



# Expressed sequence tag analysis of functional genes associated with adventitious rooting in *Liriodendron* hybrids

Y.D. Zhong\*, X.Y. Sun\*, E.Y. Liu, Y.Q. Li, Z. Gao and F.X. Yu

The Key Laboratory of Horticultural Plant Genetic and Improvement of Jiangxi, Institute of Biology and Resources, Jiangxi Academy of Sciences, Nanchang, China

\*These authors contributed equally to this study.

Corresponding author: F.X. Yu

E-mail: fxyu2000@126.com

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**ABSTRACT.** *Liriodendron* hybrids (*Liriodendron chinense* x *L. tulipifera*) are important landscaping and afforestation hardwood trees. To date, little genomic research on adventitious rooting has been reported in these hybrids, as well as in the genus *Liriodendron*. In the present study, we used adventitious roots to construct the first cDNA library for *Liriodendron* hybrids. A total of 5176 expressed sequence tags (ESTs) were generated and clustered into 2921 unigenes. Among these unigenes, 2547 had significant homology to the non-redundant protein database representing a wide variety of putative functions. Homologs of these genes regulated many aspects of adventitious rooting, including those for auxin signal transduction and root hair development. Results of quantitative real-time polymerase chain reaction showed that *AUX1*, *IRE*, and *FBI* were highly expressed in adventitious roots and the expression of *AUX1*, *ARF1*, *NAC1*, *RHD1*, and *IRE* increased during the development of adventitious roots. Additionally, 181 simple sequence repeats were identified from 166 ESTs and more than 91.16%

of these were dinucleotide and trinucleotide repeats. To the best of our knowledge, the present study reports the identification of the genes associated with adventitious rooting in the genus *Liriodendron* for the first time and provides a valuable resource for future genomic studies. Expression analysis of selected genes could allow us to identify regulatory genes that may be essential for adventitious rooting.

**Key words:** *Liriodendron* hybrids; cDNA library; Functional genes; Adventitious rooting; EST-SSRs

## INTRODUCTION

The genus *Liriodendron* contains only two species: *Liriodendron tulipifera*, which is widely distributed in eastern North America, and *Liriodendron chinense* (Hemsl). Sarg, which is native to northern Vietnam and southern China (Parks and Wendel, 1990). *Liriodendron* hybrids, derived from sexual hybridization between *L. chinense* and *L. tulipifera*, are fast-growing hardwood trees and are widely used in fast-wood plantation construction, phytoremediation, and bioenergy industry. They are highly heterotic in many aspects, especially in having more attractive flowers, faster growth, and improved resistance to biotic and abiotic stresses (Parks et al., 1983). However, this economically valuable timber and landscaping tree is hard to breed due to difficulties in both sexual and clonal propagation.

Adventitious roots arise naturally upon adaptation to environmental stresses or from the aerial organs of plants. The formation of adventitious roots can be induced by indirect callus tissue formed because of mechanical damages. The ability to form adventitious roots can lead to rapid amplification of millions of cuttings from elite clones used for plantations. This capability is a heritable quantitative trait affected by many endogenous and environmental factors (Li et al., 2009). Two major auxin response protein families, the *AUX/IAA* (auxin/indole-3-acetic acid) and *ARF* (auxin-response factor) gene families, involved in the regulation of adventitious rooting have been identified (Gutierrez et al., 2009). *ARF6* and *ARF8* positively regulate adventitious root initiation in *Arabidopsis* whereas *ARF17* regulates it negatively (Gutierrez et al., 2009). The F-box protein TIR1 (transport inhibitor response 1) is an auxin receptor (Navarro et al., 2006), which regulates the degradation of Aux/IAA proteins and the consequent changes in the expression of auxin-regulated genes (Dharmasiri et al., 2005). The auxin receptor *NAC1*, acts downstream of *TIR1* and participates in lateral root stimulation (Xie et al., 2000; Guo et al., 2005). Auxin biosynthesis occurs mainly in young leaves, shoot and root apices, and auxin is redistributed by polar transport (Shimizu-Sato et al., 2009). The mutation of *AUX1*, which encodes an auxin uptake carrier, results in reduced lateral root formation in *Arabidopsis* (Bennett et al., 1996). In the present decade, similar mechanisms of regulation of the adventitious root development have been reported in different organs of *Arabidopsis* (Gutierrez et al., 2012). However, due to the complex structure and intricate regulation of root systems, only a few genes involved in the adventitious rooting regulation have been identified (Ramírez-Carvajal et al., 2009), and the mechanism of adventitious root development in trees remains unclear.

Expressed sequence tags (ESTs) are valuable resources for gene discovery, gene mapping, and quantitative trait analysis. They can also be a good source of simple sequence

repeats (SSRs), which are useful molecular markers in plant genetics and evolutionary studies. Recently, a large number of ESTs have been generated for many trees. Currently, there are three available EST databases (Albert et al., 2005; Liang et al., 2008; Jin et al., 2011; Liang et al., 2011) and one BAC library (Liang et al., 2007) for *L. tulipifera* and one EST database for *L. chinense* (Yang et al., 2014). Most of the cDNA libraries have been constructed from flowers, xylems, and leaves. The genomic resources for the hybrid yellow poplar are still limited, especially in terms of adventitious root development. Moreover, few DNA sequences are available in the NCBI database for *Liriodendron* hybrids with the exception of 232 miRNAs associated with somatic embryogenesis (Li et al., 2012). Considering the economic and ecological value of *Liriodendron*, the availability of transcriptome data of hybrid yellow poplar will contribute to *Liriodendron* breeding and related genetic research.

