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Isolation and characterization of an Aux/IAA gene (LaIAA2) from Larix

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The phytohormone auxin controls many aspects of plant development. Auxin/indole-3-acetic acid (Aux/IAA) transcriptional factors are key regulators of auxin responses in plants. To investigate the effects of auxin on gene expression during the rooting process of Larix cuttings, a subtractive cDNA library was constructed and 272 UniEST were obtained by using suppression subtractive hybridization (SSH). Based on a fragment of 272 UniEST, the full-length cDNA of LaIAA2, an Aux/IAA gene from Larix was isolated. Then, the response expression of LaIAA2 to auxin was determined by treating with different sources and concentration of auxin and cycloheximide and the expression patterns of LaIAA2 were examined in different tissues. The results show that LaIAA2 appears to be the first response gene of auxin and LaIAA2 gene was involved in the root development and auxin signaling. The express pattern of LaIAA2 gene indicated that it might play a central role in root development, specially regulated lateral and adventitious root production.

Key words: Aux/IAA gene family, auxin, LaIAA2, Larix

INTRODUCTION

The plant hormone auxin, in particular indole-3-acetic acid (IAA), is a key regulator of virtually every aspect/process of plant growth and development, including apical dominance, phyllotaxy, lateral and adventitious root initiation, flower and fruit development, embryogenesis, cell elongation, cell division and vascular tissue differentiation (Muday, 2001; Jenik and Barton, 2005; Leyser, 2005). Auxin induces the transcription of several classes of genes as a rapid, primary response, in which one of the best characterised is the Aux/IAA family (Knox et al., 2003). Full-length cDNAs belonging to this large superfamily have been cloned from several species, including Arabidopsis thaliana, pea, Pisum sativum L., soybean, Glycine max (L.) Merrill, mung bean, Vigna radiata (L.) R. Wilcz, tobacco, cucumber, lobolly pine and tomato (Goldfarb et al., 2003; Zhang et al., 2007).

The precise biochemical functions of Aux/IAA gene family members are not fully understood, but genetic and biochemical evidence suggests that they are transcriptional factors that act as either positive or negative regulators of other auxin-responsive genes (Abel et al., 1994). Aux/IAAs encode low abundance, nuclear proteins with extremely short half-lives, some as short as 5 min. Aux/IAA proteins are characterised by a highly conserved four domain structure called Domains I, II, III and IV and contain functional nuclear localization signals (Abel and Theologis, 1995). Domain I is responsible for the repressing activity of proteins (Tiwari et al., 2004).

Abbreviations: IAA, Indole-3-acetic acid; IBA, indole-butyric acid; NAA, -naphthalene acetic acid; DAG, days after germination; AUX1, auxin resistant 1; Aux/IAA, auxin/indole-3-Acetic Acid (Aux/IAA) transcriptional factors; TIR1, transport inhibitor response 1; SCFTIR1, Skp1-Cullin-F-box complex; SSH, suppression subtractive hybridization; ARFs, auxin-response factors; ABA, abscisic acid; 2, 4-D, 2, 4-dichlorophenoxyacetic acid; BA, 6-benzylaminopurine; GA, gibberellic acid.
Domain II acts as a regulatory domain that confers instability to the Aux/IAA proteins (Worley et al., 2000; Ouellet et al., 2001; Ramos et al., 2001; Tiwari et al., 2001). Domain II interacts with an F-box protein, TIR1, which is a component of the SCF\textsuperscript{TIR1} ubiquitin ligase complex (Gray et al., 2001). Auxin increases this interaction in a dose-dependent manner, promoting the rapid degradation of Aux/IAA proteins through the ubiquitin-proteasome pathway (Gray et al., 2001; Zenser et al., 2001, 2003). Domains III and IV of Aux/IAA proteins serve as protein-protein interaction domains that promote both homodimerization with other Aux/IAA gene family members and heterodimerization with the auxin response factors (ARFs) (Ulmason et al., 1997a, b; Ouellet et al., 2001). The Aux/IAA proteins regulate auxin-mediated gene expression by controlling the activity of ARFs by protein-protein interactions (Ulmason et al., 1997b; Tiwari et al., 2003). In plant cells containing low levels of auxin, Aux/IAA-ARF heterodimers would be predicted to persist, resulting in inhibition of ARF-dependent transcription. By contrast, cells experiencing high levels of endogenous auxin would be expected to maintain little Aux/IAA protein, having ARFs dissociated from Aux/IAA proteins. This allows for expression changes in typically auxin-regulated genes.

