**Aux/IAA gene family is conserved in the gymnosperm, loblolly pine (Pinus taeda)**

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Received January 23, 2003; accepted May 10, 2003; published online November 3, 2003

**Summary** We isolated five members of the Aux/IAA gene family in loblolly pine (Pinus taeda L.). Degenerate primers complementary to conserved regions of angiosperm Aux/IAA genes were used to amplify fragments that were, in turn, used as probes to screen a cDNA library constructed from auxin-treated hypocotyls. The five unique clones, named PTIAA1–5, contain the four highly conserved domains that are characteristic of the Aux/IAA proteins. All clones contain the bipartite nuclear localization signal (NLS) betweenDomains I and II that is predicted in most angiosperm Aux/IAA genes, but only one, PTIAA2, contains the conserved NLS in Domain IV. The five invariant residues in Domain II that have been found to constitute part of a protein destabilization element in Arabidopsis thaliana (L.) Heynh. are conserved in all the PTIAAs. A postulated phosphorylation site located between Domains I and II and proximal to the conserved bipartite NLS was conserved in 20 out of 36 genes in this analysis, including the pine genes. Transcripts of all five PTIAAs accumulated specifically in the hypocotyls in response to exogenous auxin treatment and were induced by all auxins tested. Transcript abundance above basal levels in response to 1-naphthaleneacetic acid treatment was first detected after 10 min (PTIAA5) to 3 h (PTIAA2) in the different genes and remained above basal levels throughout 7 days. Induction of PTIAA2 was inhibited by the protein synthesis inhibitor cycloheximide, indicating that PTIAA2 is a secondary response gene. Phylogenetic analysis showed that all five pine genes clustered within a single class (Class I) of the dendrogram. Clone PTIAA2 has a sequence that is relatively distinct from the other four and is the most closely related to the angiosperm genes of Class I. Class I contains both primary and secondary auxin response genes, suggesting that it is the original lineage and that other gene classes have evolved subsequent to the angiosperm/gymnosperm divergence.

**Keywords:** adventitious roots, auxin, auxin-induced genes, early response genes, multi-gene family.

**Introduction**

The phytohormone auxin controls many aspects of plant development including cell elongation, cell division, apical dominance, vascular tissue differentiation, tropic growth and lateral and adventitious root initiation (Went and Thimann 1937, Hoad et al. 1987). Auxin-stimulated adventitious root formation can be an economically important process (Davies et al. 1994, Kovar and Kuchenbuch 1994, Ritchie 1994), because it is required for vegetative propagation from rooted stem cuttings. However, many tree species have stem cuttings that are recalcitrant to rooting, limiting the use of vegetative propagules. Root formation can be better understood by determining the effects of auxin on gene expression.

Several classes of auxin-induced genes have been identified in plants (reviewed in Abel and Theologis 1996, Hagen and Guilfoyle 2002). One is a large superfamily that has been referred to as the early, auxin-responsive Aux/IAA gene family (Abel and Theologis 1996). Full-length cDNAs belonging to this multigene family have been cloned from several angiosperm species, including Arabidopsis thaliana (L.) Heynh. (Conner et al. 1990, Abel et al. 1995); pea, Pisum sativum L. (Oeller et al. 1993); soybean, Glycine max (L.) Merrill (Ainley et al. 1988); mung bean, Vigna radiata (L.). Wilcz. (Yamamoto 1992); tobacco, Nicotiana tabacum L. (Dargeviciute et al. 1998); and cucumber, Cucumis sativus L. (Fujii et al. 2000, GenBank accession number AB029148). The Aux/IAA gene family in Arabidopsis (Abel et al. 1995) contains up to 29 members (Liscum and Reed 2002).

The precise details of the biochemical functions of members of the Aux/IAA gene family are not fully understood, but genetic and biochemical evidence suggests that they are transcription factors that act as either positive or negative regulators of other auxin-responsive genes. Members of the gene family code for short-lived nuclear proteins (Abel et al. 1994) that share four conserved amino acid sequence motifs called Domains I, II, III and IV and contain functional nuclear localization signals (Abel and Theologis 1995). Domain I is a leucine-rich region, suggestive of the leucine-rich repeat motif that is believed to be involved in protein–protein interactions in a number of diverse proteins (Kobe and Deisenhofer 1994), and the region that includes Domains I and II has been found to be phosphorylated by recombinant phytochrome A in vitro (Colon-Carmona et al. 2000). Domain II contains a strongly conserved region that is important for the destabilization of
Aux/IAA proteins (Worley et al. 2000, Ramos et al. 2001). Gray et al. (2001) found that interaction of the ubiquitin-ligase SCF$^{TIR}$ complex with this region of Domain II was auxin-stimulated and required for Aux/IAA protein degradation. Domain III contains a $\beta\alpha$-motif similar to the $\beta$-ribbon DNA recognition motif of the prokaryotic Arc family repressor proteins (Abel et al. 1994, 1995). Biochemical analysis has shown that Domain III can fold and dimerize in vitro in a manner that is both structurally and functionally similar to the $\beta\alpha$-fold of ArcB-like proteins (Morgan et al. 1999).