In the present study, we characterized the adventitious root transcriptomes of hybrid yellow poplar using the normalized cDNA library. Specifically, we aimed to identify the transcripts involved in adventitious rooting. The gene-based SSRs identified in the unigene assemblies can be applied to gene mapping and function analysis in the genus *Liriodendron*. To the best of our knowledge, this is the first report on the adventitious root transcriptome of *Liriodendron* hybrids, and the findings of this study could make significant contributions in improving the rooting of recalcitrant genotypes and in functional genomics studies of *Liriodendron*.

## MATERIAL AND METHODS

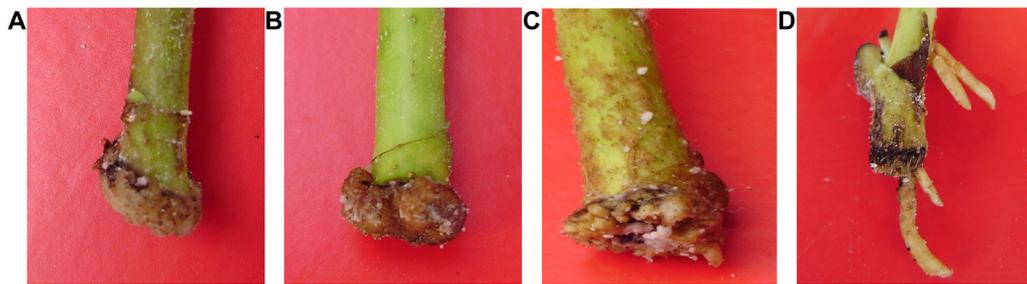
### Plant materials

Adventitious roots of hybrid yellow poplar (*L. chinense* x *L. tulipifera*) were derived from one ramet of clone 210. All the hardwood cuttings were obtained from an annual twig and dipped in a solution containing 300 ppm indole-3-acetic acid (IAA) and 300 ppm 3-indolebutyric acid (IBA) for 15 s. The trees were kept in a hotbed at 25°-30°C during the day and rooted after 25-40 days. The process of adventitious rooting in hybrid yellow poplar cuttings was performed as described previously (Zhang and Fang, 2003). Tissues of three independent biological replicates were collected at four different phases (Figure 1): I) proliferation of vascular cambium cells at the base of the cuttings (callus was translucent at the early stage); II) dedifferentiation of vascular cambium cells and formation of root initials (callus looked milky white); III) growth and development of root primordium (adventitious roots elongated by approximately 1 cm); IV) emergence of adventitious roots from the lenticel or callus of the cuttings (adventitious roots elongated by more than 2 cm). All the harvested samples were washed with 70% ethanol, immediately frozen in liquid nitrogen, and stored at -80°C, until RNA extraction.

### RNA extraction, library construction, and EST generation

Total RNA was extracted from different stages of adventitious roots according to a modified Trizol (Invitrogen, Shanghai, China) method and was purified using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA was dissolved in RNase-free (diethylpyrocarbonate-treated) water. The isolation and purification of mRNA was conducted using FastTrack® 2.0

Kit (Invitrogen, Shanghai, China) according to the manufacturer protocol. An uncut cDNA library was constructed using CloneMiner (Invitrogen) according to the manufacturer instructions. The first strand of cDNA was synthesized by priming poly(A) RNA with Biotin-*attB2*-Oligo(dT) primer [5'-Biotin-GGCGGCCGCACAACCTTTGTACAAGAAAGTTGGGT (T)<sub>19</sub>D-3'] using SuperScript™ II reverse transcriptase (Invitrogen), and the second strand was synthesized using the first cDNA strand as a template by *E. coli* DNA polymerase (TaKaRa, Dalian, China). An *attB1* adapter was ligated to the 5'-end of the cDNA, while biotin prevented ligation of the *attB1* adapter (5'-TCGTCGGGGACAACCTTTGTACAAAAAAGTTGG-3', and 3'-CCCCTGTTGAAACATGTTTTTTCAACCP-5') to the 3'-end of the cDNA that had been selected and blunt ended. Thereafter, GatewayR technology, which constituted two recombination reactions including the BP reaction and LR reaction in the cDNA library construction kit, was used. The library constructed in pDONE™ 222 (Invitrogen) was amplified in ElectroMAX™ DH10B™ T1 Phage-Resistant cells. Finally, the cDNA library titer was calculated by performing the plating assay for an insert size greater than 500 bp.



**Figure 1.** Four developmental phases during adventitious rooting of *Liriodendron* hybrids. **A.** proliferation of vascular cambium cells at the base of the cuttings; **B.** dedifferentiation of vascular cambium cells and formation of a root initial; **C.** growth and development of root primordium; **D.** emergence of adventitious roots from the lenticle or the callus of the cuttings.