The functions of Aux/IAA proteins in growth and development are determined by mutations in Arabidopsis or other plants. The gain-of-function mutations, including shy2/iaa3 (Tian and Reed, 1999), shy1/iaa6 (Kim et al., 1996; Reed, 2001), axr2/iaa7 (Timpfe et al., 1994; Nagpal et al., 2000), bdll/iaa12 (Hamann et al., 1999, 2002), solitary root (slr)/iaa14 (Fukaki et al., 2002), axr3/iaa17 (Leyser et al., 1996; Rouse et al., 1998), iaa18 (Reed, 2001), msg2/iaa19 (Tatematsu et al., 2004) and iaa28 (Rogg et al., 2001), have pleiotropic effects on plant growth. Some of the mutations suggest the potential roles of Aux/IAA proteins in root formation. For example, the shy2-2/iaa3 mutant has reduced numbers of lateral roots (Tian and Reed, 1999) and the iaa28-1 mutant forms fewer lateral roots (Rogg et al., 2001). In contrast, the axr2-1/iaa7 mutant has increased numbers of lateral roots (Nagpal et al., 2000), while the axr3-1/iaa17 mutant has increased numbers of adventitious roots (Leyser et al., 1996). These varied phenotypes in root formation might reflect the functional differences among the various Aux/IAA proteins and/or the transcriptional differences in Aux/IAA genes regarding organ/tissue specificity and responsiveness to auxin.

Auxin-stimulated adventitious root formation is required for vegetative propagation from stem cuttings; therefore, this process has an important application role in tree species transplantation. 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. Here, we report the isolation and characterization of a member of Aux/IAA genes in Larix, named LalAA2, which is involved in adventitious root formation in Larix cuttings.

**MATERIALS AND METHODS**

**Plant materials**

Two clones of *Larix leptolepis* × *Larix olgensis*, which had a significant difference in rooting ability, were chosen as plant materials (clone 31-6 with high ability of rooting and clone 15-4 with low ability of rooting) for establishing the subtractive hybridization library. The stems (clone 31-6 and clone 15-4) were treated with 1×10\textsuperscript{-3} M 1-naphthal-eneacetic acid (NAA) for 30 min, callus and adventitious roots during the rooting process were used to isolate total RNA and to construct subtractive cDNA library.

Seeds of *Larix*, which were chilled at -20°C for at least 1 month, were dipped in 45°C water for 30 min, followed by a room temperature water soaking for 24 h. Then, seeds were sown between two wetness gauze and grown in a greenhouse. Seedlings were harvested at 2 weeks and hypocotyl cuttings were prepared by severing the root at a point 2.5 cm below the cotyledons, but above the hypocotyl/root junction.

**RNA extraction**

Total RNA were extracted from stems, callus, adventitious roots using TRIzol (Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s manual. Poly(A)\(^+\) RNA was isolated from the total RNA with the polyATract mRNA isolation system kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

**SSH**

The subtractive library was constructed with ds cDNA of clone 31-6 (Tester) and 15-4 (Driver) based on BD PCR-select cDNA subtract kit (Clontech, mountain view, CA, USA) according to the protocols provided by the manufacturer. Positive clones were sequenced with it (Clontech, mountain view, CA, USA).

**Reverse transcription and clone of LalAA2 gene**

To a 0.2 ml thin-walled PCR tube on ice, add 2 μg of total RNA, 1 μl of Oligo-(dT) 18 primer and 1 μl of 10 mM dNTP mixture, and 1 μl of MLV reverse transcriptase (Promega), 2 μl of RNase inhibitor and 2 μl of M-MLV reverse transcriptase (Promega). The mixture was mixed by gently pipetting and spins briefly to collect the content. The reverse transcription reaction was performed at 42°C for 1 h to synthesize the first strand cDNA.

