and PaLAX1, we transformed aux1 mutant plants with a vector carrying PaLAX1 under control of the strong CaMV 35S promoter. The expression of the PaLAX1 transgene was confirmed by RT-PCR. In the 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 medium 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 3–6, leaf 6 being the oldest) of *NtPaLAX1-5* and *NtPaLAX1-8* and wild-type control plants regenerated from apical cuts (3 weeks after the transfer of the apical cuts to fresh medium). The amount of IAA in *NtPaLAX1-5* and *NtPaLAX1-8* lines was almost 4 times higher than that in control leaves of wild-type plants (Fig. 5A).

In 8-d-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 8-d-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, columnella, 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 Arabidopsis**

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 radio-labeled 2,4-D was, therefore, measured in segments of tobacco leaf midribs. The accumulation of radiolabeled 2,4-D was 2 to 3 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 pretreatment to approximately one-half compared to the controls without 1-NOA pretreatment (Fig. 7B). The accumulation of permeable auxin 1-NAA remained unchanged in transgenic lines compared to the 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 pretreated with 10 μM 1-NOA in comparison with nontreated 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 PaLAX1 Permease into Subfamily AUX

Based on expected nucleotide sequence similarity to the previously isolated and cloned gene AUX1 from Arabidopsis (Maer and Martindale, 1980; Bennett et al., 1996), a member of the amino acid/auxin permease family (Young et al., 1999), cDNA of a new gene PaLAX1 has been cloned from the wild cherry tree 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; Péret et al., 2007; for review, see Parry et al., 2001b). 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, ClustalW) revealed two subfamilies (Fig. 1A). Within these two subfamilies, the members differ only in minor branching parameters (compare with Péret et al., 2007). We suggest naming those two emerging subfamilies AUX (consisting of AtAUX1, AtLAX1, CsAUX1, MtLAX1, MtLAX2, PaLAX1, PsAUX1, PsAUX1, PtLAX1, and PtLAX2) and LAX (consisting of AtLAX2, AtLAX3, MtLAX3, OsLAX, PtLAX3, and ZmAUX1).

Representatives of both protein subfamilies are present in Arabidopsis, Medicago truncatula, and hybrid aspen. 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 alterations 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 find and distinguish other members of the 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 PaLAX1 gene under the control of strong constitutive promoters. Whereas the viral promoter CaMV 35S promotes strong gene expression in all cell types, bacterial promoter rolC has been reported to direct the expression preferentially to vascular tissues (Schmittling et al., 1989). We assumed that vascular tissue-driven expression of the auxin transport-related gene PaLAX1 might have a more pronounced effect on the phenotypical parameters that we were studying. However, we have not noticed any major differences that would reflect this difference. Generally, the phenotypical changes (Figs. 2–4) 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 also be 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 PaLAX1. Interestingly, the distribution of free IAA between leaves and whole roots is remarkably changed in transgenic Arabidopsis plants, which contain a much higher proportion of free IAA in leaves than in roots (Fig. 5, A and 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 Arabidopsis and tobacco. Our data on free auxin content are very 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., 2005). Even though the IAA levels were significantly increased in all transformed plants (40–300 pmol g⁻¹ fresh weight for wild type and 3–1,000 pmol g⁻¹ fresh weight 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 (1) higher rate of IAA biosynthesis and/or release from conjugates; and (2) 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 could also be 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 PaLAX 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 PaLAX1 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 transporters. The intensified development might well result from the abundance of the presumptive influx carrier PaLAX1 in outer layers of stems, which would allow more pronounced auxin gradient formation. Cooperation 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 PaLAX1 transgenic plants the function of the endogenous auxin efflux carriers either is not affected or can be stimulated by even 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 carriers, 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 static 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 medium containing both 1-NOA and 1-NAA, the auxin entering the cell readily even in the absence of an auxin transporter in both the control and transgenic samples showed an improved regeneration capacity, even if it was not as prominent as that on standard regeneration medium. Remarkable difference was achieved when the stem segments were placed on medium with the uptake inhibitor and 2,4-D, the auxin preferentially delivered into cells by a carrier (Delbarre et al., 1996). Whereas control wild-type segments did not react to the presence of this synthetic auxin and their regeneration remained completely inhibited, the transgenic segments expressing \textit{PaLAX1} readily regenerated.

