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The *PIN* and *LAX* families of auxin transport genes in *Medicago truncatula*

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Abstract Auxin transport proteins may be involved in nodule development. As a prelude to investigating the roles of these proteins in nodule development, we took advantage of the genetic and molecular resources available in the legume *Medicago truncatula* to characterize the gene families encoding auxin efflux and influx carriers. We identified ten auxin efflux carrier sequences (*MtPIN*s) and five auxin influx/permease sequences (*MtLAX*s). The genomic sequence of each of these fifteen genes was determined, the genes were mapped on the publicly available map of *M. truncatula*, and their expression was examined in shoot and root tissue of nodulating plants. With one exception, transcripts of all *MtPIN* genes were detected. The expression of *MtPIN2* was limited to nodulating roots, while transcripts of all other expressed genes were detected in both shoots and roots. Both the *PIN* and *LAX* gene families contain more members in *M. truncatula* than in *Arabidopsis*, but the gene families are not significantly expanded. Sequence comparison of the *M. truncatula PIN* and *LAX* genes with *PIN* and *LAX* genes from other dicots and monocots indicates that both gene families share a common overall structure, with areas of high homology both within *M. truncatula* and across species boundaries. Molecular phylogenies of both the *PIN* and *LAX* gene families were constructed. Combined with intron position and expression data, the phylogenies were used to assign relationships between *MtPIN* and *MtLAX* genes and the orthologous *Arabidopsis PIN* and *LAX* genes. *MtPIN2* and *MtPIN7* appear to be the result of a recent gene duplication with subsequent divergence of expression patterns. These results set the stage for the use of these genes in research on the role of auxin in nodulation.

Keywords Auxin · *LAX* genes · Legumes · *Medicago truncatula* · *PIN* genes

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Introduction

Auxin has been implicated in almost every growth response in plants. Polar auxin transport (PAT) moves auxin from its sites of synthesis to its sites of action in other portions of the plant, allowing auxin to influence various plant developmental processes via long-distance signaling (Leyser 2001). Auxin is synthesized in the shoot apex and in young leaves, and directionally transported toward basal parts of the plant (Lomax et al. 1995). PAT has been linked to the initiation and maintenance of polarized growth in developing embryos (Newcomb and Wetherell 1970; Fry and Wangermann 1976; Schiavone and Cook 1987; Steinmann et al. 1999), but also plays a role in the more general establishment and maintenance of the plant axis (Scheres and Berleth 1998; Friml et al. 2003), the patterning of the vascular tissues (Sachs 1993; Galweiler et al. 1998; Reinhardt et al. 2003), the development of inflorescences (Sessions et al. 1997; Nemhauser et al. 1998), and responses of plant organs to directed stimuli (tropisms) (Philippar et al. 1999; Rashotte et al. 2000).

Uptake transporters [*AUX1*and *LAX* (*Like-Aux1*) proteins] allow protonated auxin to enter the cell. The higher pH inside the cell favors the anionic form of auxin, which accumulates about 20-fold over the outside concentration. Efflux carriers at the base of the cell transport anionic auxin out of the cell down auxin's chemical concentration gradient, with the directionality being provided by the location of the carriers (Galweiler et al. 1998). Asymmetrically localized PIN proteins (efflux carriers) are required to generate the auxin

gradients, influencing many aspects of plant development, especially organ initiation (Benkova et al. 2003; Reinhardt et al. 2003). While other genes also affect auxin transport (Gil et al. 2001; Noh et al. 2001; Rashotte et al. 2001), the PIN and LAX proteins play an especially important role, and localization of transporters to the membrane appears to be a dynamic process (Estelle 2001; Geldner et al. 2001, 2003; Muday and Murphy 2002; Muday et al. 2003). Accumulating evidence suggests that members of the efflux gene family are expressed in specific cells and tissues, and are cycled between compartments within the cell and the plasma membrane in a regulated manner. Although *AtAUX1* and *AtPIN1* are both expressed throughout the plant and often occur in the same cell, specific pairing of *PIN* and *LAX* gene expression within cell types has not been reported. Regulation includes PIN protein relocalization and expression changes in response to gravity (Friml et al. 2002b) and the application of auxin transport inhibitors such as NPA and TIBA (Geldner et al. 2001), as well as endogenous transport inhibitors such as flavonoids, and auxin itself (Peer et al. 2004). Both flavonoids and auxin have also been implicated in nodule development.

Plant hormones are postulated to play a role in nodule development (reviewed in Hirsch and Fang 1994) because they have been shown to regulate development and growth responses, and nodule formation is both a developmental event (the formation of a new plant organ) and a growth response to stimuli (rhizobia). The localized application of auxin transport inhibitors to legume roots induces nodule-like structures in several legumes and promotes the expression of some nodulation genes (Allen et al. 1953; Hirsch et al. 1989; van de Weil et al. 1990; Scheres et al. 1992). Both direct measurements of auxin (Boot et al. 1999) and measurements of the expression of an auxin responsive *GH3:GUS* reporter gene fusion (Mathesius et al. 1998; Pacios Bras et al. 2003) are consistent with a change in endogenous auxin transport ability during nodulation in response to bacterial Nod factors. In addition, Mathesius et al. (1998) observed an accumulation of flavonoids, natural inhibitors of auxin transport (Jacobs and Rubery 1988), at the site of application of Nod factor. Taken together, these results suggest that changes in auxin concentration within the nodulation zone are part of the plant signal cascade that regulates early symbiotic development.

Given that auxin and flavonoids influence both PIN distribution/expression and nodule formation, we suspected that auxin transport proteins might play a role in nodule development. We also wondered if there were PIN or LAX proteins specific to nodule development or if nodule development merely co-opted developmental processes that already existed. Answering these questions requires identifying *PIN* and *LAX* genes in a legume, correlating them with Arabidopsis orthologs of known function through sequence and expression analysis, and investigating the function of PINs and LAXs that show evidence of expression in nodulating roots. We took advantage of the molecular resources now available in the model legume *Medicago truncatula* (Cook et al. 1997; Cook 1999; Harrison 2000; Frugoli and Harris 2001; Oldroyd and Geurts 2001) to complete the first two parts of this investigation. We identified ten *MtPIN* genes and five *MtLAX* genes. We determined the genomic sequence for each of these fifteen genes, mapped them on the publicly available map, and examined their expression patterns. With the exception of one *MtPIN* gene, all these genes are expressed in nodulating roots of *M. truncatula*.

The *PIN* and *LAX* gene families are not significantly expanded in *M. truncatula* relative to *Arabidopsis*. We identified *PIN* and *LAX* genes from other species besides *Arabidopsis* through an analysis of unannotated sequence data available in GenBank. Sequence comparisons of the *M. truncatula PIN* and *LAX* genes with plant *PIN* and *LAX* genes identified by cDNA sequences indicate that each gene family possesses a common overall structure with several conserved areas both within *M. truncatula* and across species boundaries. We used these conserved segments to construct a molecular phylogeny. By combining the phylogenetic data with information on intron position and expression, we assigned orthologous relationships between *Arabidopsis* genes and *M. truncatula* genes where possible. Our results set the stage for investigating the function of these genes in nodulation.