Yeast two-hybrid assays have shown that Domains III and IV can mediate homo- and heterodimerizations of the Aux/IAA and auxin response factor (ARF) proteins (Kim et al. 1997). Auxin response factor polypeptides are not induced by auxin (Ulmasov et al. 1999$a$), but the varying interactions between Aux/IAA and ARF proteins have been found to play critical roles in the repression and activation of auxin-responsive genes (Ulmasov et al. 1999$b$). In a model proposed by Tiwari et al. (2001), Aux/IAA proteins interact by dimerization as repressors of ARF transcriptional activity at low auxin concentrations. At elevated auxin concentrations, the Aux/IAA proteins dissociate and are degraded through ubiquitination. Auxin response factor proteins are released to bind as transcription factors at auxin response elements (AuxREs) (Ballas et al. 1993) found in many auxin-regulated gene promoters (Abel et al. 1996, Ulmasov et al. 1997$a$). Because many Aux/IAA promoters contain AuxREs, their transcription is thus activated. Degraded Aux/IAA molecules are replenished through this process, and at lowered auxin concentrations, Aux/IAA and ARF proteins dimerize again, restoring homeostasis to the auxin signaling pathways.

The potential roles of Aux/IAA proteins in root formation are suggested by mutations in Arabidopsis. Mutant lines with single amino acid substitutions in Domain II of five distinct members of the Aux/IAA gene family have phenotypes with altered lateral or adventitious root formation. All result in gain-of-function mutations, presumably because of increased protein stability (Gray et al. 2001), but the phenotypic effects on root formation are varied. Gains of function result in decreased lateral root formation in the slr-1,iaa14 (Fukaki et al. 2002) and iaa28-1 (Rogg et al. 2001) mutants, and in decreased lateral and adventitious root formation in the shy2/IAA3 mutant (Tian and Reed 1999). These results support the hypothesis that Aux/IAA proteins function as transcriptional repressors. However, the arr3 gain-of-function mutation in IAA17 caused increased adventitious root formation (Leyser et al. 1996, Rouse et al. 1998). Moreover, the arr2/IAA7 mutant showed decreased adventitious root formation, but increased lateral root formation (Nagpal et al. 2000). These variations in phenotype demonstrate the complexity of gene actions within and among the Aux/IAA family members.

Because of the central role that auxin plays in plant development, it is likely that plant taxa that are phylogenetically distant contain members of this multigene family. The study of genes in more distant taxa may provide insight into the evolution of the gene family and the growth and developmental processes that they control. In this paper, we report the cloning, sequence analysis and expression characterization of five members of the Aux/IAA gene family from loblolly pine (Pinus taeda L.), a woody, perennial gymnosperm.

### Materials and methods

#### Plant materials

Open-pollinated seeds from a single mother tree of loblolly pine were soaked for 10 min in 10% (v/v) commercial bleach with three drops of dishwashing soap, rinsed in distilled water and chilled at 4 °C for at least 1 month. Following stratification, seeds were again treated with bleach and rinsed. Seeds were sown in vermiculite and grown in a 16/8 h, 26/23 °C, light/dark regime, or were grown in a greenhouse. Seedlings were harvested at 2 weeks and hypocotyl cuttings prepared by severing the root at a point 2.5 cm below the cotyledons, but above the hypocotyl/root junction.

#### Induction of PTIAA expression

The entire hypocotyl cuttings, up to but not including the cotyledons, were immersed in 5 or 10% ethanol (v/v) or 0.04 N NaOH solutions with or without growth regulators for 10 min. After treatment, cuttings were placed in moist sand in the same conditions described for seedling growth and harvested at determined times. Hypocotyl samples were prepared by severing the cotyledons, followed by rapid freezing in liquid nitrogen, and stored at −70 °C until RNA extraction.

#### Isolation of RNA

Total RNA was isolated using either an adaptation of the procedures from Hughes and Galau (1988) and Chang et al. (1993), or the Chang procedure alone. In the adaptation, the Hughes and Galau procedure was followed, except that 5 M potassium acetate (pH 6.5) was used instead of 8.5 M potassium acetate (pH 6.5). After the lithium chloride precipitation step, samples were resuspended in 1 M NaCl, 0.5% SDS, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, according to Chang et al. (1993). The Chang procedure was then followed to completion.

#### Amplification of PTIAA fragments

First strand cDNA was synthesized from total RNA, extracted from hypocotyls treated for 10 min with 1.6 mM 1-naphthaleneacetic acid (NAA), with reverse transcriptase (Promega, Madison, WI) using the oligo(dT)-15 primer (Promega). Taq polymerase (Promega) and degenerate polymerase chain reaction (PCR) primers were used to amplify cDNA fragments corresponding to conserved regions in Domains II, 5'-RTIGTIGGIGGCCICIRT-3' and IV, 5'-ACRTCICCCIRC-ARCA TCCA-3' from the Aux/IAA genes of pea, soybean, mung bean and Arabidopsis (Oeller et al. 1993). Nested primers from Domains III, 5'-TWAYYTIMGIAARRTIGAYYT-3' and IV, 5'-CICCRTTYTTRCTYTCRTA-3' were used to eliminate nonspecific amplification.