Reporter assays in \textit{PaLAX1} 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 Casimiro et al. [2001] and Swarup et al. [2005]).

Our results revealed that, under some circumstances, \textit{PaLAX1} expression (see below) could also complement the agravitropic phenotype of the Arabidopsis loss-of-function \textit{aux1} mutant. Moreover, this phenotypical complementation of \textit{AUX1} function could be reverted back to agravitropic phenotype by the auxin uptake inhibitor 1-NOA. Interestingly, only transgenic lines homozygous in \textit{PaLAX1} 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 \textit{PaLAX1} as an auxin influx carrier, we have proved that strong expression of \textit{PaLAX1} is accompanied by an increase in the net auxin uptake in both tobacco and Arabidopsis plants.

For comparisons of 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 \textit{aux1} mutant could not be rescued by 2,4-D, whereas 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

Figure 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 (\textit{NtPaLAX1-5}, gray column; \textit{NtPaLAX1-8}, black column) measured in mature leaves (leaves 3–6) 8 weeks after clonal propagation of the apical cuts. Vertical bars indicate SD (n = 3 per one plant; three plants measured). B, Free IAA content in Arabidopsis wild-type (wt) and transgenic plants (\textit{AtPaLAX1-5} and \textit{AtPaLAX1-15}) measured in leaf rosettes (white columns) and whole roots (black columns) of 8-week-old plants. Vertical bars indicate SD (n = 3 per one plant; three plants measured).

Figure 6. Auxin-responsive \textit{IAA2::GUS} reporter expression in Arabidopsis. Auxin-responsive \textit{IAA2::GUS} expression in root apices of wild-type (wt) and \textit{AtPaLAX1-15} lines. Scale bars = 0.5 mm.
standard labeled 2,4-D uptake assay (Parry et al., 2001a; 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 plants of approximately equal size were always used.

In all experiments performed, the transgenic explants accumulated about 3 times more radiolabeled 2,4-D compared to the control wild-type explants. The accumulation rate of transformed segments, when pretreated 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

**Figure 7.** Net accumulation of [³H]2,4-D and [³H]1-NAA in tobacco (A–C) and Arabidopsis (D–F). A, Net accumulation of [³H]2,4-D in midribs of tobacco leaves (mix of leaves at various growth stages, 8-week-old plants). White column, Wild-type control; gray and black columns, transgenic plants NtPaLAX1-5 and NtPaLAX1-8, respectively, at the time 5 min after addition of radiolabeled auxin. B, Time course of the net accumulation of [³H]2,4-D in midribs of tobacco leaves (8-week-old plants after pretreatment (20 min) with 1-NOA (10 µM). Squares, Wild type; triangles and diamonds, transgenic plants NtPaLAX1-5 and NtPaLAX1-8, respectively; circles, wild-type control without 1-NOA pretreatment. C, Time course of the net accumulation of [³H]1-NAA in midribs of tobacco leaves (8-week-old plants). Squares, Wild type; triangles and diamonds, transgenic plants NtPaLAX1-5 and NtPaLAX1-8, respectively. D, Time course of the net accumulation of [³H]2,4-D in Arabidopsis inflorescence stem segments (5-week-old plants). Squares, Wild type; triangles and diamonds, transgenic plants AtPaLAX1-5 and AtPaLAX1-15, respectively. E, Time course of the net accumulation of [³H]2,4-D in Arabidopsis inflorescence stem segments (5-week-old plants) after pretreatment (20 min) with 1-NOA (10 µM). Squares, Wild type; triangles and diamonds, transgenic plants AtPaLAX1-5 and AtPaLAX1-15, respectively; circles, wild-type control without 1-NOA pretreatment. F, Time course of the net accumulation of [³H]1-NAA in Arabidopsis inflorescence stem segments (5-week-old plants). Squares, Wild type; triangles and diamonds, transgenic plants AtPaLAX1-5 and AtPaLAX1-15, respectively. Vertical bars indicate SD (n = 3 per one plant; three plants measured). Where invisible, SDs were smaller than the symbol.
supported the direct involvement of PaLAX1 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 PaLAX1 by means of the biochemical methods available, our 2,4-D accumulation results, showing increased auxin uptake in PaLAX1 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 PaLAX1 to the single-component amino acid permeases (Young et al., 1999; Swarup et al., 2004), provide quite a solid background for marking PaLAX1 protein, together with other permeases of the 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. [3H]IAA and [3H]2,4-D (both of specific radioactivity 20 Ci mmol–1) were produced by American Radiolabeled Chemicals, Inc.