Materials and methods

Isolation of *MtPIN* genes

An alignment of three *A. thaliana* PIN proteins and a rice PIN protein (Luschnig et al. 1998) was used to develop a degenerate primer pair for use in PCR amplification of *PIN* gene fragments from *M. truncatula* genomic DNA: AEC1 (5′ CKTTCCAYTTCATCTCCWCCA 3′) and AEC2 (5′ TGSTGCTAGACGTGGTAMTCC 3′). Amplification products were cloned into pBS-KS(+) (Stratagene, La Jolla, Calif.) and sequenced with primers from the polylinker. The insert from one clone with homology to an auxin transporter as determined by BLAST (Altschul et al. 1997) (Genbank Accession No. AQ629001) was used to screen a *M. truncatula* BAC library with 5× genome coverage [MT_ABa; 30,720 clones; Nam et al. 1999), available to order at http://www.genome.clemson.edu]. The insert was labeled with [^{32}P]dCTP by random priming, and hybridization was performed according to protocols from the Clemson University Genomics Institute Web site (http://www.genome.clemson.edu/protocols/). Hybridizing clones were grouped into five contigs based on the sizes and/or sequences of the PCR products generated with primers AEC1 and AEC2 and fingerprinting of *Hin*dIII digested BAC DNA. The following prototypes for each contig were selected: 28J21 (*MtPIN1*), 25F22 (*MtPIN2*), 71F7 (*MtPIN3*), 10J7

(*MtPIN4*), and 30I16 (*MtPIN5*). These BACs were analyzed by Southern hybridization with a probe from the 5′ end of *MtPIN3* (positions 676–856 of Genbank Accession No. AY115838), and single hybridizing bands were detected for the *MtPIN1*, *MtPIN3* and *MtPIN4* BACs. The *MtPIN5* BAC 30I16 failed to yield a signal. The *MtPIN2* BAC yielded two strongly hybridizing bands, one of approximately 4 kb and the other >10 kb. Because the *MtPIN2* sequence (determined as described below) was predicted to have no internal *Hin*dIII sites, we suspected that the BAC contained another *PIN* gene. The 4-kb hybridizing fragment was cloned into pBS-KS(+), sequenced, and indeed found to encode an additional *PIN* gene, designated *MtPIN7*.

In an effort to identify more *PIN* genes, a screen was performed on a portion (36,864 clones) of a second publicly available *M. truncatula* BAC library with 20× genome coverage (MtH2; available to order at http://www.genome.clemson.edu) using the *MtPIN3* 5′ end probe. Twenty-five hybridizing BACs were identified, and Southern analysis of *Hin*dIII-digested DNA from these BACs yielded two groups of BACs with a hybridizing *Hin*dIII fragment profile distinct from the *MtPIN1 -4* bands previously identified. A *PIN* gene was sequenced from representatives of each of these groups —BAC 1A14 (*MtPIN6*) and BAC 71A5 (*MtPIN10*).

Two additional putative *PIN* genes were found by BLAST analysis of *M. truncatula* sequences from the ongoing *M. truncatula* Genome Sequencing Project. MtH2 BAC 23F15 (AC137994) carries a complete putative *PIN* gene (named *MtPIN8*). MtH2 BAC 30B20 carries the 5′ end of a putative *PIN* gene; overlapping adjacent BACs were selected from data provided by the *M. truncatula* physical mapping project (http://www.medicago.org/genome/) and MtH2 BAC 64A5 was used to sequence the rest of this *PIN* gene, which was designated *MtPIN9*.

Isolation of *MtLAX* genes

The complete sequences of *MtLAX1*, *MtLAX2* and *MtLAX3* cDNAs were published previously (de Billy et al. 2001). Full-length cDNA clones for these genes, and partial cDNA clones of *MtLAX4* and *MtLAX5* from the same library, were obtained (de Billy et al. 2001). The partial sequences of *MtLAX4* and *MtLAX5* were determined from the cDNA library clones. A portion of genomic library MtH2 (36,864 clones) was screened with a probe mixture consisting of labeled *MtLAX1*, *2*, *3*, and *5* cDNA clone inserts, and subsequently with a probe representing the last 555 bp of the coding region plus 260 bp of the 3′ untranslated region of the *MtLAX4* cDNA. Thirty-two BACs were identified and grouped based on amplification of PCR products with pairs of *MtLAX* gene-specific primers. All but two of these were found to carry one of the five known *MtLAX* genes. Analysis of the remaining two BACs by PCR using degenerate primers based on a highly conserved region of the *LAX* genes corresponding to codons 49–55 and 141–148 of *MtLAX1* (5′-GAYGCNTGGTTYWSITGYGC-3′ and 5′-CCRAAIARIARRAAIGTRCARTT-3′) failed to yield products. Because of the location of the primers with respect to known *Hin*dIII sites in the genes, if these two BACs contained partial sequences of *MtLAX1*, *2* or *3* they would not be expected to yield PCR products.

Sequencing

The sequences of the PCR fragments were used as the starting point for sequencing the putative transporter genes found in the initial five *PIN* contigs. Sequencing was performed directly from BAC DNA or from PCR products of BAC DNA generated by (1) pairing contig-specific forward primers with primers designed to recognize the 3′ untranslated regions of ESTs identified as putative auxin transporter transcripts, (2) pairing contig-specific reverse primers with a degenerate primer (5′-TGYTCCGGIATIAAYCGITTYGT-3′) designed to recognize a conserved amino acid motif (CSGINRFV) found near the N-terminus of auxin transporters reported from other species, and (3) using other contig-specific primers. The *LAX* genes were sequenced from PCR products generated from BAC DNA using cDNA-based gene-specific primer pairs and gene-specific primers paired with a degenerate primer designed to recognize a conserved amino acid motif (DAWFSCA) near the N-terminus of auxin permeases. For *MtLAX4* and *5*, the 5′ ends of the genes were sequenced directly from BAC DNA. All sequencing was performed on an ABI 370 Sequencer using Big Dye Terminator 2 or 3 according to the manufacturer's standard protocols.

Mapping

Chromosomal locations of *PIN* and *LAX* genes from BACs whose location had not already been determined as part of the *M. truncatula* Genome Project were determined by scoring CAPS (Cleaved Amplified Polymorphic Sequence) and dCAPS (derived Cleaved Amplified Polymorphic Sequence) markers developed from BAC end sequences or from intron sequences in the genes [e.g. in Choi et al. (2004)] (Table 1). The CAPS and dCAPS markers were used to determine genomic locations by scoring them on DNA from an established mapping population consisting of individuals obtained from a cross between the *M. truncatula* lines A17 and A20 (Kulikova et al. 2001). The *PIN* and *LAX* markers were placed with respect to markers on the public map (Choi et al. 2004) via Colormapping. Colormapping is a non-mathematical method which uses a color matrix to display the genotypes of a core set of markers in members of a mapping population, allowing new markers to be placed in relation to known markers by visual analysis (Kiss et al. 1998; Kalo et al. 2000).