The cDNA library was derived from the uncut cDNA library by saturation hybridization with genomic DNA of hybrid yellow poplar on magnetic beads. Firstly, the genomic DNA was extracted using an Axygen genomic DNA miniprep kit (Axygen Biosciences, USA). DNA was incubated with *Hind*III, purified, and ligated with Biotin-dUTP. Then, the DNA was incubated with *Bam*HI and purified. The immobilized affinity system was constructed by adsorbing the magnetic beads using the DYNAL M280 magnetic bead kit (Invitrogen). The driver DNA of the immobilized affinity system was pre-hybridization at 65°C for 1 h in a hybridization oven (Hb-1000, UVP, Upland, CA, USA). Thereafter, single-stranded plasmids of the denatured cDNA library were hybridized with the driver DNA in the presence of 0.5% SDS (w/v), 6X SSC, and 5X Denhardt's solution (Invitrogen). The reaction was performed for 16 h at 65°C to achieve a calculated  $C_{ot}$  value of 5 s-M. The hybridization mixture was washed four times with washing buffer (1% SDS (w/v) and 2X SSC) and separated from the magnetic beads using a magnetic separation rack (Invitrogen) by incubating at 75°C for 10 min before being renatured. The plasmids eluted from the magnet were concentrated by butanol extraction and ethanol precipitation. The recombinant plasmid DNA was transformed into ElectroMAX™ DH10B™ T1 Phage Resistant cells by electroporation using the BTX

ECM 630 electroporator (Genetronics, San Diego, CA, USA). The plasmid DNA prepared from these bacteria represents the amplified normalized cDNA library. The titer of this library was determined by XGal/IPTG screening. ESTs were generated by sequencing the cDNA library and approximately 5252 clones were randomly picked by Sanger sequencing.

## Data processing, assembly, and annotation

All the sequences were trimmed of low quality segments at the 5'- and 3'-ends by Phred score 20 prior to assembling the ESTs and then the vector and adaptor sequences were removed by Cross-match. Sequences longer than 100 bp were clustered into unigenes, including the contigs and singletons using Phrap. Unigenes were imported and compared against the NCBI database using BLASTX and TBLASTX (minimum match identity  $\geq 25\%$  and E value  $\leq 1E^{-10}$ ). The NCBI non-redundant nucleotide and protein databases were used for general annotation. The conserved domain information with potential functions of gene and gene ontology were predicted using Gene Ontology (GO; <http://www.geneontology.org>).

## Gene expression analysis

Seven ESTs (Table 1) for adventitious rooting, including one gene for auxin transport (*AUX1*), three genes for auxin signal response (*ARF1*, *NAC1*, *FBI*), and three genes for root hair development (*RHD1*, *RHL1*, *IRE*), were selected for the study. Using the partial sequences obtained from the EST sequencing, we designed primers for detecting the expression patterns of these genes.

Total RNA extraction from tissues in four developmental phases of adventitious rooting (Figure 1) and various organs (adventitious roots, stems, and leaves) was performed as described above. One microgram DNase treated RNA was then reverse transcribed into cDNA using SuperScript III First-Strand Synthesis System (Invitrogen) with random primers. For quantitative real-time polymerase chain reaction (RT-PCR), specific primers ( $T_m$ , 58°-61°C) were designed to amplify a PCR segment of 50-150 bp using Primer Express Software version 3.0 (Applied Biosystems, Foster City, CA, USA) and Oligo6.6 (Molecular Biology Insights, Cascade, CO) (Table 1). Real-time PCR was conducted using the ABI 7500 Real-time PCR system (Applied Biosystems) and SYBR Green Realtime PCR Master Mix (Toyobo, Co., Ltd., Osaka, Japan) according to the manufacturer protocol. For each sample, the reactions were performed in a final volume of 20  $\mu$ L containing 10  $\mu$ L 2X SYBR Green Realtime PCR Master Mix, 1  $\mu$ L (10  $\mu$ M) each primer, 3  $\mu$ L cDNA, and 5  $\mu$ L Milli-Q H<sub>2</sub>O; three technical replicates were taken for each sample.

**Table 1.** Primers and PCR product size of the genes used for RT-PCR analysis.

Gene name	Unigene number	Forward primer	Reverse primer	Product size (bp)
<i>AUX1</i>	Contig89	AAGGAAGCCGAGAAGGTTAG	CCGTTCAAGAGATCCATCA	224
<i>ARF1</i>	E11-K04302389-K2389	GCTCTCATCTCCCTTGT	CCTCCGTTCAAGAAGCC	108
<i>NAC1</i>	Contig669	AGATGTTGGGTGGTGGAG	TGGCATCAGTGGCAGAG	120
<i>RHD1</i>	Contig1663	GCCACGGAAGGTCAAG	GGAAGCGGAGGTAGGAAG	131
<i>IRE</i>	Contig535	TCCTCTCCGCCGTTGATT	GCCATCATAGCCCTTCTTCC	218
<i>RHL1</i>	H11-K04304600-K4600	AGAAGGGCCAGAGGAAGAA	TGCGGGAATCAATGTAGAG	130
<i>FBI</i>	C03-K04304147-K4147	GGTCCGCAACAAGATACAA	ATCTGTCCCAACATCCCTAA	122
<i>Histone H3</i>	Contig924	TCCACAGGAGGAAAGGCTC	GTGCAACAGTTCAGGGCG	115

The amplification was conducted with 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min, along with initial denaturation at 95°C for 1 min. The *histone H3* gene was used as an internal control. Data from the quantitative RT-PCR analysis for gene expression were transformed using the  $2^{-\Delta\Delta Ct}$  method.