A forward primer LalAA2F1 (5'-ATGKAGAGGGTTGGGCAAYGA-3') based on Aux/IAA gene sequences of other plants and a reverse primer LalAA2R1 (5'-CTATTCTGGACTTGGATT-3') based on Aux/IAA gene sequence of Larix SSH library were designed for LalAA2 amplification. Total volume of PCR reaction system was 50 μl, including 5 μl of 10 × reaction buffers, 2.5 μl of template, 2.5 μl of 10 μM primers of LAIAA2RF1 and LAIAA2RR1, respectively, 1 μl of 10 mM dNTP, 2 μl of Taq polymerase (TIANGEN) and 33.5 μl of PCR-Grade water. Thermocycling was performed at 94°C × 30 s, 60°C × 30 s and 72°C × 1 min for 36 cycles. The PCR product (10
metabolism
Information
pathways
Transport
Perception and
response to stimuli
Developmental
processes
Localization
Unknown proteins
No hits found

Figure 1. Distribution (as percentage) of predicted functions represented among the 272 genes.

Result was separated by 1% agarose gel electrophoresis, staining with GoldViewTM nucleic acid stain.

The PCR amplified segment was inserted into pGEM-T-easy vector according to the manufacturer’s instructions. Competent cells of Escherichia coli stain DH5α were prepared for transformation of the LalAA2-T-easy vector recombinant. IPTG/X-gal blue-white screening was carried out under ampicillin selection (Sambrook et al., 1989). The white E. coli colonies were first confirmed by PCR and then, the positive clones were sequenced.

Sequence analyses

The sequence analyses of LalAA2 cDNA were performed by DNA star (http://www.dnastar.com) and bioXM 2.2 (http://bioxm.go.nease.net). Other AUX/IAA gene sequences were retrieved at the National Center for Biotechnology Information (NCBI) at the web site (http://www.ncbi.nlm.nih.gov). Properties and structures of LalAA2 protein were predicted by the software tools on scan prosite web site (http://au.expasy.org/psort).

Real-time quantitative PCR expression analyses

The relative quantification was performed using the SYBR Premix Ex Taq™ (Takara). The real-time quantitative PCR was carried out on the ABI 7500 system with the LalAA2 primers 5'-GCTCCTGGGCTTTCTATGTTC-3' and 5'-CCATCGTCTGCTAGGCTTTGA-3'. The program for quantitative PCR was as follows: initial denaturation step of 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 60°C for 34 s. Specificity of the reaction was verified by both melting curve analysis and sequencing of amplification products. Real-time PCR experiment was performed in triplicate and normalized to 18S rRNA (with primer 5'-TTTCTGCGCATACACTTTCT-3' and 5'-CGATGAGCCAGTATTGTA-3') by the ΔΔ Ct method.

Semi-quantitative PCR expression analyses

Total RNA were extracted from roots, stems, leaves, stems which were treated with 200 ppm IBA, callus and callus with adventitious root primordium. The cDNA synthesis was performed as described earlier. The cDNA pools from these tissues were then, amplified using the primers 5'-GCTCCTGGGCTTTCTATGTTC-3' and 5'-CCATCGTCTGCTAGGCTTTGA-3', respectively. These primers span the two introns and the amplified fragment was easily distinguished from genomic DNA contamination. The amplification reaction was as follows: initial denaturation step of 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. A 218 bp fragment of the β-actin gene was amplified as a control using the primer set 5'-TGCTCCAGAAGAACACCTCA-3' and 5'-GGAACCTGCTTGCCGCTGCT-3'. The PCR products were separated on a 1.2% agarose gel.

Statistical analysis

Each experiment was independently performed at least three times. The data from real-time PCR were analyzed by one way analysis of variance (ANOVA) using SigmaStat 3.5 software (Systat Software, Inc., Richmond, CA). Differences between experimental groups were considered significant at P < 0.05.

RESULTS

Characterization of up-regulation genes for rooting of Larix cuttings

To reveal different expression of genes in cuttings of Larix which had significant difference in rooting ability, an SSH approach was used. The driver cDNA was synthesized from mRNA isolated from clone 15-4 of which rooting ability was low and the tester cDNA was produced from mRNA isolated from clone 31-6 of which rooting ability was high.