Table 1 Primers used for mapping genes

Marker	Primers (5′ to 3′)	Annealing temperature	Enzyme	Band sizes (bp)	
				A-17	A20
MtPIN1	ACCAAGCTTGACCCTTATGTTTC and GAGTTCAAGAGATCTTTGCACGCTG	55	*Mbo*II	80 + 17	42 + 36 + 17
MtPIN2 and *MtPIN7*	GGATTAACGGGTGAGGGAAAAAG and CAAATAAATAGCATGCAAGACAAA	55	*Hinf*I	406	292 + 119
MtPIN3	GAATGATATTGTTCACTTGTTGAGCATAAT and ACACATAGTGAGACAGGGTGTGACATCTAG	50	*Xba*I	212 + 30	242
MtPIN4	TGTAGCAATTCTTAAGAGAGCC and CTTACACATCAATATAAGTGTG	55	*Fok*I	521	392 + 192
MtPIN5	AACATGATTTTTGAGTTCCCATAAGA and ACAGCTAACATGATTCGCCACAC	55	*Tsp* 509I	110 + 56 + 29	164 + 29
MtPIN6	AAACGTAAATTAGTAAGTTGATTGT and GCGGTGCGTCGTGGCTGTAG	55	*Ssp*I	234	186 + 56
MtLAX1	AAACAAGGCGAAGAAACAATGATG and ATGATGAGACTGATTAGATGTG	50	*Hph*I	243 + 61 + 47	290 + 61
MtLAX2	ACTCACCGTGAATCCAAATATCAGTAT and ACCCAGTAACCCATCAAGCACTT	50	*Bsr*I	225 + 1	185 + 40 + 1
MtLAX3	GACATTGCCATATTCCTTTTCA and GGTTCCTCCAGTGTTTTCCTAA	50	*Bsm*AI	300 + 166	467
MtLAX5	CCCCTCATGGTATTTCACTTG and CATTATTTCCCTGTACCAACCAAC	50	*Bgl*II	687 + 387	1012

PIN and *LAX* gene expression analysis

Intron-spanning, gene-specific PCR primer pairs were chosen to differentiate between possible products amplified from genomic DNA contaminants and products amplified from cDNA (Table 2). The primers were tested for gene specificity using *PIN* gene BACs or *LAX* gene cDNA clones as template. PCRs (10 μl) consisted of PCR Mastermix (Eppendorf AG, Hamburg, Germany), each primer at 0.5 μM, and 2 ng of template DNA. Thermocycling conditions were 95°C for 4 min, followed by 30 cycles of 95°C for 20 s, 61°C (*PIN* genes) or 63°C (*LAX* genes) for 20 s, and 72°C for 45 s. Products were analyzed on 1% or 2% (w/v) agarose gels in 0.5× Tris-Borate-EDTA buffer and visualized by staining with ethidium bromide. In each case, only the intended target yielded PCR products.

RNA was purified from plant tissues using the RNeasy Plant Mini Kit (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. Roots (minus 1 mm of root tip) and shoots of 12-day old nodulating plants were separated for extraction. RNA (20 μg) was treated with 10 U of RQ1 RNase-free DNase in (Promega, Madison, Wis.) in a 100-μl reaction at 37°C for 30 min. Following phenol and chloroform extractions and ethanol precipitation, the RNA was resuspended in 20 μl of distilled water and quantified by measuring the absorbance of a 1:50 dilution at 260 nm. cDNA was synthesized in 20-μl reactions from 1-μg aliquots of RNA, using random hexanucleotide primers (Invitrogen, La Jolla, Calif.) and Superscript Reverse Transcriptase II (Invitrogen) following the manufacturer's recommendations. PCRs were performed and analyzed as above using 0.5 to 4.0 μl of cDNA as

Table 2 Primers used for RT-PCR

Gene	Forward primer	Reverse primer	Predicted product size	
			cDNA	Genomic DNA
MtPIN1	ATGGCTCTGCTGCTGCTGCTAA	GTTCCACTTTCCCTACCTCTTCTTCTACC	327	418
MtPIN2	GCATGGGCGGTGGAAGTGGTAA	GCTGGTGGCATCTGTGTATTTTTG	385	566
MtPIN3	CTACTACTAAACCTGTGAAGAATC	GGTGGAGCTCCGGCGTTGAA	375	800
MtPIN4	TTCAAAAACTATCCTGCACCTAATCCA	ATCTCCACCTTTCTCACCTCCTTCA	366	757
MtPIN5	CACCACCGAGGACTTGTAAT	CATCGTTTTTGCCCCACCTT	245	330
MtPIN6	AAAGGCTCATTGGATTGGGTTAT	GCGGTGCGTCGTGGCTGTAG	292	895
MtPIN7	GAAAGGGGCAACACCTCAGTA	GTCTCTTTCTCTTCGACTATCACTCA	124	298
MtPIN8	TTGCTAAAGAATATAATGTCCA	TCCACCGGCAAGGCAATGA	79	173
MtPIN9	ATTGGCATTTCTTGGGCTTTCATA	TTAAAACCAAACCAAATACAGTCAAAC	190	422
MtPIN10	AAGATCTTCATATGTTTGTTTGGAGTT	TTCCTCCACACCATAATCAAAAT	334	640
MtLAX1	AAACAAGGCGAAGAAACAA	AAGGCCAAGGAAAGACCAA	588	1252
MtLAX2	GGTGGTTCTGTTTGGGAT	ATACGGTAATTGTGGAAGGATGGT	452	1058
MtLAX3	ATGGAAAGGGAAGAGGAAGGT	TGGAAAGAAGGGATGAAAACTG	503	946
MtLAX4	CATTCCAATGCTTTCTCT	TACACTAACTTAACTGACCTATT	815	3689
MtLAX5	CAGTTTATTGGGCATTTGG	CATGCATCCCTACTGTTTTCTCC	177	250

template. The quantity used depended on the primer set, but was always the same for shoots and roots.

Phylogenetic analysis

Additional *PIN* and *LAX* sequences from other species where obtained by searching the Genbank Non-Redundant Database (nr-July 2004) with TBLASTN. *PIN* and *LAX* sequences for which a genomic sequence and/or a full length cDNA was found in Genbank or the TIGR Gene Index of Tentative Consensus sequences (TCs) were included in the analysis. The presence of a TC alone was not considered sufficient to include a gene in the analysis because the compilation program separates multiple TCs when only a few ESTs or short ESTs from different parts of the sequence are present, confounding the phylogeny with partial length sequences.

Protein sequence alignments were generated using the CLUSTAL method in MegAlign (DNASTAR, Madison, Wis.) with minor adjustments being made manually. Positions in gaps in the alignments were eliminated from the analyses. Unrooted most parsimonious trees were produced from the results of 100 bootstrap replicates using the PROTPARS and CONSENSE algorithms of Phylip 3.6 with default parameters. The corresponding nucleotide alignments were used to generate unrooted maximum likelihood trees from 100 bootstrap replicates using DNAML and CONSENSE (Phylip 3.6) with default parameters. Trees were viewed and edited with TreeView (Page 1996) and show only branches supported by more than 50% of the replicates.

Results

Identification and mapping of members of a *PIN* gene family in *M. truncatula*

Ten members of the *PIN* gene family of auxin efflux transporters (*MtPIN1–MtPIN10*) were identified by a combination of genomic library screening and analysis of publicly available sequences of BAC library clones (see Materials and methods). The ten predicted proteins range in size from 357 to 660 amino acids and are 28 to 79% identical to each other. PIN proteins comprise a conserved N-terminal region of transmembrane segments, a variable middle region thought to be a cytoplasmic domain, and a conserved C-terminal region of transmembrane segments. The differences between the proteins arise mainly from differences in the length and sequence of the central variable region. The genes can be broadly grouped into two categories based on the length of this middle region, with one group of genes having a much shorter sequence than the other. Use of only conserved regions of the proteins in phylogeny analysis resulted in the proteins with similar variable regions clustering together, even though this portion of the sequence was not included in the analysis.