After amplification, the PCR products were gel purified using the JetSorb Kit (Genomed, Research Triangle Park, NC)
and ligated into the pGEM-T plasmid vector (Promega) for transformation of JM109 high efficiency cells (Promega). Eleven clones were analyzed using restriction digests and four independent fragments were sequenced. The four independent fragments showed homology to the angiosperm auxin-induced genes and were used as probes for cDNA library screening.

**cDNA construction and screening**

Poly(A+) RNA was isolated from hypocotyls treated with 1.6 mM NAA and, after 10 min, harvested (as described above) using a CsCl modification of the Hughes and Galau (1988) procedure. Briefly, after isopropanol precipitation, total RNA samples were resuspended in 25 ml TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), heated at 65 °C to dissolve the pellet and re-pelleted through a cushion of 5.7 M CsCl in a swinging bucket rotor at 113,000 g for 24 h at 20 °C. The pellets were resuspended in TE and precipitated with sodium acetate and ethanol. Poly(A+) RNA was purified using oligo(dT)-cellulose. A cDNA library was synthesized using 5 µg of the poly(A+) RNA with the ZAP-cDNA Gigapack II Gold Cloning Kit (Stratagene, La Jolla, CA). The primary library contained 8.3 × 10^5 pfus. After amplifying, the library contained 1.5 × 10^12 pfus. Only 5 × 10^5 plagues were sequentially screened using the four independent fragments previously as probes. The fragments were 32P-random prime-labeled using the Prime-A-Gene Kit (Promega) with the subsequent removal of unincorporated nucleotides using the JetNick Kit (Genomed). Screening and excision were performed according to the manufacturer’s instructions (ZAP-cDNA Cloning Kit), except that the prehybridization and hybridization solutions contained 5× SSC, 5× Denhardt’s solution, 1% SDS and 0.8 mg ml−1 sheared herring sperm DNA. Thirteen clones were excised and five independent clones were isolated. Four of the cDNAs were full length and one was missing the 5′ end. The 5′ end of the truncated cDNA was subsequently isolated from the cDNA library using PCR as described by Schraml et al. (1996), resulting in a full-length clone.

**DNA sequencing and analysis**

The cDNAs were sequenced in both directions by automated sequencing at the DNA Sequencing Facilities of the University of North Carolina at Chapel Hill, North Carolina State University and Iowa State University. Sequence data were analyzed using the GCG sequence-analysis system (Genetics Computer Group, Madison, WI). Homology studies were performed in January 2000 using BLAST programs (Altschul et al. 1990) at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/). Sequences were aligned using ClustalW (Thompson et al. 1994), and phylogenetic analyses were conducted using MEGA Version 2.1 (Kumar et al. 2001).

**Southern blot analyses**

Enriched nuclear DNA was isolated from needles of the same loblolly pine mother tree. Nuclei were isolated by grinding leaf tissue in liquid nitrogen and suspending in cold isolation buffer (CIB) (10 ml g−1 of tissue) consisting of 0.33 M sorbitol, 10 mM Tris (pH 9.5), 10 mM EDTA (pH 8.0), 3 mM spermine tetrachloride, 4 mM spermidine free base, 2% (w/v) polyvinylpyrrolidone, 10% (w/v) polyethylene glycol (MW 1000), 0.5% (v/v) Triton X-100, 0.08 M KCl, 0.1% (w/v) bovine serum albumin, 0.5% (v/v) β-mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride. The suspension was homogenized for a few seconds and filtered through two layers of sterile cheesecloth and one layer of 100 µm nylon mesh that had been boiled in 1 mM EDTA and cooled. The nuclei were pelleted at 1600 g and gently resuspended in repeated changes of CIB until the supernatant was no longer green. The final pellet was resuspended in CIB, the nuclei were lysed, and the DNA was extracted using a hexadecltrimethylammonium bromide (CTAB) procedure. Briefly, one tenth of the volume of cold lysis buffer (0.5 M Tris (pH 9.5), 0.2 M EDTA and 10% sarkosyl (w/v)) was added, followed by one seventh of the volume of 5 M NaCl and one tenth of the volume of 10% CTAB with 0.7 M NaCl and 10 µg RNase. After incubation at 65 °C for 30 min, DNA was extracted with chloroform:IAA (CIA) (24:1, v/v) twice and precipitated with isopropanol. The DNA pellets were resuspended in TE, and the CIA extractions were repeated. The DNA was then reprecipitated with sodium acetate and ethanol and resuspended in TE.