### SSR development from ESTs

SSRs were mined from the non-redundant ESTs by Simple Sequence Repeat Identification Tool (SSRIT) database (<http://www.grameme.org/db/searches/ssrtool>). The minimum number of repeats was nine for di-nucleotide repeats, six for tri-nucleotide, five for tetra-nucleotide, and four for penta- and hexa-nucleotide repeats. SSR primer pairs were designed from the flanking sequences, using the PRIMER3 software ([www.vgenome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www.vgenome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)).

## RESULTS AND DISCUSSION

### Library construction and characterization

An uncut cDNA library was constructed from four developmental phases of adventitious roots of hybrid yellow poplar. The titer of this library consisted of  $7.34 \times 10^6$  clones with more than 95% recombinant clones and the titer of the library was  $3.72 \times 10^5$  with more than 95% recombinant clones after normalization. Four widely distributed housekeeping genes were then selected and specific primers for real time polymerase chain reaction (RT-PCR) were designed ([S1 Table](#)). The abundance of transcripts decreased notably in the normalized cDNA library compared to the uncut samples. These results suggested that the normalized cDNA library had been established successfully, which facilitated further studies to identify the expressed genes in the developmental phases of adventitious rooting in hybrid yellow poplar.

In the present study, a normalized cDNA library was constructed from the developing adventitious roots of hybrid yellow poplar. The normalization efficiency was identified using the four housekeeping genes. There was a 35- to 85-fold reduction in abundance after the normalization. This normalization would greatly facilitate the enrichment of the library for rare genes, reduce the cost of sequencing, and increase the recovery rate of unigenes by avoiding redundant clones.

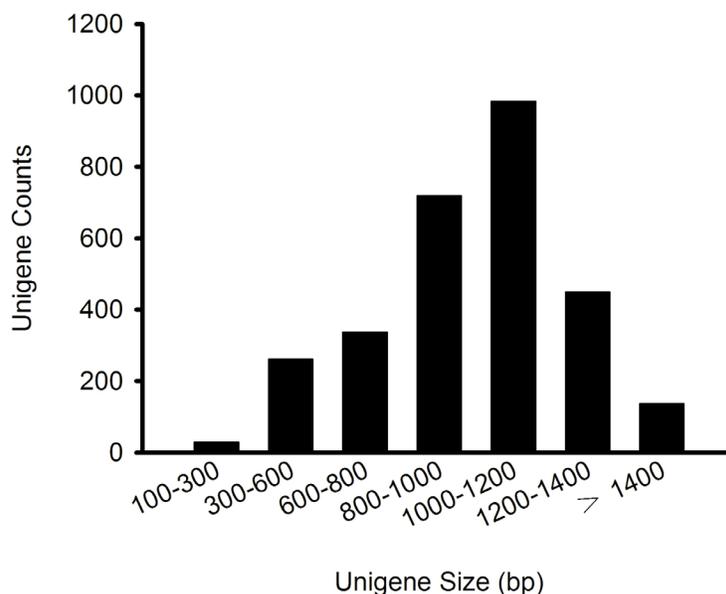
### Library sequencing and contig assembly

A total of 5176 high quality ESTs were obtained after screening for vector sequences and excluding low quality sequences shorter than 100 bp (Table 2). The read length of the ESTs ranged from 59 to 2349 bp, with an average length of 1001 bp, which was longer than floral bud ESTs of *L. tulipifera*, and petal and leaf ESTs of *L. chinense* (Liang et al., 2008; Liang et al., 2011; Yang et al., 2014) but was smaller than the secondary xylem ESTs of *L. tulipifera* (Jin et al., 2011). The GC content of 46.74% was comparable to that of other transcriptomes in *Liriodendron* (Liang et al., 2008; Yang et al., 2014), but was different from those of *Arabidopsis* (42.7%) and rice (51.1%; Kuhl et al., 2004).

**Table 2.** Summary of ESTs from adventitious roots of *Liriodendron* hybrids.

	Number
No of high quality EST sequences	3229
No of contigs	2055
No of singletons	866
No of unigenes sequences	2921
Range length of unigenes (bp)	59-2626
Average unigenes size (bp)	1000
Range length of contigs (bp)	149-2626
Average contigs size (bp)	1,010
Range length of singletons (bp)	59-2349
Average singletons size (bp)	977

An assembly of overlapping, contiguous sequences yielded 2921 unigenes, including 2055 contigs and 866 singletons (Table 2). The average length of the unigenes was 1000 bp (contigs averaged 1010 bp and singletons averaged 977 bp), with 59 bp being the shortest and 2626 bp being the longest. Of the final unigene set, 720 unigenes were 800-1000 bp and 984 unigenes were 1000 bp or longer (Figure 2).