A large-scale screening of up-regulated genes in clone 31-6 was carried out by analysis of clones obtained from the SSH library. Out of 500 isolated ESTs, 272 were sequenced successfully and analyzed for their sequence.
Based on the documented functions of the genes from which these ESTs were derived, these ESTs were classified into 8 categories (Figure 1). In all the 272 UniESTs, 10 of them were related to the hormones. Furthermore, of these 10 UniESTs, 6 had relationship with auxin, 2 with gibberellin, 1 with ABA and 1 with jasmonic acid. In the 6 UniESTs related to auxin, 2 of them belonged to AUX/IAA family, 1 belonged to NAC gene family and another 3 belonged to the auxin response genes.

**Cloning of LaIAA2 gene**

The open reading frame (ORF) of LaIAA2 comprised 891 base pair, encoding a 296 amino acid protein (Figure 2). The LaIAA2 amino acid polypeptide was predicted with molecular weight (Mw) of 32.1 kD and isoelectric point (pl) of 5.94 using the software of DNAStar (http://www.dnastar.com). The derived protein comprised the four conserved domains characteristic of the Aux/IAA gene family, Domains I to IV and two nuclear localization signal (NLS) sequences. The predicted bipartite NLS comprised an invariant basic doublet KR in interdomain II and basic amino acids in Domain II. A second SV40-type NLS was located at Domain IV (Figure 2). Comparison of the LaIAA2 amino acid sequence against GeneBank database revealed a high degree of consensus over other Aux/IAA proteins. The highest score (83% identity) was found with the sequence of Pinus pinaster Aux/IAA protein (GeneBank, accession number CAC85936.1). The higher score (82% identity) was found with the sequence of Pinus taeda Aux/IAA protein (GeneBank, accession number AAP44405.1). Lower identities were detected with Aux/IAA amino acid sequences from other species: Populus tremula x Populus tremuloides Aux/IAA protein, Zinnia elegans Aux/IAA protein and G max GH1 protein (GeneBank, accession number CAB84706.1, AAM12952.1 and AAB70005.1) (50, 50 and 48%). In all 29 Aux/IAA proteins of Arabidopsis, only AtIAA9 protein (GeneBank, accession number NP_569017.2) (46%) had higher degree of identity with LaIAA2 protein. These results show that LaIAA2 protein had higher degree of identity with gymnosperm Aux/IAA protein, instead of angiosperm Aux/IAA protein. Furthermore, the results indicate that there was large variation in LaIAA2 gene during evolution.

Furthermore, the predicted results of LaIAA2 protein secondary structure showed that a βsheet (183~188aa), two alpha helix α1 (200~219 aa) and α2 (232~239 aa) sequentially formed a βαα motif, which was thought as a DNA binding domain in prokaryotic and in Arabidopsis. Ten conserved protein kinase C (PKC) phosphorylation sites (T-56, S-66, S-96, T-128, S-161, T-166, T-205, S-211, T-221 and S-292), 3 casein kinase I (CKI) (S-28, S-79 and S-227), 4 casein kinase II (CKII) phosphorylation sites (S-12, T-205, S-210 and S-211), 5 cAMP-dependent protein kinase (PKA) (S-44, S-66, S-135, T-166 and S-279) and 2 cdc2 protein kinase (Cdc2) (S-188 and S-204) were also present in LaIAA2 protein (Figure 2). The occurrence of these phosphorylation sites also implied that LaIAA2 was a short-lived protein, similarly to Arabidopsis Aux/IAA members.

**Deduced phylogeny of the LaIAA2, other gymnosperm and angiosperm members of the Aux/IAA gene family**

An alignment of the amino acid sequence of LaIAA2 protein with other homologies was analyzed using DNAMAN software (Figure 3). Previous phylogenetic analyses of angiosperm Aux/IAA genes revealed four main classes in the family (Abel and Theologis, 1995; Dargenvitute et al., 1998; Liscum and Reed, 2002). However, the clustering pattern of the LaIAA2 amino acid sequences was different from that in angiosperm species. The LaIAA2 and other gymnosperm Aux/IAA genes (PtIAA1, PtIAA2) were clustered in a single class (class I) and formed a discreet subgroup within that class.