The genes all have a similar overall intron/exon structure, with most having a large first exon corresponding to the N-terminal transmembrane segments and the majority of the variable segment, followed by a second exon corresponding to the rest of the variable segment and the beginning of the C-terminal transmembrane region. Four small exons of 86, 158, 77, and 64 bp, respectively, encode the remaining transmembrane segments. This same overall gene structure is observed in the *PIN* genes from *Arabidopsis* (Fig. 1C).

Comparison of the *M. truncatula* PINs reveals that *Mt* PIN2 and *Mt* PIN7 are highly similar to each other, with 81% amino acid sequence identity, 87% nucleotide sequence identity throughout the coding regions, and 73% identity when intron sequences (excluding a region missing from the variable segment of *MtPIN7*) are included in the comparison. The two genes are located within 20 kb of each other. The first intron of *MtPIN7*, which occurs earlier in the sequence than that of *MtPIN2*, contains several small insertions, but 45 of the remaining 86 bp in the *MtPIN7* intron are identical to the coding sequence of *MtPIN2*.

MtPIN1 and *MtPIN3* show 79% amino acid sequence identity and 74% nucleotide sequence identity within their coding regions; *MtPIN4* and *MtPIN5* have 73% amino acid identity and 69% nucleotide sequence identity within the coding regions (excluding short stretches of the *MtPIN4* variable region which are missing from *MtPIN5*). *MtPIN6* possesses an intron near the 5′ end which is not present in other *M. truncatula PIN* genes but is found at an identical position in *AtPIN6*. *MtPIN8* and *MtPIN9* are predicted to encode shorter proteins than the other *PIN* genes, lacking most of the variable segment; such a structure is also observed in *AtPIN5* and *AtPIN8*.

The genomic locations of *MtPIN1 – MtPIN7* were determined by developing BAC-specific markers that were polymorphic between the related *M. truncatula* ecotypes A17 and A20, and testing these markers on a mapping population on which several dozen markers have already been mapped (Table 3). *MtPIN8*, *MtPIN9* and *MtPIN10* are contained in contigs that have been anchored on the *M. truncatula* physical map during the course of the genome sequencing project. Four of the genes (*MtPIN2*, *MtPIN3*, *MtPIN6*, and *MtPIN7*) map to an 8-cM region on the short arm of chromosome 1, two to the long arm (*MtPIN9 and MtPIN10*) and one to the short arm (*MtPIN8*) of chromosome 7, and one each to chromosomes 4 (*MtPIN1*; long arm), 6 (*MtPIN4*; near centromere), and 8 (*MtPIN5*; long arm).

PIN genes from other plant species

BLAST analysis of the complete *Arabidopsis* genome sequence indicates that there are eight *PIN* genes in this species. Expression of each of these has been demonstrated (Galweiler et al. 1998; Muller et al. 1998; Zhong and Ye 2001; Friml et al. 2002a, 2002b; Benkova et al.

2003; Reinhardt et al. 2003). Expressed *PIN* genes have also been described in other dicot species, including hybrid aspen (*Populus tremula* × *Populus tremuloides*; Schrader et al. 2003), pea (*Pisum sativum*), bitter gourd (*Momordica charantia*) and *Brassica juncea*. We have identified by BLAST analysis two putative *PIN* genes in *Lotus japonicus* from completed TAC clone sequences, and named these *LjPIN1* (Chr2; clone LjT06K23) and *LjPIN2* (Chr4; clone LjT05D12).

Among the monocots, one *PIN* gene each had previously been identified in rice (*OsREH1*; Luschnig et al. 1998) and wheat (*TaPIN1*). BLAST analysis of rice BAC sequences and the full-length cDNA database (http://cdna01.dna.affrc.go.jp/cDNA/) yielded sequences of seven additional *PIN* genes (left unnamed pending annotation by the Knowledge-based Oryza Molecular Biological Encyclopedia). An additional wheat *PIN* gene was found among cDNA sequences in GenBank and has been named *TaPIN2*.

Relationships between *PIN* genes from *M. truncatula* and other plant species

PIN protein sequences and coding sequences were used to resolve evolutionary relationships within this gene family and aid in determining orthology. The aligned N- and C-terminal conserved regions of the sequences (representing 302 amino acids) were used in parsimony analysis (PROTPARS; Phylip 3.6) to generate a phylogenetic tree (Fig. 1A). An attempt was made to further refine the tree by using the corresponding nucleotide sequences for maximum likelihood tree generation. Examination of the alignment, however, showed codon bias in the monocot sequences, with high G or C preference in the third codon position. Such codon bias is common in rice genes (Yu et al. 2002) and was also noted in the catalase gene family in maize (Guan and Scandalios 1996). This bias would skew the phylogenetic analysis, and therefore only dicot species were used in the nucleotide sequence analysis (Fig. 1B) with DNAML (Phylip 3.6).

The sequences fell into two major groups. One group contained all the shorter proteins plus AtPIN6 and MtPIN6. The other group contained the remaining longer proteins. When nucleotide sequence identities are compared within species, a similar range is observed. The *Arabidopsis PIN*s share 36.5–84.5% identity, while the *Medicago* genes range from 38.9 to 81.4% identity. When nucleotide sequence identities are compared within groupings however, identities within clades are higher than identities within species.

For instance, *PsPIN2* and *MtPIN3*—two legume genes—are 92.8% identical, while the closest *M. truncatula* gene to *MtPIN3* (*MtPIN1*) shares only 74.2% identity with it. Likewise, two other legume genes (*LjPIN1*and *MtPIN8*) are 75.6% identical to each other, but only ~56% identical to *AtPIN8*. Thus within-species identity is often lower than identity within classes of *PIN* sequences.