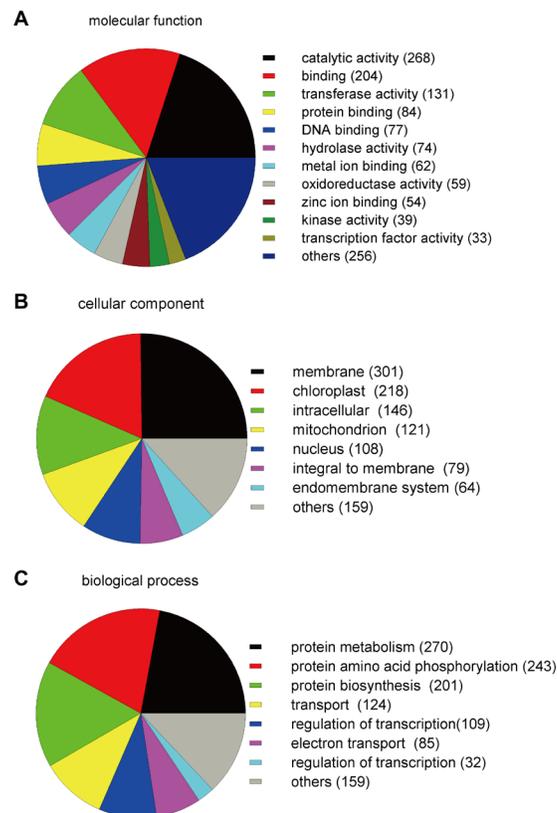
**Figure 2.** Distribution of unigene size.

### Functional classification of transcriptome

To identify the unigenes that were potentially encoding proteins with known functions, BLASTx analysis was performed against the non-redundant protein database. A total of 2547 (87.2%) unigenes showed significant similarity with at least one expressed, hypothetical, or unknown protein, including 1796 contigs and 751 singletons. Among those unigenes, 468 unigenes (184 singletons and 284 contigs) had high similarity with unknown proteins. However, 374 (12.8%) of the unigenes did not display significant similarity to any protein

in the database. Those unigenes could be expressed specifically during the development of adventitious roots of the *Liriodendron* hybrids. When compared to model species, the best BLASTX matches were with sequences from grape, poplar, rice, and *Arabidopsis*. Woody angiosperm species had more hits than non-woody species (Liang et al., 2011).

Detailed functional annotation of the unigenes was performed following the GO scheme. The terms were organized into three categories representing molecular functions, cellular components, and biological processes. A total of 2921 unigenes were annotated with 3760 annotations distributed in three categories (Figure 3), among which, 1341 (45.9%) unigenes could be assigned putative molecular functions, 1196 (40.9%) putatively contributed to cellular components, and 1,223 (41.9%) were predicted to be involved in biological processes. A wide variety of putative functions were shown in this database. With regard to the molecular functions, classes were mainly involved in catalytic activity (7.13%), protein binding (5.43%), and transferase activity (3.48%; Figure 3A). Unigenes related to cellular components were mostly assigned to membrane (8.01%), chloroplast (5.80%), and cellular component (3.88%; Figure 3B). Simultaneously, the biological process category mainly comprised of unigenes for metabolism (7.18%), amino acid phosphorylation (6.46%), and biosynthesis (5.35%; Figure 3C).



**Figure 3.** Pie chart representation of gene ontology (GO) annotation of EST functions in *Liriodendron* hybrids. **A.** Putative molecular functions; **B.** putative cellular components; **C.** putative biological process.

## Genes for adventitious rooting

Previous studies showed that adventitious rooting was regulated by auxin-responsive or some other genes. In the present study, many genes associated with adventitious root development were identified ([S2 Table](#)). Thirty seven contigs and 11 singletons were auxin signal transduction-related genes induced during adventitious rooting. *Aux/IAA*, *GH3* (Gretchen Hagen 3), and *SAUR* (small auxin up RNA) gene families are considered to be primary auxin-response genes (Abel and Theologis, 1996), most of which were induced immediately by auxin. *AUX/IAA* is a transcription suppressor and its expression level increases instantly when induced by auxin. *SAUR* may be involved in the auxin signal transduction pathway of calcium and calmodulin (Yang and Poovaiah, 2000). *GH3* regulates the IAA level by conjugating amino acids to IAA. When IAA is added *in vitro*, primary root growth in the mutants, *gh3.1-1*, *gh3.17-1*, *gh3.2-1*, and *gh3.5-1* was much slower than that in wild type *Arabidopsis* (Staswick et al., 2005). Furthermore, the expression of the *GH3* gene was positively correlated with the number of adventitious roots (Sorin et al., 2006). We identified six contigs (Contig89, Contig129, Contig891, Contig524, Contig1358, and Contig1861), one contig (Contig818), and one singleton (C05-K04305603) to be highly homologous to the *Aux/IAA*, *GH3*, and *SAUR* genes, respectively.