**The expression patterns of LaIAA2 in Larix**

**The expression patterns of LaIAA2 in different tissues**

Organ-specific expression patterns of LaIAA2 in Larix were investigated with RT-PCR. The amplified product was a 0.2 kb fragment in length. The results show that LaIAA2 was expressed at low levels in the leaf and root and yet at high levels in stem. The LaIAA2 transcripts were detected in different phases of adventitious root formation in Larix cutting. Furthermore, after the cuttings were treated with 200 ppm IBA, the expression of LaIAA2 increased obviously (Figure 4).

**Timing of LaIAA2 expression**

To monitor the kinetics of gene expression, we measured mRNA accumulation for up to 5 days with total RNA extracted from Larix hypocotyls treated with a control solution or 1.6 mM NAA for 10 min. The results show that expression of LaIAA2 increased following NAA treatment. LaIAA2 mRNA began to accumulate in abundance at 10 min after treatment and reached maximal levels at 1 day after treatment. The abundance of LaIAA2 mRNA persisted above the basal levels for 3 days in NAA-treated hypocotyl cuttings (Figure 5).

**NAA dose response of LaIAA2 expression**

To analyze the effect of NAA concentration on the
expression of LaIAA2, we performed a dose-response experiment using solutions with NAA concentrations ranging from 0 to $1 \times 10^{-3}$ M. The highest levels of LaIAA2 mRNA resulted from $1 \times 10^{-3}$ M NAA treatment. This result was different from Arabidopsis seedling, in which $1 \times 10^{-3}$ M IAA was supra-optimal for IAA mRNA abundance (Abel et al., 1995). However, the two experiments were different, whole seedlings were treated with IAA for 2 h in Arabidopsis experiment, whereas hypocotyl cuttings were treated with NAA for only 10 min in our experiment (Figure

Figure 2. Sequence alignment of LaIAA2 and other Aux/IAA genes based on their amino acid sequences. Alignment was limited to full-length sequence and was analyzed using DNAMAN.
**Figure 3.** The phylogenetic tree of *LaIAA2*. The phylogenetic tree of 31 Aux/IAA proteins from other plants and 1 from Larix. The tree was constructed based on the amino acid alignment with selected sequences found in GenBank/EMBL bank.

**LaIAA2 expression in response to different hormones and cycloheximide**

To determine the changes of *LaIAA2* mRNA abundance after being treated with different hormones and cycloheximide, hypocotyls were cuttings were treated by 6-benzylaminopurine (BA)(1.6 mM), abscisic acid (ABA)(3.2 mM), gibberellic acid (GA)(1.6 mM) or cycloheximide (CHX)(with or without NAA (1.6 mM))(0.08 mM), then real-time quantitative PCR was performed.

In the same way, mRNA abundance of *LaIAA2* following NAA treatment increased. Treatment with ABA did not increase mRNA levels of substantially greatly when compared with the controls for *LaIAA2*. The BA and GA treatment caused a slight increase in mRNA abundance in *LaIAA2*. Expression of *LaIAA2* was unaffected by CHX (Figure 8).

**DISCUSSION**

Previous researches of rooting ability of cuttings in conifer and other plants revealed that rooting ability of cuttings were affected by auxin and its family members (Greenwood and Weir, 1994; Syros et al., 2004; Wiesman et al., 1989). The ability of auxins to stimulate adventitious root formation is well documented (Hartmann and Kester, 1983; Sircar Chatterjee, 1973). Exogenously supplied auxins, especially IAA and IBA, could improve rooting quantity of cuttings. At the same time, rooting success varied markedly with genotype and 5-fold differences among full-sib families had been observed (Foster, 1990); whereas, its poor understanding of the molecular basis for auxin and genotype in rooting ability is desirable. In molecular level, does the research about the genes which related to rooting have big significance? It can explore the rooting mechanism about *Laix* cutting and it may also improve the rooting efficiency. In our research, by the SSH method, we successfully constructed the cDNA library between two *Laix* clones which have difference rooting ability (Feng, 2010). The result shows that some of early auxin response genes (AUX/IAA family) that is found in the cDNA library were important for us to understand the function of auxin in rooting of *Laix* cuttings. So based
Figure 4. The expression of LaIAA2 in root, stem, leaf and rooting of Larix cuttings.