Sequence Analysis

Identification of AIAUX1 homologs was carried out by a BLAST search at the National Center for Biotechnology Information (Alschul 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 homologous from protein amino acid sequence alignment by maximum likelihood (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 Fig. S1).

Isolation of PaLAX1 cDNA, Construction of Transformation Vectors, PCR, and RT-PCR

For the isolation of AUX1-related clones from wild cherry tree (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 (A. thaliana) AUX1 gene (the gene was kindly provided by Prof. M. Bennett, University of Nottingham, UK). The sequence of AUX1-like amplification products was used to design wild cherry tree-specific primers. A combination of a 5′ SK vector sequence primer (CCCTCTAGAACTTAGGATC) and a 3′ PauxI3 wild cherry tree-specific primer (GTTGAGAAGCTCATCACCAAA) was found to amplify approximately 1 kb of the 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 rearayed 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 manufacturer’s 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). Sequence data were analyzed using Genetics Computer Group software. Nucleotide and protein alignments were made using ClustalW software (Thompson et al., 1994).

A full-length PaLAX1 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 (PaLAX1 cDNA placed under the control of the CaMV 35S promoter), By/hI-Sacl fragment of pBChaxfull (PaLAX1 cDNA) was ligated into pROK2 vector (Baulcombe et al., 1986) digested with BamHI/Sacl. This vector was used for transformation of Arabidopsis plants. In pCPaLAX1 (PaLAX1 cDNA placed under the control of the nos promoter), the initial vector pBlpoc (L. Perry, unpublished data) was constructed by replacement of the HindIII/Smal CaMV 35S promoter region of pBlpoc (CLONTECH) by a HindIII/EcoRV polynucleotid of pUC57 (Fermentas). Agrobacterial nos gene promoter from the pBlpoc plasmid of Agrobacterium rhizogenes was amplified. The SaI/BgII fragment of the PCR product, together with SacI/BgII-digested PaLAX1 cDNA, was ligated into pBlpoc digested with SalI/SacI. This vector was used for transformation of tobacco (Nicotiana tabacum) plants.

Detection of transgene and its expression was performed by PCR and RT-PCR, respectively (CHAX, 5′-TACACAGCTTGACTGTCG-3′; CHAXr, 5′-ATCCAACTTGAAGCTTA-3′. PaLAX1-specific primers; Ta = 52°C). Amplification from specific primers did not result in visible product when performed on control transgenic material. The DNA techniques were performed according to the usual laboratory protocols described in Ausubel et al. (1995) with modifications (Krizkova and Hrouda, 1998).