For Southern blot hybridization analysis, 25 µg of digested DNA was electrophoresed in a 1% agarose gel, transferred to uncharged nylon membranes (MagnaGraph, Micron Separations, Westborough, MA) by standard alkaline capillary transfer procedures (Ausubel et al. 1998) and affixed to the membrane by UV irradiation (Stratalinker, Stratagene). Membranes were prehybridized at 65 °C for 3 h in 4× SSPE, 10× Denhardt’s solution and 1% SDS with 250 µg ml−1 of denatured herring sperm DNA and hybridized sequentially to each of the five 32P-labeled cDNA clones overnight at 65 °C in 4× SSPE, 5× Denhardt’s solution and 0.5% SDS with 75 µg ml−1 of denatured herring sperm DNA. Probes were prepared as for the cDNA screening. Membranes were washed twice in 2× SSPE and 0.1% SDS for 5 min each, and twice with 0.5× SSPE and 0.1% SDS at room temperature. They were then washed twice at 42 °C and twice at 65 °C for 15 min each in 0.5× SSPE and 0.1% SDS. The membranes were exposed to BioMax film (Eastman Kodak, Rochester, NY) with two intensifying screens for approximately 6 days at ~70 °C.

**Northern blot analyses**

Total RNA (10–20 µg) was separated on 1% agarose gels containing 2.2 M formaldehyde (Ausubel et al. 1998) and transferred to uncharged nylon membranes (MagnaGraph) by standard capillary transfer methods (Ausubel et al. 1998). Transferred RNA was affixed to membranes by UV cross-linking (Stratalinker, Stratagene). Hybridization probes were the same as for Southern blot analyses. Membranes were prehybridized and serially hybridized following standard formamide hybridization procedures (Ausubel et al. 1998). Membranes were washed twice for 5 min each with 2× SSC, 0.1% SDS and 0.2× SSC, 0.1% SDS at room temperature, twice for 15 min with 0.2× SSC, 0.1% SDS at 42 °C, and if necessary, once or twice for 15 min with 0.1× SSC, 0.1% SDS at 68 °C.
Membranes were exposed to Kodak BioMax MS or X-Omat film for 1–5 days (depending on signal intensities of the membranes) with two intensifying screens at –70 °C. The blots were then stripped (Ausubel et al. 1998), hybridized with a larch ribosomal DNA probe (Hutchison et al. 1990), exposed to film for 1 h and signal ratios quantitated by densitometric scanning (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA).

Results and discussion

Molecular cloning of five auxin-induced cDNAs from pine

To identify genes of the Aux/IAA gene family from loblolly pine, we used degenerate PCR primers complementary to conserved regions from the Aux/IAA genes of pea, soybean, mung bean and Arabidopsis. Eleven amplified fragments were cloned, analyzed by restriction digestion and sequenced. Four unique fragments ranging from 165 to 177 nucleotides in length were found. The deduced amino acids of the four fragments ranged from 67 to 89% in identity with each other and 33 to 69% with the angiosperms. Of the 13 to 16 amino acids (depending on amplified fragment length) that were identical in the eight angiosperm sequences, 86 to 100% were conserved in the four pine fragments.

Each independent fragment was used to screen 5 × 10^5 cDNAs from an auxin-induced library. Thirteen clones were excised, analyzed by restriction digestion and sequenced, and five unique clones were isolated. These were named PTIAA1 through 5 (Pinus taeda IAA). All clones included the poly-A tail, and four were found to contain full-length predicted coding sequences. A truncated coding sequence was recovered for PTIAA1, and the 5′ end was subsequently isolated from the cDNA library using anchored PCR.

PTIAA1–5 are separate members of a multigene family

The seedlings contributing mRNA for the cDNA library synthesis were from an open-pollinated family (one mother tree, numerous pollen parents). Thus, Southern blot analyses were conducted to determine whether any of the cDNAs corresponded to different alleles at the same locus. Nuclear enriched DNA of the mother tree was digested with Aralase ribosomal DNA probe (Hutchison et al. 1990), exposed to film and revealed that the genes were single- or low-copy in the genome (data not shown). The clones, with 302 amino acids, is the longest of the predicted pine polypeptides, containing 50 more amino acids than the next largest PTIAAs (PTIAA3 and PTIAA5). PTIAA1 is the shortest polypeptide, because it contains a partially truncated carboxy-terminus (Figure 1).

All five PTIAA polypeptides contain the four highly conserved domains (I, II, III and IV) that are characteristic of the Aux/IAA protein family in angiosperms (Figure 1). Of the 37 amino acids listed as invariant among the 21 known angiosperm genes (Abel et al. 1995), 31 are identical in the five PTIAAs and 29 are identical in these 36 (31 angiosperm and five pine) sequences. All five PTIAAs contain basic residues that comprise the bipartite nuclear localization signal (NLS) found in Domains I and II in PS-IAA4/5 and PS-IAA6 and that are predicted in most angiosperm Aux/IAA proteins (Abel and Theologis 1995). However, the NLS in Domain IV that is conserved in the angiosperm proteins is present only in PTIAA2 among the pine genes.