Figure 5. NAA dose response of LaIAA2.

Figure 6. The hormone response of Lal
Figure 7. The auxin response of LaIAA2.

Figure 8. The expression of LaIAA2 since NAA treatment.

on the segment of AUX/IAA family in cDNA library, we isolated and characterized an Aux/IAA gene from Larix, named LaIAA2. LaIAA2 gene is identified as a member of the Aux/IAA family based on its amino acid sequence similarities to Aux/IAA proteins and its rapid and relatively specific induction by auxin. The deduced amino acid sequence of LaIAA2 gene has all four domains of a canonical Aux/IAA protein. Compared with other Aux/IAA proteins, however, they are highly conserved for the characteristic domains I-IV. This property suggests that LaIAA2 gene might have similar regulatory mechanisms to its homologous proteins in auxin-regulated events. In contrast, less conservation is observed between the LaIAA2 amino acid sequence and its homologous proteins within the interdomain regions. These divergent domains might be the trace of evolutions and they might...
mediate interactions with other proteins and confer a specific function on different members of the Aux/IAA family in different plants. Based on its fast induction kinetics and the non-inhibiton of its induction by cycloheximide treatment, LalIAA2 appears to be the first response gene to auxin in Larix.

Auxins inhibit root elongation, increase lateral root production and induce adventitious roots. Abundant lateral and adventitious root is found in few mutants that overproduce auxin. Conversely, long primary roots, few lateral roots are found in mutants that were deficient in auxin responses (Zhang et al., 2007). As a big auxin response gene family, Aux/IAA genes are also expressed differentially in different plant tissues, organs and development stages (Abel et al., 1995). In Arabidopsis, gain-of-function mutations cause either enhanced (Leyser et al., 1996) or reduced (Rogg et al., 2001) root formation. In tomato, the Sl-IAA3 promoter drives GUS expression predominantly in root cap and developing lateral roots (Chaabouni et al., 2009). In this study, we characterized that LalIAA2 gene was expressed differentially in various organs or development stages. The results indicate that transcriptional levels of LalIAA2 gene were regulated by some development-related elements. Semi-quantitative RT-PCR showed that LalIAA2 was highly expressed in all phases of adventitious root formation in Larix cuttings. Especially, the expression of LalIAA2 gene increased obviously after the cuttings were treated with 200 ppm IBA. All of these results indicate that LalIAA2 gene was regulated by auxin and might play a central role in root development. Then, a series of experiments to test LalIAA2 gene expression patterns were done. In the test of timing of LalIAA2 expression, LalIAA2 mRNA reached maximal levels at 1 day after treatment; this differed from Aux/IAA genes in tobacco seedlings, which declined to near basal levels within 24 h of NAA treatment (Dargevicutte et al., 1998). The persistence of elevated mRNA levels of the LalIAA2 could be the result of the continued presence of NAA in its active form in Larix cuttings. In the test of NAA dose response of LalIAA2 expression, the highest levels of LalIAA2 mRNA resulted from 1×10^{-3} M NAA treatment. This result is same with physiological experiment that the quantity of roots of Larix cuttings treated with 1×10^{-3} M NAA was most (Wang et al., 2006). In the test of LalIAA2 expression in response to auxins from different sources or different hormones, LalIAA2 was most strongly induced by NAA. These four experiments indicate that LalIAA2 gene was induced by exogenous auxin treatment of Larix cuttings, prior to the morphological changes that led to adventitious root formation and at auxin concentrations that cause root formation. Lastly, the alignment showed that the amino acid sequence of LalAA2 protein was more similar with PTIAA1 and PTIAA2 genes. Both PTIAA1 and PTIAA2 genes are possibly enhanced adventitious root formation (Goldfarb et al., 2003). All of these results indicate that LalIAA2 gene is involved in adventitious root formation and enhanced root formation.

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