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ORIGINAL ARTICLE

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Analysis of expressed sequence tags in developing secondary xylem and shoot of *Acacia mangium*

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Abstract *Acacia mangium* is a fast-growing tree widely planted in tropical countries because of its rapid growth, high wood density, high fiber quality, and good adaptability. Despite its importance as a fiber source in the pulp and paper industry, a large-scale analysis of expressed sequence tags (ESTs) has not been performed in *A. mangium*. In this study, we sequenced 10752 clones of a normalized complementary DNA (cDNA) library prepared from *A. mangium* developing secondary xylem and shoot, and obtained a total of 8963 ESTs. The ESTs were assembled into 6220 unigenes comprising 1614 contigs and 4606 singletons. The unigene set was then subjected to various bioinformatic analyses. BlastN searches of the unigene set against the Gene Index Databases of soybean, *Medicago truncatula*, *Lotus japonicus*, grape, poplar, spruce, and pine demonstrated that the largest number of unigenes shared homologies with the soybean Gene Indices. BlastX searches against the TAIR9 peptide database enabled us to annotate the unigenes. Based on the annotation, we discussed whether the unigenes involved in the cell cycle, cell growth, shoot apical meristem development, and cell wall biosynthesis were present. This new genomic resource will accelerate the functional genomics of wood formation and molecular breeding to improve the wood properties of *A. mangium*.

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Introduction

Wood is mostly made up of secondary xylem produced in the vascular cambium of tree stems and shoot. Secondary xylem comprises various cells, such as tracheids, vessel elements, fibers, and parenchyma cells. Among these cells, fibers, tracheids, and vessel elements (which occupy most of the secondary xylem in angiosperm trees and are responsible for structural support and water transportation,[1,2]) deposit a thick cell wall, called the secondary wall, composed of laminated cellulose microfibrils covered with hemicelluloses and lignin–hemicellulose matrices.[3]

Wood is a commercially important feedstock for the timber, furniture, engineered wood, and pulp and paper industries. As a renewable and environment-friendly resource, wood has recently attracted much attention as feedstock for biofuel.[4] Furthermore, wood also serves as a major CO_2 sink in the global carbon cycle.[2,4] Sustainable production of high-quality wood is an important objective for human society.

For the purpose of the production of wood and reforestation, fast-growing trees have been planted worldwide. Nevertheless, breeding programs, which mostly target the improvement of wood properties, are still at a primitive stage.[5] This is because forest trees usually take a long time to reach sexual maturity; consequently, conventional tree breeding programs require a very long period of time. In contrast, molecular breeding, such as marker-assisted selection and genetic transformation based on genomic information such as expressed sequence tags (ESTs), could significantly benefit the improvement of wood properties.

Acacia mangium (Fabaceae) is a fast-growing tree that is widely planted in Southeast Asia, including Indonesia, Malaysia, Vietnam, Laos, and Thailand, due to its high growth rate, high wood density, good fiber quality, and

ability to thrive in poor soils and acidic soils in tropical areas.[6–9] In spite of the importance of *A. mangium* as a plantation tree in tropical countries, only one report detailing 1123 ESTs representing 576 unique genes (unigenes) expressed in the flowers has been published for *A. mangium*.[9] Thus, the production and analysis of ESTs from developing secondary xylem and shoot could make a significant contribution to the molecular breeding of *A. mangium* and increase our understanding of the genes involved in wood formation.

Here, we report the construction and analysis of ESTs comprising 8963 sequences obtained by sequencing a normalized cDNA library from developing secondary xylem and shoot of *A. mangium*. The sequences were assembled into a unigene set of 6220 sequences. Each unigene was annotated and compared with the genome and ESTs of other plant species. Based on the annotation, we discuss whether unigenes involved in the cell cycle, cell growth, shoot apical meristem development, and cell wall biosynthesis were present.

Materials and methods

Plant materials and total RNA extraction

A. mangium seed, gifted from Musi Hutan Persada, Sumatra, Indonesia, was germinated in June 2006. The young tree was cultivated in a pot filled with an equivalent mixture of garden soil [Hanasaki Monogatari (Akimoto Tensanbutsu, Iga, Japan)] and vermiculite outside at our campus in Uji, Japan, except between November 2006 and April 2007. During that period, we maintained it in a growth chamber at 25°C with a photoperiod of 16 h light and 8 h dark. A shoot (10 cm from the shoot tip) was collected from a young tree (about 60 cm tall) by excision with scissors in October 2007. The developing secondary xylem was collected from a young tree (about 50 cm tall) in September 2007 by scratching the juicy tissue with a blade after peeling off the bark. The collected samples were immediately frozen and stored in liquid nitrogen until use.

The total RNAs from developing secondary xylem and shoot were independently extracted using the method of Bugos et al.,[10] and then purified by an RNeasy Plant Mini Kit (Qiagen). The RNAs were further purified by repetitive ethanol precipitation. The concentration of total RNAs was estimated by spectrophotometric measurement and the quality of RNAs was checked using a BioAnalyzer (Agilent Technologies).