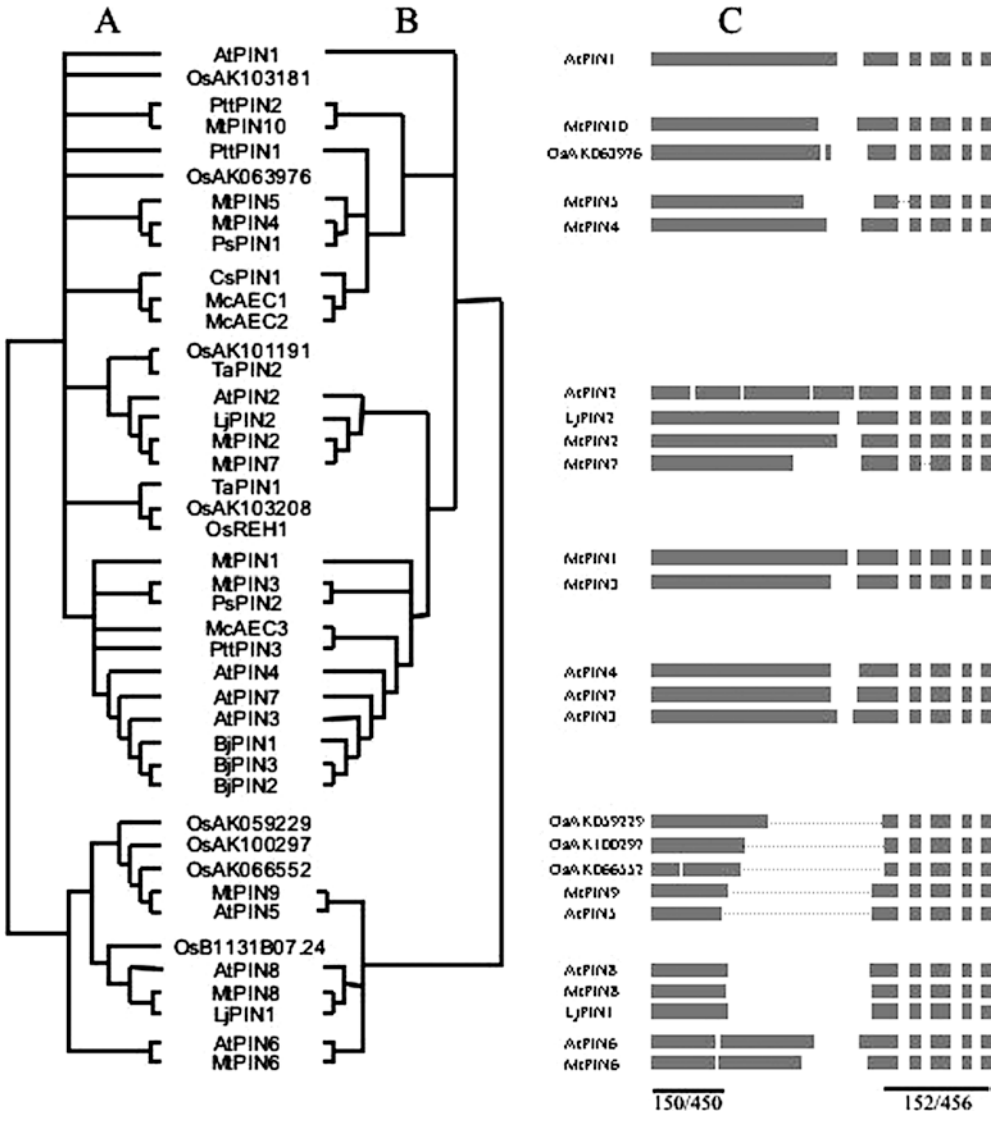
Identification and mapping of *LAX* genes in *M. truncatula*

Five *MtLAX* genes were identified from cDNA clones and BAC libraries (see Materials and methods). Four of the genes were localized on the *M. truncatula* genetic map using intron-based markers (Table 3). *MtLAX1* mapped to a 5.5-cM region on the long arm of chromosome 5, *MtLAX2* to a 22-cM region in the middle of chromosome 7 and *MtLAX3* to a 3-cM region on the long arm of chromosome 3, while *MtLAX5* is tightly linked to the markers DNABP and NUM1 on the long arm of chromosome 4. *MtLAX4* was mapped by analysis of available *M. truncatula* genome fingerprinting and sequence data. Sequences from the ends of two BACs carrying *MtLAX4* (4L2 and 62N11) are found near the end of the completely sequenced BAC MtH2 35F15 (AC138016), which carries the marker TE016. TE016 has been mapped to the telomeric region of the short arm of chromosome 4 (Choi et al. 2004). Various pairs of gene-specific primers for *MtLAX4* failed to yield PCR products from the A20 and R108 ecotypes (data not shown), suggesting that *MtLAX4* may be absent in these ecotypes.

All of the *M. truncatula LAX* genes are highly similar at the protein level (72.8–85.6% identity; PAM250 residue weight table), although parsimony analysis splits them into two major groupings, with *MtLAX1*, *2* and *4* forming one branch and *MtLAX 3* and *5* another (Fig. 2A). Examination of sequence pairs shows that *MtLAX2* and *MtLAX4* show the highest level of sequence identity (85.6%), but *MtLAX2* displays even greater similarity to a pea sequence, *PsAux1* (93.8%)—so again, within-species identity is often lower than identity within classes. Examination of their nucleotide sequences shows that all of the *M. truncatula LAX* genes have seven introns within the coding region.

LAX genes in other plant species

Four members of the auxin influx carrier family are present in the *Arabidopsis* genome, and these vary in intron number (having 5–7 introns within the coding region) with the variation occurring at the C-terminal end of the genes (Parry et al. 2001). Expressed auxin influx carrier genes have been described in hybrid aspen, pea, maize (*Zea mays*), and cucumber (*Cucumis sativus*). BLAST analysis of the full-length rice cDNA database yielded sequences of four auxin influx carriers. One additional putative auxin influx carrier gene was identified among *Lotus japonica* TAC clone sequences and was named *LjLAX1*. The TIGR Gene Index for *L. japonica* (version 3.0) lists four partial *LAX* TCs, which represent three separate genes. Each of these se-



quences is different from the *LjLAX1*, suggesting the number of *LAX* sequences in *L. japonica* is at least four, but without full-length sequence the *Lotus* sequences could not be included in the phylogenic analysis. Partial sequence comparison suggests that the excluded *L. japonica* sequences are most similar to *MtLAX1*, *2* and *4*.

Relationships between *LAX* genes of *M. truncatula* and other plant species

Because of their extreme similarity, nearly the entire sequence of the *LAX* genes could be used for analysis. Two groups of LAX proteins emerge from the parsimony analysis (Fig. 2A). There is insufficient information based on protein parsimony to elucidate relationships between many of the sequences in the top clade, but maximum likelihood analysis of the nucleotide sequences (Fig. 2B) allows some comparisons. For instance, the two *Arabidopsis* sequences are more similar to each other than to any other gene in this clade, an observation that does not hold for the multiple sequences from poplar or *M. truncatula*.

Expression of *M. truncatula PIN* and *LAX* genes

With the exception of *MtPIN5*, all *M. truncatula PIN* genes could be amplified from cDNA obtained from nodulating plants (Fig. 3). *MtPIN2* could only be amplified from roots. The bands seen in *MtPIN5* reactions were also observed in no-template controls (not shown) and are assumed to be primer dimers, since they do not match the expected size of the product from the predicted cDNA. The major bands observed in other reactions migrate at sizes expected based on predicted intron/exon structures (Table 2).

Further evidence that *MtPIN5* is not expressed comes from its absence from the public collection of almost 190,000 ESTs (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species = medicago). There is no obvious

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Fig. 1A–C Phylogenetic analysis and intron exon structure of *PIN* genes. **A** Parsimony analysis based on an alignment of the conserved N- and C-terminal regions of the PIN proteins (representing 302 amino acids-see Materials and methods). Only branches with greater than 50% support are displayed. **B** Maximium likelihood tree of dicot nucleotide sequences encoding conserved N- and C-terminal regions of *PIN* gene products. **C** Deduced intron/exon structure of *PIN* genes for which genomic sequences were available. The *dotted lines* indicate gaps in the nucleotide sequence. The numbers at the *bottom* indicate the number of amino acids and nucleotides aligned from each section. Sequences used in these alignments were the following: *MtPIN1* (AY115836), *MtPIN2* (AY115837), *MtPIN3* (AY115838), *MtPIN4* (AY115839), *MtPIN5* (AY115840), *MtPIN6* (AY553209), *MtPIN7* (AY553210), *MtPIN8* (BK005119), *MtPIN9* (AY553211) and *MtPIN10* (AY553212) from *Medicago truncatula*); *AtPIN1* (AF089085), *AtPIN2* (AF086907), *AtPIN3* (AF087818), *AtPIN4* (AF087016), *AtPIN5* (AB005242), *AtPIN6* (AF087819), *AtPIN7* (AF087820) and *AtPIN8* (AL391146) from *Arabidopsis thaliana*; *CsPIN1* (AB08589) from *Cucumis sativus*; *BjPIN1* (AJ132363), *BjPIN2* (AJ249297) and *BjPIN3* (AJ249298) from *Brassica juncea*; *LjPIN1* (BK005120) and *LjPIN2* (BK005121) from *Lotus japonicus*; *MCm-AEC1* (AF246995), *MCm-AEC2* (AF247004) and *MCm-AEC3* (AF247005) from *Momordica charantia*; *OsREH1* (AF056027), AK059229, AK103181, AK063976, AK101191, AK066552, AK100297, AK103208 and AP003408c from *Oryza sativa* (the coding sequence of this gene has been reanalyzed; the start site has been redefined to include an additional 198 bp of upstream sequence); *PsPIN1* (AY222857) and *PsPIN2* (AB112364) from *Pisum sativum*; *PttPIN1* (AF190881), *PttPIN2* (AF515435) and *PttPIN3* (AF515434) from *Populus tremula x P. tremuloides*; and *TaPIN1* (AY496058) and *TaPIN2* (BK005137) from *Triticum aestivum*