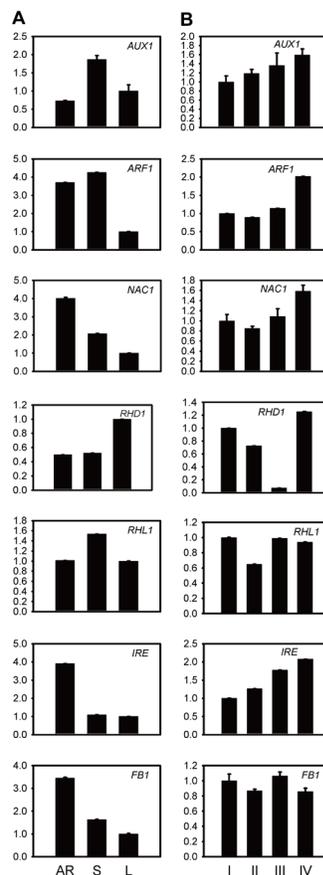
Additionally, eight contigs (Contig524, Contig1095, Contig1131, Contig1358, Contig1451, Contig1482, Contig1860, and Contig2053) and two singletons (C12-K04301531-K1531 and E11-K04302389-K2389) were highly homologous to the *ARF* (auxin response factor) genes. *ARF* regulates auxin signaling by mediating the expression of auxin-responsive genes at the transcriptional level (Guilfoyle and Hagen, 2007). *ARF17* is a negative regulator, whereas *ARF6* and *ARF8* are positive regulators of adventitious rooting (Gutierrez et al., 2009). They regulate the expression of the *GH3.3*, *GH3.5*, and *GH3.6* genes, which are required for fine-tuning the adventitious root initiation in the *Arabidopsis* hypocotyl (Sorin et al., 2005; Gutierrez et al., 2012). Two contigs (Contig490 and Contig1073) and one singleton (C03-K04304147-K4147) were highly homologous to the *TIR1* gene. *TIR1* is an auxin receptor which mediates Aux/IAA degradation and finally the expression levels of the auxin-regulated genes (Dharmasiri et al., 2005). In *Liriodendron* hybrids, many auxin-response genes were expressed during adventitious root development, suggesting that the interaction of auxin signal transduction related genes regulated the adventitious rooting.

Both *SCR* (Scarecrow) and *SHR* (Short Root) are members of the GRAS family of computative transcription factors. The SHR-SCR signaling pathway plays a key role in stem cell maintenance and quiescent center specification in *Arabidopsis* (Sabatini et al., 2003). The expression of homologs of *SCR* and *SHR* genes (Contig228, Contig1131, and D05-G0907296065-996) implies that some cells may serve as stem cells in the adventitious rooting of *Liriodendron* hybrids. However, the existence of stem cells in hardwood cuttings is still not clear and needs further research. In *Arabidopsis*, *NAC* (NAM/ATAF/CUC) is a transcription factor, which is expressed specifically in the roots. Its expression was induced by IAA to increase the number of lateral roots (Xie et al., 2000; Guo et al., 2005). One contig and five singletons were homologous to *NAC* in our EST database, which might have functions in the lateral root formation during adventitious rooting.

## Expression analysis by RT-PCR

As shown in Figure 4, different genes were expressed constitutively at different tissues

but had different expression patterns. For example, *NAC1*, *IRE*, and *FBI* genes were all highly expressed in the adventitious roots. The expression of *IRE* in the adventitious roots was more than three times higher than that in the stems and leaves. However, the expression of *AUX1* and *RHD1* genes in the adventitious roots was much lower than that of stems and leaves. *ARF1* and *RHL1* were more highly expressed in stems, than in adventitious roots and leaves. The expression of *AUX1*, *ARF1*, *NAC1*, *RHD1*, and *IRE* genes increased with the development of the adventitious roots, except for slight reduction in the expression of *ARF1* and *NAC1* genes during the phase II and rapid reduction in the expression of the *RHD1* gene during the phase III. These results suggested that the expression of auxin-related genes was induced by adventitious rooting. However, the gene expression profiles of *RHL1* and *FBI* remained nearly the same in the four phases. The increment of *RHL1* and *FBI* gene expression levels in phase I and phase III suggested that the induction of adventitious root primordium was regulated by the exogenous auxin and that the elongation of the adventitious roots was controlled by endogenous auxin.



**Figure 4.** Expression of different genes by RT-PCR. **A.** Expression of seven genes in adventitious roots (AR), stems (S), and leaves (L); **B.** Expression of seven genes in four different developmental phases (I, II, III, IV) of adventitious roots.

Auxin is synthesized in specific tissues and is transported by carriers. The redistribution of different auxins is carried out by different influx (*AUX1* gene family) and efflux carriers (*PIN* gene family; Teale et al., 2006). In trees, auxin is transported actively through the vascular cambium, whereas it is actively transported through the vascular parenchyma in *Arabidopsis* (Marchant et al., 2002). This is further supported by the fact that *AUX1* mRNA existed in stems and leaves, and accumulated rapidly with the adventitious root development. Consistent with the *aux1* mutation in *Arabidopsis* (Bennett et al., 1996), these results revealed that auxin induced *AUX1* expression, and might eventually control the root formation in *Liriodendron* hybrids.