Transformation, in Vitro Cultivation, and Regeneration of Tobacco Plants

Tobacco ‘Maryland Mammoth’ plants were used for the leaf disc transformation via Agrobacterium tumefaciens as described by Clark (1997). All cultivars were performed at 20°C/23°C, 16-h-light/8-h-dark photoperiod cycles. Regenerated transformants were grown on modified solid Murashige and Skoog medium (4.3 g L–1 Murashige and Skoog basal salt mixture, 104 mg L–1 Murashige and Skoog vitamins, 4% Suc, 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 sulfate; Sigma) and 1 mg mL–1 cefotaxim (Claforana).

For regeneration experiments, the stems of 6-week-old transformed and control plants (i.e. 6 weeks after the transfer of the apical cuts to the fresh medium) were cut into 5-mm internodal segments. The segments were placed on regeneration medium Murashige and Skoog medium (six segments per Matra box): 4.3 g L–1 Murashige and Skoog basal salt mixture, 104 mg L–1 Murashige and Skoog vitamins, 5% Glc, 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 sulfate; Sigma), and cultivated for 3 weeks. 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-NOA were added to regeneration medium, respectively. For the leaf shape description, the circularity (4π×area/perimeter)2, maximal circularity = 1, and elongation (maximal Feret/min Feret, e = 0 [0, 180]; LUCIA, α = 0, 10, 20, 30, … 180) factors were calculated (image analysis software LUCIA G, version 4.71, designed by Laboratory Imaging).

Transformation of Arabidopsis Plants, Derivation of Homozygous Lines, and Genetic Crosses

Arabidopsis L. Heynh., ecotype Columbia, plants were grown in a greenhouse for 4 to 5 weeks. All cultivars were performed at 20°C/23°C, 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 the floral-dip method (Clough and Bent, 1998).

Collected T0 seeds were surface sterilized using 70% ethanol for 2 min. Selection of transformed plants was performed on modified solid Murashige and Skoog medium (Murashige and Skoog half dose, 1% Suc) supplemented with 100 μg mL–1 kanamycin (kanamycin sulfate; Sigma) and 1 mg mL–1 cefotaxim (Claforana). Selected plants were transferred to nonsterile conditions. The segregation ratio of kanamycin-resistant and kanamycin-sensitive plants was scored on 3-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, University of Nottingham, UK). For complementation experiments (transformation with pRKopalAXI), the mutant aux1-7 line (Maher and Martindale, 1980) was used (seeds of line N3074 obtained from Nottingham Arabidopsis Stock Centre). For GUS qualitative assay, transgenic seedlings were infiltrated for 15 min with 20 mM 

Measurement of the Net Auxin Uptake (Auxin Accumulation) in Leaf Midribs of Tobacco and in Inflorescence Stems of Arabidopsis

For studies of 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. Because the stems of transgenic tobacco were too short to provide suitable material for the measurements of 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., 2001a).

The segments were placed into the ice-cold uptake buffer (1.5% Suc, 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.

One hundred milligrams of segments were dropped into 5 mL of 2 mM [3H]2,4-D or 2 mM [3H]1-NAA for 0, 5, 10, and 15 min, respectively, in three 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 1 mL of 96% ethanol for 30 min, and afterward 5 mL of scintillation solution (Ecolite) were added and the sample was incubated overnight (Morriss and Robinson, 1998). Radioactivity was then measured using the liquid scintillation analyzer Packard tri-Carb 2900TR (Packard Instruments). When the effect of 1-NOA was examined, the 20-min pretreatment with 10 μM 1-NOA preceded the incubation with [3H]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 to 6 (leaf 6 being the oldest one; tobacco) or the whole rosettes (Arabidopsis) homogenized in liquid nitrogen. The extract was purified using a dual-mode solid-phase extraction method as described by 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 an online fluorescence detector with excitation and emission wavelengths adequate for IAA (Dobrev et al., 2005). The identity of IAA peak was verified using gas chromatography-mass spectrometry with deuterated IAA standard. Internal standard of [3H]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 SDs and variations. Image analysis software LUCIA G, version 4.71 (Laboratory Imaging), was used for phenotype data evaluation.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AJ862887.
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