defect in the sequence of the coding region of *MtPIN5*, including the intron/exon boundaries, that would prevent the gene from being expressed, but the intron/exon boundaries have shifted (Fig. 1C).

The availability of the TIGR Medicago Gene Index made it possible to assemble "electronic Northerns" for *PIN* and *LAX* genes represented in the EST database. *MtPIN5*, *6*, *7*, *8*, *9* and *10* do not appear as ESTs in release 7.0 of the database. *MtPIN2*, *3* and *9* appear as single ESTs from root tips (TC91788), phosphate-starved leaves (TC83905), and seedling roots 24 h after inoculation (TC81304), respectively. Only *MtPIN1* is represented by more than one EST: three independent *MtPIN1* ESTs come from a developing flower library (TC80729).

The original report identifying the initial members of the *MtLAX* gene family reported expression of *MtLAX1*, *2* and *3* in nodulating roots by in situ hybridization (de Billy et al. 2001). RT-PCR of the same cDNAs used for the *PIN* analysis with primers from Table 2 demonstrated amplification of transcripts of all five *LAX* genes from both shoots and roots of nodulating plants (data not shown). "Electronic Northerns" based on release 7.0 of the TIGR Medicago Gene Index were more informative. *MtLAX4* does not appear among the ESTs, and *MtLAX5* is represented by a single EST from an early seed development library (TC84343). *MtLAX1* is represented twice, once from developing roots and once from a library made from leaves subjected to insect herbivory (TC89992), confirming the RT-PCR results. *MtLAX2* is represented five times —twice from the early seed development library, and once each from phosphate starved leaves, developing leaves and nematode infected roots (TC88482). *MtLAX3* is by far the most widely/abundantly expressed of the *LAX* genes with 12 independent ESTs: two from phosphate starved leaves, one from developing flowers, two from roots colonized with *Glomus versiforme* (mycorrhizae), and one each from stems, developing stems, developing seeds, *Colletotrichum trifolii* infected leaves, seedling roots, root tips, and a mixed tissues library (TC86757).

Table 3 Genomic locations of *Medicago truncatula PIN* and *LAX* genes

Gene[a]	Chr.	Flanking markers (map position in cM)	
MtPIN1	4	NUM1 (49.5)	Bottom of Chr (56.5)
MtPIN2	1	DK340R (2.9)	ppPF (10.9)
MtPIN3	1	DK340R (2.9)	ppPF (10.9)
MtPIN4	6	DK321L (33.2)	R-44D11L (36.1)
MtPIN5	8	DK412L (52.0)	EST671 (53.2)
MtPIN6	1	DK340R (2.9)	ppPF (10.9)
MtPIN7	1	DK340R (2.9)	ppPF (10.9)
MtPIN8	7	DK225L[b] (2.4)	
MtPIN9	7	PPGM (40.9)	DK427R (46.8)
MtPIN10	7	CAK[b] (48.6)	
MtLAX1	5	DK139L (67.5)	DK006R (73.0)
MtLAX2	7	PESR1 (18.9)	PPGM (40.9)
MtLAX3	3	DK201R (59.1)	PRTS (62.1)
MtLAX4	4	TE016 [c] (0.6)	
MtLAX5	4	DNABP (49.5)	NUM1 (49.5)

[a]Markers tightly linked to the indicated genes were used to determine genomic locations (see Materials and methods). Indicated flanking markers are from the mapping data provided in Choi et al. (2004)
[b]The marker is on the same BAC as the *PIN* gene
[c]The marker is on a BAC which overlaps BACs carrying *MtLAX4*

Discussion

The combination of protein and DNA sequence comparison, mapping, analysis of gene structure, and expression data support some direct orthologies between *Arabidopsis* and *M. truncatula* auxin transport genes. For six expressed *Medicago truncatula* auxin transport genes we were able to find a direct ortholog in Arabidopsis, while for seven auxin transport genes the relationships were more complex (Table 4).

PIN genes

Of the ten *M. truncatula PIN* genes isolated, nine are expressed (Fig. 3). Two pairs of *MtPIN* genes appear to be the result of gene duplications. *MtPIN4* and *MtPIN5* are closely related in sequence, but the change in intron