The ARF and AUX/IAA proteins regulate auxin-mediated transcriptional activation/repression in *Arabidopsis* (Teale et al., 2006). They control adventitious root initiation by regulating *ARF* transcripts and miRNA abundance (Gutierrez et al., 2009). In *Arabidopsis*, *ARF1* was expressed at low levels in all the organs tested, including roots, stems, leaves, flowers, and siliques. In mango tree wood, *MiARF1* was cloned from the cotyledon section during the adventitious root formation, and was expressed in both rooting and non-rooting tissues (Xiao et al., 2004). However, our results showed that *ARF1* was more highly expressed in stems and adventitious roots than in leaves. Moreover, in contrast to the earlier reports, its expression increased with the adventitious root formation. These results implied that *ARF1* might serve different functions in the adventitious rooting in *Liriodendron* hybrids and other species.

*NAC1* is a transcription activator in the auxin signaling pathway in *Arabidopsis* lateral root development. *NAC1* is expressed mainly in roots, and its mRNA levels positively correlate with the lateral root number (Xie et al., 2000; Guo et al., 2005). Similar expression patterns were also found in hybrid yellow poplar, which suggested a similar function of the *NAC1* gene in lateral root formation in adventitious rooting.

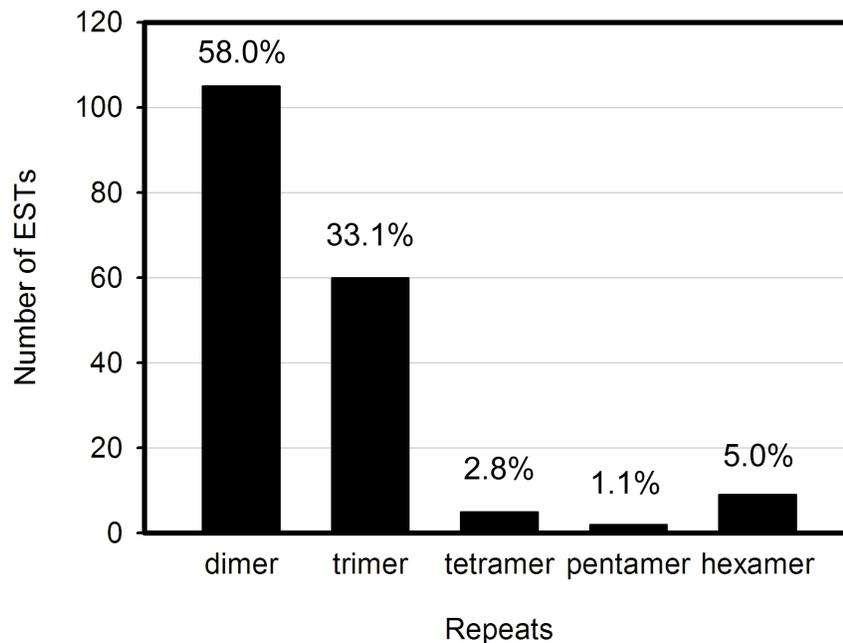
Several studies on root hair initiation had previously been reported (Seifert et al., 2002). For example, *RHD1* is ubiquitously expressed in roots, leaves, stems, and flowers, and controls normal root hair initiation (Seifert et al., 2002). The *rh11* mutation, which led to extremely low transcript levels throughout the plant, inhibited primary root hair formation and produced a lethal seedling phenotype (Schneider et al., 1998). In *Liriodendron* hybrids, *RHD1* and *RHL1* transcripts were detected in each tissue at very low levels. Moreover, auxin induced *RHD1* gene expression during adventitious rooting and might control proper root hair initiation. However, there was a rapid reduction in *RHD1* expression in phase III of adventitious rooting, suggesting the necessity of *RHD1* inhibition during adventitious rooting especially during the emergence of adventitious roots from the lenticle or the callus of the cuttings. *IRE* was strongly expressed in the elongating root hair cells, and the root hairs of the *ire* mutant were about 40% shorter than the wild type in *Arabidopsis* (Oyama et al., 2002). In *Liriodendron* hybrids, *IRE* gene transcripts appeared to accumulate at relatively higher levels in roots than in other organs, and the expression of *IRE* was strongly induced by auxin, suggesting its importance in root hair elongation.

We isolated the full length *FBI* gene in *Liriodendron* hybrids. The putative protein had high similarity with the F-box proteins of other species (Sun XY and Yu FX, unpublished results). The F-box families, such as *TIR1* and *AFB3* (auxin signaling F-box 3), act as auxin receptors in the auxin signaling pathway, implying their key roles in adventitious rooting. The *FBI* transcript level was slightly higher in phase I and II, which suggested that *FBI* was involved in the early induction of adventitious root primordium by auxin and regulated the elongation of adventitious roots by endogenous auxin in the hybrid yellow poplar. The

characteristics of their expression patterns will provide crucial information for the identification of functional mechanisms of adventitious rooting in *Liriodendron* hybrids and facilitate the vivid comprehension of the functional contribution of genes to metabolic pathways.