Five predicted amino acid residues that are invariant in Domain II among all angiosperm Aux/IAA proteins, VGWPP, are also identical in the pine proteins. These residues constitute a portion of a protein destabilization element in Arabidopsis (Ramos et al. 2001). Reporter gene and immunological studies with IAA7 and IAA17 demonstrated that these proteins were substrates of the SCF^{TIR1} ubiquitination complex and that Domain II destabilizes these proteins by targeting

<table>
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<th>GenBank accession number</th>
<th>Insert size (bp)</th>
<th>Integrity of cDNA</th>
<th>Amino acids (no.)</th>
<th>Calculated molecular mass (kDa)</th>
<th>Predicted isoelectric point</th>
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<td>Poly(A)</td>
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<td>27.8</td>
<td>7.97</td>
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</table>

\(^1\) Stop codon within Domain IV.

\(^2\) 5′ End isolated from the cDNA library by PCR.
AUX/IAA GENE FAMILY IN PINE

Figure 1. Sequence alignment of the primary structures of five pine (indicated in bold) and 31 representative predicted angiosperm Aux/IAA proteins. Alignment is limited to full-length sequences and was obtained using ClustalW (Thompson et al. 1994) with manual adjustment. Identical and conserved amino acid residues (at least 19 out of 35 matches) appear in the consensus (capital and small letters, respectively). Conserved domains are underlined and shown in bold. Conserved basic residues that comprise the nuclear localization signals (NLS) in PS-IAA4/5 and PS-IAA6 (Abel and Theologis 1995) are indicated by boxes. Conserved core amino acids of the protein destabilization element (Ramos et al. 2001) are indicated by asterisks. A conserved serine residue between Domains I and II that is a predicted phosphorylation site is boxed and in bold.
them to SCFTIR1 for degradation in response to auxin (Gray et al. 2001). The core amino acids found by Ramos et al. (2001) to be essential for low protein accumulation, GWPPv, are conserved in PTIAA1, 3, 4 and 5, but the variable valine is replaced by isoleucine in PTIAA2, as it is in some other angiosperm Aux/IAA proteins. The substitution of isoleucine for valine is not predicted to have an effect because of the presence of a large hydrophobic R group in both residues (Ramos et al. 2001).

Twenty of the 36 proteins included in this sequence alignment have a conserved serine residue between Domains I and II, proximal to the conserved bipartite NLS. In these 20 proteins, including the pine proteins, the neural network method of Blom et al. (1999) (http://www.cbs.dtu.dk/services/NetPhos) predicts this serine to be a likely phosphorylation site with a score of 0.931 or greater (score range 0.000–1.000). Nuclear protein transport has been found to be regulated by phosphorylation close to the NLS in many eukaryotes (Jans 1995). The conservation of a putative phosphorylation site in this area is also noteworthy because the N-terminal region encompassing these domains is a substrate for phytochrome-dependent phosphorylation of Aux/IAA proteins in vitro (Colon-Carmona et al. 2000).

Deduced phylogeny of the PTIAAs and angiosperm members of the Aux/IAA gene family

We subjected the sequence alignment (Figure 1) to phylogenetic analysis using MEGA Version 2.1 software (Kumar et al. 2001). A neighbor-joining, bootstrapped phylogenetic tree (Figure 2) was generated using complete-deletion to ignore regions containing gaps. Pairwise distance estimations were computed using a gamma parameter of 2.25, bootstrap resampling with 500 replications and a starting random seed of 64238. All interior branches with a bootstrap value less than 50% were collapsed.

Previous phylogenetic analyses of angiosperm Aux/IAA genes revealed four main classes in the family (Abel et al. 1995, Dargeviciute et al. 1998, Liscum and Reed 2002). Despite the addition of these five gymnosperm sequences, the 36 sequences analyzed here fall into four classes that are largely similar to the previous groupings. However, the clustering pattern of the pine sequences is different than that seen in angiosperm species. The five PTIAAs cluster in a single class (Class I) and form a discreet subgroup within that class. The other six species in this analysis have family members in at least two classes, except cucumber, which has two family members in separate subgroups of Class I.