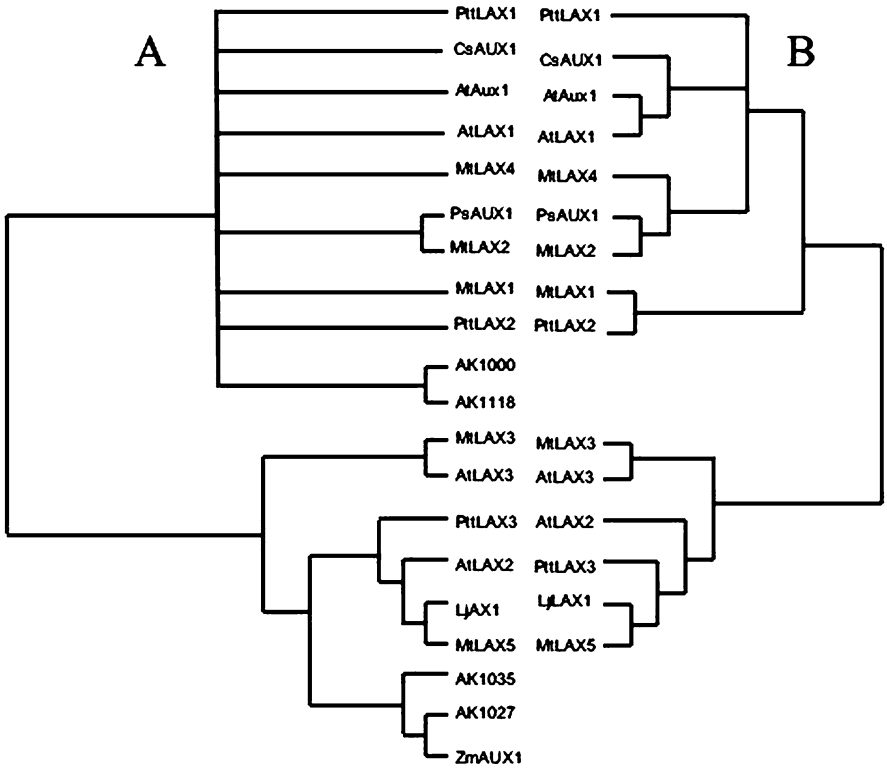

Fig. 2 A, B Phylogenetic analysis of LAX proteins. **A** Parsimony analysis of LAX proteins. Only branches with greater than 50% support are displayed. **B** Maximum likelihood tree based on nucleotide sequences of *LAX* genes. The sequences used in these alignments were the following: *MtLAX1* (AY115841), *MtLAX2* (AY115843), *MtLAX3* (AY115842), *MtLAX4* (AY115844) and *MtLAX5* (AY115845) from *Medicago truncatula*, *AtAUX1* (X98772), *AtLAX1* (AJ249442), *AtLAX2* (AJ243221) and *AtLAX3* (AY127575) from *Arabidopsis thaliana*, *AK102729*, *AK103524*, *AK100090*, and *AK111849* from *Oryza sativa*, *PttLAX1* (AF115543), *PttLAX2* (AF190880), and *PttLAX3* (AF263100) from *Populus tremulus*, *PsAUX1* (AB107919) from *Pisum sativum*, *LjLAX1* (BK005138) from *Lotus japonicus*, *ZmAUX1* (ZmAUX1) from *Zea mays*, and *CsAUX1* (AB085896) from *Cucumis sativus*

structure and the lack of expression of *MtPIN5* suggest that it may be silenced—one possible fate of duplicated genes (Lynch and Conery 2000).

A second gene duplication event is evident in the *MtPIN2*/*MtPIN7* pair of efflux transporter genes. *MtPIN7* is located on the same BAC as *MtPIN2* (Table 3), is highly homologous to *MtPIN2* at both the DNA and protein sequence levels, contains intron sequences with homology to the coding sequence of *MtPIN2* (which could result from the intron boundary shifting in *MtPIN7* when it duplicated and the coding sequence degenerated), and is part of a clade in which all other species examined contribute only one member (Fig. 1), suggesting that *MtPIN7* is the result of a duplication of *MtPIN2* that occurred recently in the *Medicago* lineage. Since *MtPIN2* is expressed in the roots and not in the shoots while *MtPIN7* is expressed in both the shoots and roots (Fig. 3), the functions of the two genes may have diverged since their duplication. Under this scenario, *MtPIN2* and *MtPIN7* could be considered as a pair of paralogs, sometimes termed a derived and an ancestral ortholog (Koonin 2001). Although the half-life of a duplicated gene has been estimated at only 3–7 Myr (Lynch and Conery 2000), genes are more likely to survive duplication if they undergo subfunctionalization (including changes in levels of expression) or partitioning of functions (Wolfe 2001). The *MtPIN2*/*MtPIN7* pair provides an excellent example of such a case, and detailed expression and mutational analysis are ongoing in our laboratory.

Several direct orthologies are suggested by the DNA and protein comparisons and the intron/exon analysis in Fig. 1 and summarized in Table 4. *AtPIN6* appears to be the ortholog of *MtPIN6* based on their sequence identity and the conserved structure of their intron/exon boundaries. Little has been published about the expression pattern of *AtPIN6*, except that transcripts are present in lateral root primordia (Benkova et al. 2003), which is consistent with *MtPIN6* expression in roots. Likewise *AtPIN8*/*MtPIN8* and *AtPIN5*/*MtPIN9* appear to be orthologs. These three pairs occur in a separate clade from the rest of the *PIN* genes and, except for the *PIN6* grouping, contain members from both monocots and dicots, suggesting that they are ancestral groupings. Both *M. truncatula* genes are expressed in roots and shoots, but the expression pattern of neither *Arabidopsis* gene has been reported. If the expression data for *MtPIN7* is taken into account, *AtPIN2* and *MtPIN2* in the upper clade could also be orthologs, because *AtPIN2* is expressed only in roots, and *MtPIN2* shares the same expression pattern (Fig. 3), despite the unusual intron structure of *AtPIN2* (Fig. 1C).

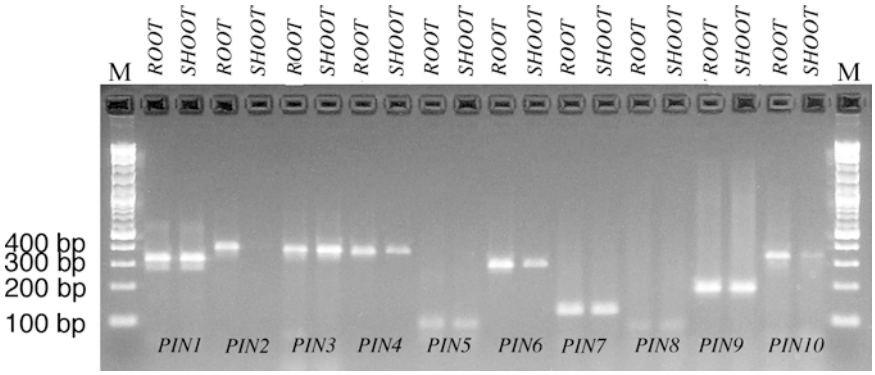

Fig. 3 Confirmation of expression of *MtPIN1* – *MtPIN10* in nodulating roots and shoots of the wild-type *M. truncatula* ecotype A17. Total RNA was isolated from 12-day old nodulating plants, and used to test for the presence or absence of gene expression by RT-PCR. Primers specific for each gene (Table 2) were used to generate the products in the indicated lanes. Bands seen in the *MtPIN5* reactions were also observed in the no-template control (not shown) and are assumed to be primer dimers. The major bands observed in other reactions migrate at sizes expected based on the predicted intron/exon structures listed in Table 2

Other relationships are not as clear. In one case, three *Arabidopsis* genes (*AtPIN4*, *AtPIN7* and *AtPIN3*) are in the same clade with two *M. truncatula* genes (*MtPIN1* and *MtPIN3*). In another case, three *M. truncatula* genes (*MtPIN4*, *MtPIN5* and *MtPIN10*) are in a clade to which *Arabidopsis* contributes only one member (*AtPIN1*). In the first case, the *Arabidopsis* genes appear to be more closely related to each other at both the DNA and protein level than to either of the *M. truncatula* genes, and all of the intron structures are similar. The three *Arabidopsis* genes have all been shown to have roles in root growth and responses to gravity (Friml et al. 2002a, 2002b; Benkova et al. 2003), but their expression is not specific to roots (Benkova et al. 2003) and neither is that of the *M. truncatula* genes (Fig. 3). In the second case, if *MtPIN5* is assumed to be silent because of its structure and lack of detectable expression, the relationships within this clade at the DNA level (Fig. 1B) suggest that *MtPIN4* and *MtPIN10* are closer to each other in sequence than to *AtPIN1*. *Arabidopsis* stands out as contributing only one member to the clade, while *M. truncatula*, poplar, pea, wheat and rice all contribute multiple members. Since the *Arabidopsis* genome has been completely sequenced, the presence of multiple genes from both monocots and dicots in this clade implies that *Arabidopsis* lost a *PIN* gene after its evolutionary divergence from the organisms represented in the tree rather than that a *PIN* gene was duplicated in *M. truncatula*. In addition, there is no evidence for subfunctionalization or partitioning of functions in the *M. truncatula* genes: *AtPIN1* is expressed in seedlings, flowers, roots, leaves and stems (Galweiler et al. 1998) and *MtPIN4* and *MtPIN10* are expressed in both roots and shoots.