### SSR mining

SSR data sets may facilitate marker development and can be applied to molecular breeding, linkage map construction, evolutionary, phylogenetic, and population genetic studies in *Liriodendron* hybrids (Kantety et al., 2002). In the present study, SSRs longer than 18 bp were identified in 2,921 ESTs and 181 potential SSRs were identified in the EST data set of *Liriodendron* hybrids, with 166 (5.7%) ESTs containing at least one of the considered (di-, tri-, tetra-, penta-, and hexa-) SSR motifs. This is consistent with the frequency of SSR-containing ESTs found in *L. tulipifera* (5.6%; Xu et al., 2010), but is higher than the frequency reported in rice (4.7%), wheat (3.2%), and maize (1.5%; Kantety et al., 2002). This result suggests that there are abundant SSRs in the genome of hybrid yellow poplar. On an average, one SSR was found per 16.16 kb among the 2.92 Mb ESTs that were sought. Among the 156 ESTs containing SSRs, 155 ESTs had one SSR and 15 ESTs had two SSRs. The frequency of SSR unit sizes was not evenly distributed (Figure 5): 58.0% were dimers and 33.1% were trimers. Interestingly, there were only five (2.8%) tetramers and two (1.1%) pentamers. Dimer repeats were the most abundant SSRs that were also observed in *L. tulipifera* (Liang et al., 2008; Liang et al., 2011); in contrast, trimers mainly contributed to SSR-containing ESTs in rice, soybean, and *Arabidopsis* (Scott et al., 2000).



**Figure 5.** Frequency of SSRs in the *Liriodendron* hybrids unigene sets.

Among the dimer repeats, GA/TC (56.9%) was the most frequent dinucleotide repeat and GC/GC was the least frequent in the *Liriodendron* hybrid EST dataset. Similar results had been reported in many other plant species including citrus, *Arabidopsis*, barley, wheat, maize, and rice (Li et al., 2012). The rarity of GC repeats in eukaryotic genomes might be a result of the tendency of CpG sequences to be methylated, which could inhibit transcription (Gonzalez-Ibeas et al., 2007). The most frequent repeats among the trimers were AAG/CTT (14.9%), followed by GCA/TGC, and ATC/GAT (11.0 and 2.76%), respectively, and the least frequent was ACA/TGT (Table 3). This is consistent with previous findings that indicated AAG/AGA/GAA was the most dominant trinucleotide repeats in *Arabidopsis* and *Rosa* (Jung et al., 2005).

**Table 3.** Expression frequency of 2055 adventitious root contigs of *Liriodendron* hybrids.

Frequency	No of contigs	No of EST	Percentage (%)
2	1098	2196	50.95
3	29	87	2.02
4	9	36	0.84
5-16	13	109	2.53
24-48	6	231	5.36
50-119	4	318	7.38
130	1	130	3.02
173	1	173	4.01
204	1	204	4.73
250	1	250	5.80
284	1	284	6.59

One hundred and twenty-two ESTs containing 127 SSRs were selected for SSR primer modeling and for primer generation (S3 Table). Among those primers, 115 (90.6%) produced clear bands in *Liriodendron* hybrids, *L. chinense*, and *L. tulipifera*. Sixty one (48.0%) EST-SSR markers were found to be polymorphic in eight *Liriodendron* hybrids, suggesting a high level of cross-species transferability. This is in agreement with the report that the *L. tulipifera* SSRs may be similarly vital in related species of the same family (Xu et al., 2010).

To date, there has been no EST library for *Liriodendron* hybrids. Only a few miRNAs have been reported during somatic embryogenesis. The present research generated and characterized a normalized uncut cDNA library for *Liriodendron* hybrid adventitious roots, identified 5176 ESTs, and then obtained 2921 unigenes (2,055 contigs and 866 singletons, ranging from 59 to 2626 bp). Most of the unigenes (87.2%) had similarity to the publicly available plant protein sequences, and 2921 unigenes were annotated with 3760 annotations distributed among the three categories using the GO scheme, representing a wide variety of putative functions. Many genes might play important roles in the development of adventitious roots in *Liriodendron* hybrids, such as genes related to auxin signal transduction, transcription factors, root meristem maintenance, and root hair development. Furthermore, the spatial expression patterns indicated that *AUX1*, *IRE*, and *FBI* were highly expressed in the adventitious roots, and that the expression of *AUX1*, *ARF1*, *NAC1*, *RHD1*, and *IRE* was induced during the adventitious rooting, suggesting the potential functions of related genes during adventitious rooting of hybrid yellow poplar. In addition, 181 SSRs were identified based on EST sequences and 115 EST-SSR markers produced clear bands in the genus *Liriodendron*, implying that these markers could be applied in genetic mapping and genetic diversity among *Liriodendron*. This study is the first to identify genes associated with adventitious rooting in *Liriodendron* and provides numerous cues for future genomic studies on *Liriodendron* hybrids.

## Conflicts of interest

The authors declare no conflict of interest.

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## Supplementary material

[Table S1.](#) CT values of RT-PCR of housekeeping genes.

[Table S2.](#) Genes associated with adventitious roots development in Liriodendron hybrids.

[Table S3.](#) EST-SSRs sequences identified during SSRs mining.