The clustering of the pine sequences may be the result of incomplete sampling of the Aux/IAAs in the pine genome. The five PTIAAs reported here were cloned from a cDNA library constructed from a single organ (hypocotyls) given a single treatment (1.6 mM NAA for 10 min). The existence of additional family members is suggested by the Southern analyses, in which cross-hybridizing bands not attributable to PTIAA1–5 were observed. A search of the expressed sequence tag (EST) database (Blast Gene2EST server http://

woody.embl-heidelberg.de/gene2est/) revealed 14 ESTs from pine with substantial homology to Aux/IAA proteins. Out of these 14 ESTs, accession number BE431390 (231 nt) appears...
to encode a protein distinct from the five PTIAAs. This EST was not included in our analysis because it contains only the area of Domain IV. Of the remaining EST sequences (296–600 nt), eight are identical to PTIAA2, two are identical to PTIAA5 and two do not appear to translate as Aux/IAA proteins. One additional EST, accession number BG275348, is identical to PTIAA2 with the exception of a single nucleotide, resulting in a single amino acid change. A complete sequence would be necessary to determine whether this EST represents a distinct locus or an allele of PTIAA2. Thus, the limited number of distinct PTIAs recovered from different tissues (primarily developing xylem) suggests that the five reported here are reasonably representative of the gene family in pine. However, we cannot rule out the possibility that an analysis with additional PTIAs, if they exist, would yield a phylogenetic tree with pine sequences distributed among the different classes.

There may, in fact, be no orthologs of the other three phylogenetic classes in pine. This would suggest that the Class I genes are more closely related to ancestral Aux/IAA genes and that the other three classes radiated subsequent to the angiosperm–gymnosperm divergence. Class I is the largest of the four classes and contains genes from all seven species in this report. If all Aux/IAA genes in pine are members of Class I, then it is possible that the angiosperm Aux/IAA genes in other classes represent the evolution of new auxin responses or new or redundant auxin signaling mechanisms.

Within the subgroup of Class I that comprises only the pine genes, PTIAA2 is distinct from the other four PTIAs and is also the most similar to the angiosperm genes. PTIAA2 is the only pine protein that contains the strongly conserved asparagine in Domain II (Figure 1). This asparagine is identical in all angiosperm Aux/IAA proteins in this analysis, apart from those in the most distant class (IV), containing the four Arabidopsis genes IAA10 to 13. Also, as previously mentioned, PTIAA2 is the only pine gene that contains the NLS in Domain IV. In addition, the angiosperm subgroup that is most closely related to PTIAA2 contains CS-IAA2, IAA9 and 8, and GH1, genes that, in addition to PTIAA2 (and with the exception of the distant Class IV), are the longest in the analysis and contain the longest intervening sequences between Domains I and II. This length similarity is in addition to those found in the phylogenetic analysis, because gaps resulting from gene-length variation were ignored by complete-deletion.

The segregation of PTIAA2 from the other pine proteins may correspond with gene function, supporting the proposition that Aux/IAA gene classes may be related to functional groups of proteins (Liscum and Reed 2002). Expression studies reported below indicate that PTIAA2 is a secondary response gene and the other four PTIAs are primary response genes. In fact, all genes that have been classified as secondary response genes to date (IAA7 and 8 (Abel et al. 1995) and Nt-iaa28 (Dargeviciute et al. 1998)), fall into Class I. This further supports the idea that Class I is an ancestral class and demonstrates that the existence of primary and secondary response genes predates the angiosperm–gymnosperm divergence.

Expression of PTIAA genes

Timing of auxin-induced gene expression To monitor the kinetics of gene expression, we measured mRNA accumulation for up to 7 days with total RNA extracted from pine hypocotyls treated with a control solution or 1.6 mM NAA for 10 min. This auxin treatment was chosen because it is effective in stimulating auxin-induced adventitious root formation (Diaz-Sala et al. 1996). Abundance of all five PTIAs mRNAs increased following hypocotyl cutting preparation, even when treated with the control solution (Figure 3). PTIAs mRNA abundance in control-treated cuttings was highest at 1 day following cutting preparation and declined to basal levels by 3 days. Presumably, the elevation of PTIA mRNA abundance following treatment with the control solution was due to endogenous auxin that was produced at the shoot apex and accumulated in the hypocotyl following cutting preparation.

Following NAA treatment, mRNA abundance of all five PTIAs increased, but the kinetics of mRNA accumulation varied among the genes. All PTIAs mRNAs except PTIAA2 showed some induction within 1 h following treatment. PTIAs3 was the most rapidly induced, with elevated levels detected within 10 min and maximal abundance by 1 h after NAA treatment. PTIAs1 and 4 showed near maximal abundance 3 h after treatment. PTIAs5 abundance gradually increased, starting at 1 h and reaching a maximum 3 days after treatment. PTIAs2 had the slowest induction of the five genes. mRNAs began to increase in abundance 3 h after treatment and reached maximal levels at 1 and 3 days after treatment. Induction of the expression of all PTIAs by NAA treatment preceded the first cell divisions leading to adventitious root formation in hypocotyl cuttings, which occur between 2 and 4 days after NAA treatment in loblolly pine (Diaz-Sala et al. 1996) and eastern white pine (Pinus strobus L.) (Goldfarb et al. 1998).

The abundance of mRNA in all five PTIAs persisted above basal levels for 7 days in NAA-treated hypocotyl cuttings. This differs from Aux/IAA genes in tobacco seedlings, which declined to near basal levels within 24 h of NAA treatment (Dargeviciute et al. 1998). In other cases, Aux/IAAs have not been tested for elevated mRNA levels beyond 8 h following treatment (Abel et al. 1995, Oeller and Theologis 1995). The persistence of elevated mRNA levels of the PTIAs could be the result of the continued presence of NAA in its active form in the pine cuttings. Pine hypocotyl cuttings do not metabolize NAA that was produced at the shoot apex and accumulated in the hypocotyl following cutting preparation.