Thus five *MtPIN* genes can be assigned one-to-one with four *Arabidopsis PIN* genes, with one gene pair (*MtPIN2*/*MtPIN7*) assigned as a metalog pair of *AtPIN2*. The remaining four expressed *MtPINs* cannot with confidence be paired on a one-to-one basis with the remaining four *AtPINs* based on the present data.

Table 4 Summary of *Arabidopsis*/*M. truncatula PIN* and *LAX* gene relationships

Arabidopsis expression data[a]	*Arabidopsis* gene	*M. truncatula* gene	*M truncatula* expression data[b]
Genes with orthologous relationships			
Lateral root primordia	*AtPIN6*	*MtPIN6*	Roots and shoots
Not reported	*AtPIN8*	*MtPIN8*	Roots and shoots
Not reported	*AtPIN5*	*MtPIN9*	Roots and shoots
Roots	*AtPIN2*	*MtPIN2*	Roots
Not reported	*AtLAX3*	*MtLAX3*	Leaves, flowers, stems, seeds, roots
Not reported	*AtLAX2*	*MtLAX5*	Early seedlings, roots, shoots
Genes with paralogous or uncertain relationships			
Roots and shoots, flowers, leaves	*At PIN1*	*MtPIN10*	Roots and shoots
		MtPIN4	Roots and shoots
Roots and shoots, flowers, leaves	*At PIN4*	*MtPIN1*	Flowers, roots and shoots
Lateral root primordia	*AtPIN7*	*MtPIN3*	Leaves, roots
Roots, stems, leaves	*AtPIN3*		
Root, shoot	*AtAUX1*	*MtLAX2*	Developing seeds, leaves, roots
Not reported	*AtLAX1*	*MtLAX4*	Roots and shoots
No corresponding gene from present data			
		MtLAX1	Roots and shoots

[a]Expression data from Galweiler et al. (1998), Swarup et al. (2001), Friml et al. (2002a), Friml et al. (2002b), Benkova et al. (2003)
[b]Data from this work

AUX / *LAX* genes

AUX / *LAX* genes comprise a smaller family than *PIN* genes in all organisms for which *LAX* gene sequences have been deposited in Genbank, although the incomplete nature of most plant genome sequences must of course be taken into account. Unlike the *PIN* gene comparisons, molecular phylogeny of the *LAX* genes suggests an evolutionary divergence in this gene family between monocots and dicots (Fig. 2). All monocot sequences analyzed thus far fall into two clades, and each of these clades is an individual branch on one of the two large branches that divide the *AUX*/*LAX* gene family into two groups.

As with *PIN* genes, *Arabidopsis* has fewer gene family members than *M. truncatula* A17 (four as opposed to five). However, because of the ecotype specificity of *MtLAX4*, the two organisms may share the same number of gene family members at the species level. *MtLAX5* and *AtLAX2* appear to be orthologs, as do *MtLAX3* and *AtLAX3*. However, the relationships between *AtAUX1* and *AtLAX1* and *M. truncatula LAX* genes are more complex. *AtAUX1* is expressed in leaves and roots and often occurs in the same cells as *AtPIN1* (Swarup et al. 2001). For many *Arabidopsis PIN* gene family members, physiological and genetic data are available to support their roles in auxin transport. However, among the *AtAUX*/*LAX* genes, only for *AtAUX1* is supporting data available to demonstrate its involvement in auxin transport (Marchant et al. 2002). The rest of the *Arabidopsis* genes have been identified and grouped in this class by sequence homology or based on unpublished observations (Parry et al. 2001). The *MtLAX* genes are expressed in both roots and shoots. The expression patterns of the *AtLAX* genes have not yet been published, but like the *AtLAX* genes, the presumed involvement of *MtLAX*s in auxin transport is based on sequence homology.

While *AtAUX1*and *AtLAX1* share close sequence identity to each other, their relationships to *MtLAX4*, *MtLAX2* and *MtLAX1* are unclear, even when DNA sequence comparisons are made (Fig. 2B). Based on a smaller number of sequences Parry et al. (2001) concluded that *AtLAX2* was the founding member of the *AUX*/*LAX* family in *Arabidopsis*, and that other family members arose by gene/genome duplication prior to angiosperm evolution. The data reported here support this conclusion, with *MtLAX5* being the ortholog of *AtLAX2* and *MtLAX3* the ortholog of *AtLAX3*. While the DNA comparison suggests that *MtLAX2* and *MtLAX4* are more closely related to *AtAUX1* and *AtLAX1* than *MtLAX1*, we were unable to discern any further relationships within this group due to the similarities in the expression patterns of the genes. In Arabidopsis, *AtAUX1* is critical for many developmental processes, and the ubiquitous expression of *MtLAX2* could imply orthology to *AtAUX1*. *MtLAX4* may be a duplication of *MtLAX2* in the A17 ecotype because, although we were able to detect *MtLAX4* sequence in the A17 ecotype of *M. truncatula* by PCR and it is represented on at least four BACs in the MtH2 BAC library from this ecotype, we were unable to amplify *MtLAX4* from the A20 ecotype or the more closely related R108 ecotype.

Thus two *MtLAX* genes (*MtLAX3* and *5*) form orthologous pairs with *AtLAX3* and *AtLAX2*, respectively. Of the remaining three *MtLAX* genes, none correspond directly with the *AtLAX1* and *AtAUX1*, but *MtLAX2* is the best candidate for an ortholog of *AtAUX1* based on expression pattern.

PIN or *LAX* genes specific to *M. truncatula*

A simplistic approach to identifying auxin transporters involved in nodulation would be to identify a *PIN* or *LAX* gene sequence specific to *M. truncatula* (i.e., lacking an orthologous counterpart in the non-nodulating *Arabidopsis*), which we were unable to do. The fact that no auxin transporters are exclusive to nodule development certainly does not preclude their involvement in the process. The *MtPIN2*/*MtPIN7* pair is the most recent duplication for which both genes are expressed in *M. truncatula*. Only one gene from another legume is present in the same clade, and since a comprehensive assessment of gene family members in other legume species besides *M. truncatula* has not been done, it is difficult to determine if the duplication is legume specific, *M. truncatula* specific, or if *Arabidopsis* has lost a *PIN* sequence. However, the extremely close sequence identity between the intron region of *MtPIN7* corresponding to a region coding for protein in *MtPIN2*, leads us to suggest a recent event. While neither gene family is dramatically expanded in *M. truncatula* relative to *Arabidopsis*, *M. truncatula* has more expressed *PIN* and *LAX* genes than *Arabidopsis* (8 *PIN*s and 4 *AUX*/*LAX*s in *Arabidopsis* versus 9 *PIN*s and 5 *LAX*s in *M. truncatula*), so it is possible that some of these sequences may represent genes used exclusively in nodule development. Indeed, we suffer from an abundance of candidates: all of the expressed genes identified in this study are expressed in nodulating roots. If nodulation simply co-opts normal plant developmental processes, we would expect several of these genes to be involved in nodulation. We are currently pursuing this investigation using antibodies and RNA interference expressed against various *PIN* sequences, and preliminary analysis suggests that at least four of the *MtPIN* genes have some role in nodule development (Huo, Schnabel and Frugoli, unpublished data).

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