Construction of a normalized cDNA library

Total RNAs from developing xylem (0.91 μg) and shoot (0.7 μg) were mixed. Normalized cDNAs were prepared using a Trimmer-Direct cDNA Normalization Kit (Evrogen) and a Creator SMART cDNA Construction Kit (Clontech), according to the manufacturers' instructions. The cDNAs ligated into a pDONR-LIB vector were electroporated into *Escherichia coli* DH10B-T1^R (Invitrogen), and the resultant original library was used for DNA sequencing.

DNA sequencing

The original *E. coli* cDNA library was plated onto Luria and Bertani (LB) media containing 20 mg/l chloramphenicol and incubated for 16 h at 37°C. The isolated colonies (10 752 colonies) were picked by sterile toothpicks and inoculated onto 384-well plastic plates containing LB medium. The plates were incubated for 24 h at 37°C. The insert of each plasmid was amplified by colony polymerase chain reaction (PCR) from the 384-well plates using M13-reverse (3′-AACAGCTATGACCATG-5′) and M13-forward (3′-GTAAAACGACGGCCAG-5′) primers. The PCR program was as follows: 94°C for 2 min; 35 cycles of 94°C for 45 s, 52°C for 45 s, and 72°C for 3 min; and 72°C for 10 min. The PCR products were purified by ethanol precipitation and sequenced as previously described using an ABI PRISM 3730xl sequencer (Applied Biosystems).[11]

Bioinformatics

The sequence trace files were submitted to the PHRED program[12] using the parameter –trim_alt 0.01 (QV 20), and sequences longer than 100 nt were selected. The selected sequences were submitted to the CROSS_MATCH[13] program using the parameters –minmatch 12 and –minscore 20.

The putative full-length clones were screened as follows. First, the sequences of all clones derived after CROSS_MATCH[13] were submitted to a BlastX search against the SWISS-PROT database.[14] Next, based on the results, the sequences which met the following conditions were chosen as those from putative full-length clones: (1) the sense query sequences matched the sense subject sequences; (2) the E value was less than 10^{-20}; (3) the alignment length was more than 100 nt; (4) the subject sequences started from an ATG start codon; (5) the 5′ ends of the query sequences started prior to the start codon of the subject sequences.

Sequence assembly was performed using the PHRAP program[13] with the default parameters and resulted in clustered sequences (contigs) and unclustered sequences (singletons). The unigene set comprising the contigs and singletons was submitted to BlastN and BlastX searches. BlastN searches were performed against the following versions of Gene Index Databases:[15] soybean (*Glycine max*), release 13; *Medicago truncatula*, release 9; *L. japonicus* (*Lotus japonicus*), release 4; grape (*Vitis vinifera*), release 6; poplar (*Populus* spp.), release 4; pine (*Pinus* spp.), release 7; and spruce (*Picea* spp.) release 3. BlastX searches were conducted against the TAIR9 peptide database.[16]

Gene ontology (GO) analysis of the ESTs was carried out as follows. The GO slim term annotation for each *Arabidopsis* gene (ATH_GO_GOSLIM09324.txt) was retrieved from TAIR.[16] Each EST was submitted to a BlastX search against the TAIR9 peptide database.[16] GO slim terms of the best-matched *Arabidopsis* gene were assigned to each EST. Finally, the number of each term was counted.

Results and discussion

Construction and characterization of a normalized cDNA library

In normalized cDNA libraries, the number of cDNAs originating from abundant mRNAs is reduced, and therefore, the chance of sequencing low-copy cDNAs, such as transcription factor genes, is increased. Thus, we constructed a normalized cDNA library prepared from total RNAs from the developing secondary xylem and shoot where wood is actively formed in *A. mangium*. The normalized *E. coli* cDNA library was constructed substantially according to manufacturers' instructions from a mixture of total RNAs. The titer of the original library was 3.25×10^9 cfu/μg. The insert size of 15 independent clones was estimated to be about 1.0 kb based on gel electrophoresis of the PCR products.

Construction and characterization of ESTs

In total, 10752 independent colonies of the cDNA library were isolated and inoculated onto 384-well plates containing culture medium. The inserts were amplified by PCR, and the PCR products were used as templates for sequencing. The resulting sequences were subjected to bioinformatic analysis (Fig. 1). As a result, 8963 sequences (average size: 618 nt) were used as the EST dataset. The ESTs were deposited in the DNA Data Bank of Japan (DDBJ) nucleotide database[17] (DDBJ accession numbers: FS583802 to FS592764).

We then parsed the 8963 sequences by submitting the sequences to BlastX searches against the SWISS-PROT[14] and NCBI nr[18] databases. The normalization process appeared to have been successfully performed, because only 38 chlorophyll a/b-binding protein family genes, which are known to be highly expressed in chlorophyll-containing tissues,[19,20] were found in the EST dataset, and a transcription factor gene, which is generally expressed at low levels in plants,[21] ranked in the top 13 genes represented in the EST dataset (data not shown). We also found 2768 putative full-length orthologs in the EST dataset by parsing the SWISS-PROT BlastX results.