NAA dose response of PTIAs gene expression To analyze the effect of NAA concentration on the expression of the PTIAs, we performed a dose-response experiment using solutions with NAA concentrations ranging from 0 to 1 x 10–3 M (Figure 4). Hypocotyls were treated as previously described and harvested at 24 h. The 1 x 10–3 M NAA treatment resulted in the highest levels of mRNAs for all five PTIAs. This differed from Arabidopsis seedlings, in which 1 x 10–3 M IAA was supra-optimal for IAA mRNA abundance (Abel et al. 1995). However, in that study, whole seedlings were treated
with IAA for 2 h, whereas in our experiment, hypocotyl cut-
ing were treated with NAA for only 10 min. Exogenous NAA
applications (10 min pulse) in the mM concentration range are
required for maximal adventitious root formation in hypocotyl

Figure 3. Kinetics of mRNA accumulation in loblolly pine hypocotyls
incubated in the presence (black bars) or absence (white bars) of
1.6 mM 1-naphthaleneacetic acid (NAA) for 10 min, placed in moist
sand and harvested at the times indicated. Total RNA (10–20 µg) was
extracted and separated on 1% formaldehyde-agarose gels, trans-
ferred to nylon membranes and hybridized with 32P-labeled PTIAA
probes. The PTIAA mRNA abundances were determined by auto-
radiographic volume measurements normalized to an 18S ribosomal
probe from larch and are plotted relative to the minus auxin control at
5 h (value of 1). The experiment was conducted twice. Original auto-
radiograms from one experiment are included in each graph.

Figure 4. Dose-response of loblolly pine hypocotyls to 1-naphthal-
enecetic acid (NAA). Hypocotyls were treated with concentrations
of NAA ranging from 0 (9.5% ethanol) to 10−3 M for 10 min, placed
in moist sand and harvested at 1 day. The PTIAA mRNA abundances
were determined as in Figure 3 and are plotted relative to the minus
auxin control. The experiment was conducted twice. Original auto-
radiograms from one experiment are included in each graph.
cuttings of loblolly (Diaz-Sala et al. 1996) and eastern white pine (Goldfarb et al. 1998).

**PTIAA gene expression in response to different auxins**

To determine if the PTIAAs are induced by a variety of naturally occurring and synthetic auxins, we carried out Northern blot analysis with RNA extracted from hypocotyl cuttings that had been treated (as previously described) with NAA (1.6 mM), indole-3-acetic acid (IAA) (8 mM), indole-3-butyric acid (IBA) (3.2 mM), 2,4-dichlorophenoxyacetic acid (2,4-D) (0.4 mM) or 9.5% ethanol, which was used to dissolve all of the auxins, and harvested at 24 h. The concentrations of the various auxins were chosen to correspond approximately to the strength of each auxin in pine tissue culture systems. In addition, some cuttings were treated with tryptophan (8 mM), an auxin analog, dissolved in ethanol.

Treatment with all auxins increased the mRNA levels of all five PTIAAs at least slightly (Figure 5). At the concentrations tested, all PTIAAs were most strongly induced by NAA, followed by IBA for four of the five PTIAAs. The lowest level of induction for all five PTIAAs was observed in the hypocotyls treated with 2,4-D and IAA. Treatment of hypocotyl cuttings with tryptophan did not result in PTIAA levels greater than the control, with the possible exception of PTIAA5.

**Specificity of auxin-induced gene expression and effect of cycloheximide**

Total RNA was extracted from hypocotyls that had been treated as previously described with the plant growth regulators 6-benzylaminopurine (BA) (1.6 mM), abscisic acid (ABA) (3.2 mM) and gibberellic acid (GA) (1.6 mM) to test hormone specificity. Some hypocotyls were also treated with the translational inhibitor, cycloheximide (CHX) (0.08 mM), with and without NAA (1.6 mM), to determine whether de novo protein synthesis is necessary for induction of the PTIAA genes. Controls consisted of cuttings treated with 0.04 N NaOH, used to dissolve BA and ABA, and 9.5% ethanol, used to dissolve GA, NAA and CHX.

As in previous experiments, there was an increase in mRNA abundance of all five PTIAAs following NAA treatment (Figure 6). Treatment with ABA did not result in mRNA levels substantially greater than the controls for any of the PTIAAs. The BA treatment caused a slight increase in mRNA abundance in PTIAA1. Induction of three Aux/IAA genes by BA treatment was also reported in tobacco seedlings (Dargeviciute et al. 1998). Similarly, GA treatment appeared to result in slightly elevated levels of PTIAA1, 2, 4 and 5 mRNA.

Cycloheximide treatment inhibited mRNA accumulation of PTIAA2 and had varied effects on the other four PTIAAs (Figure 6). Cycloheximide inhibition of PTIAA2 accumulation occurred in both NAA-treated and control-treated hypocotyl cuttings. This inhibition, combined with slower induction kinetics, strongly suggests that PTIAA2 is a secondary response gene that requires protein synthesis for induction. Accumulation of mRNAs of PTIAA1 and PTIAA3 was induced by CHX treatment alone and superinduced above NAA treatment alone when treated with a combination of CHX and NAA. Expression of PTIAA4 and 5 was unaffected by CHX. Genes induced, superinduced and unaffected by CHX have been reported for angiosperm Aux/IAAs (Abel et al. 1995, Dargeviciute et al. 1998) and genes showing any of these responses have been considered primary response genes, be-
cause their induction is independent of protein synthesis (Abel et al. 1995).

Conclusions

We have cloned and characterized five genes from loblolly pine that appear to be members of the Aux/IAA gene family. This is the first report of genes of this family in gymnosperms. The genes are identified as members of this gene family based on their sequence similarities to angiosperm Aux/IAAs and their rapid and relatively specific induction by auxin. Combined with a recent report of an Aux/IAA gene in the moss Physcomitrella patens (Hedw.) Bruch and Schimp (Imaizumi et al. 2002), our findings demonstrate a relatively ancient origin and suggest a fundamental role of the gene family in plant development across the plant kingdom. Five PTIAA genes are significantly fewer than the 29 Aux/IAAs in Arabidopsis (Liscum and Reed 2002). Whereas DNA hybridizations and EST databases indicate the potential presence of additional family members in pine, the available evidence suggests that the pine gene family is substantially smaller than in Arabidopsis. Surprisingly, phylogenetic analysis shows that all five pine genes group together within one of the four angiosperm Aux/IAA classes. Thus, it appears that the gene family has undergone substantial ramification during evolution of at least some angiosperm taxa. One of the five pine genes, PTIAA2, has a sequence that is relatively distinct from the other four and is also the most closely related to the angiosperm genes. Interestingly, PTIAA2 appears to be a secondary response gene, based on its slower induction kinetics and the inhibition of its induction by cycloheximide treatment. The angiosperm gene class that contains the pine subclass also contains both primary and secondary angiosperm response genes. This suggests that this class was the original lineage and that the other classes have evolved subsequent to the angiosperm/gymnosperm divergence. If this is true, then it raises questions about the functions of the genes in the other three classes; they may represent novel angiosperm auxin functions, distinct signaling mechanisms, or merely redundancies. The developmental functions of the five PTIAAs are not yet fully understood. In these experiments, we determined whether the expression patterns of any were consistent with a role in adventitious root formation. All five genes were induced by exogenous auxin treatment of hypocotyl cuttings, prior to the morphological changes that lead to adventitious root formation, and at auxin concentrations that cause root formation. Because there is evidence, however, that Arabidopsis Aux/IAAs may act as repressors of auxin triggered transcription (Ulmasov et al. 1997b, 1999b, Tiwari et al. 2001), it is possible that one or more of the PTIAAs function to restore auxin homeostasis, after the initial reception of the auxin signal. This would allow for the normal development and growth of the new root meristem. However, gain-of-function mutations in Arabidopsis Aux/IAAs cause either enhanced (Leyser et al. 1996, Rouse et al. 1998) or reduced (Tian and Reed 1999, Rogg et al. 2001, Fukaki et al. 2002) root formation. These different phenotypes and the lack of obvious orthology between pine and Arabidopsis, however, indicate that these mechanisms are not conserved.
dopis genes make assigning a specific root formation-related function to the PTIAAs problematic.

Alternatively, the PTIAAs could be controlling gene expression in response to light. Loblolly pine seedlings grown in the dark exhibit enhanced, presumably auxin-mediated elongation, typical of etiolation responses. Because phytochrome has been shown to phosphorylate Aux/IAA proteins in vitro (Col- lon-Carmona et al. 2000), it is possible that, in the light, phytochrome activates a PTIAA protein that in turn represses downstream genes that otherwise cause auxin-induced elongation. In addition, other light-induced developmental processes are mediated by auxin. An auxin-responsive pine gene isolated from shoots using microarray hybridizations is similar in sequence to an angiosperm gene that represses plastid development (Busov 2001). Definitive determination of specific developmental functions for the PTIAAs awaits evidence from future transgenic experiments.

Acknowledgments

Funding for this research was provided by the industrial supporters of the North Carolina State University Loblolly and Slash Pine Rooted Cutting Program and the North Carolina Agricultural Research Service. International Paper Company provided loblolly pine seed. Gary Coleman (University of Maryland) and Glenn Howe (Oregon State University) provided protocols for CsCl purification of RNA and nuclear-enriched DNA, respectively, and Keith Hutchison (University of Maine) provided the larch 18S ribosomal RNA clone.

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