To predict which biological processes the ESTs are involved in, GO analysis was performed on the EST dataset. A total of 12929 GO slim terms belonging to the Biological Process set were assigned to the ESTs. The top three major GO slim terms were "other cellular processes" (23%), "other metabolic processes" (21%), and "unknown biological processes" (10%) (Fig. 2A). These three are also the top three major GO slim terms in *Arabidopsis*, but the proportion of "unknown biological processes" was larger in

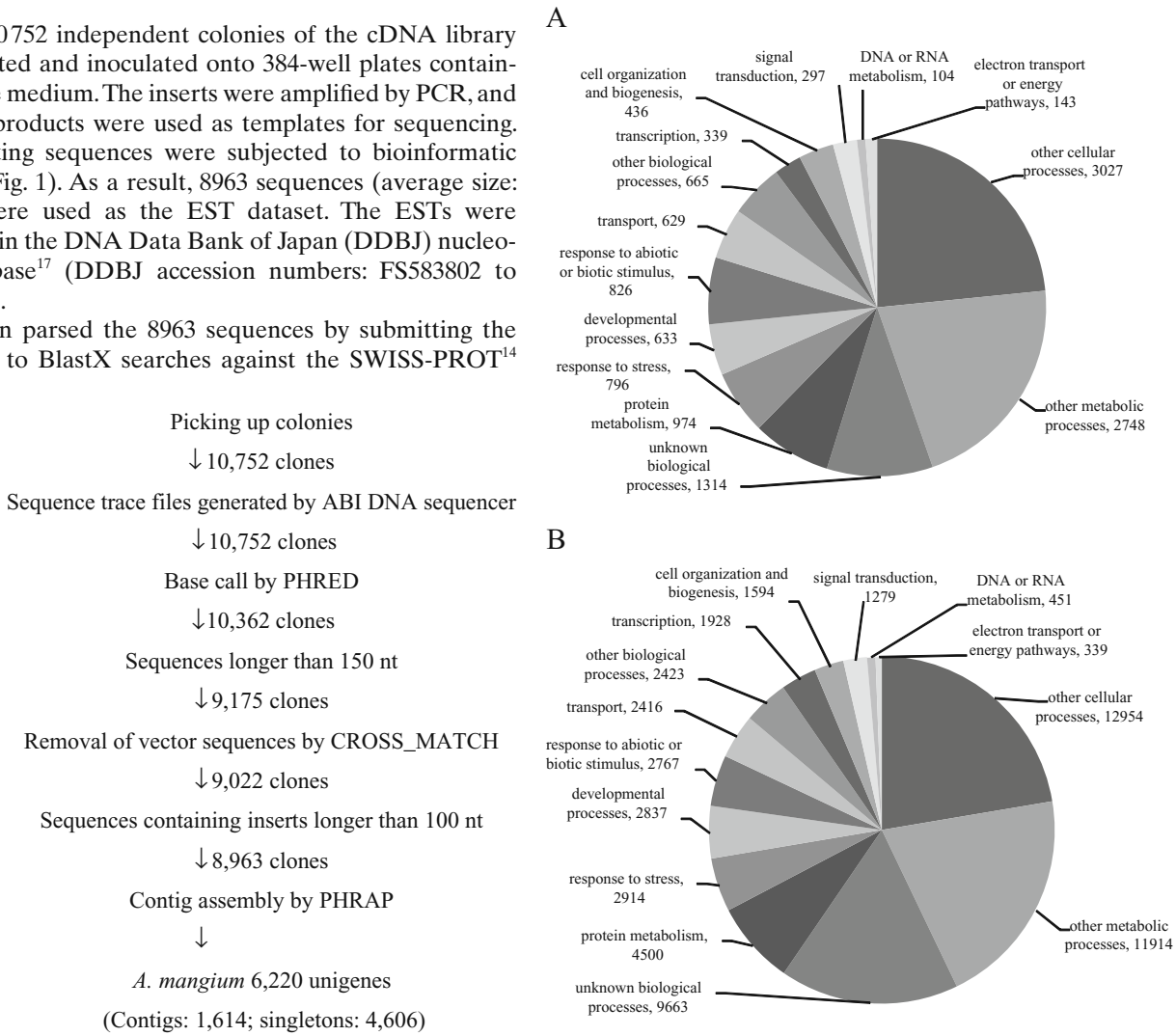

Fig. 1. Scheme for the construction of expressed sequence tags (ESTs) and unigenes

Fig. 2. The distribution of gene ontology slim term assignments for **A** the *Acacia mangium* orthologs to *Arabidopsis* genes and **B** the total genes of *Arabidopsis*

Arabidopsis (Fig. 2B). Overall, the tendency of GO slim term assignment is similar between the ESTs and *Arabidopsis*, suggesting that the EST dataset represents genes required for the biological processes involved in the developing secondary xylem and shoot of *A. mangium.*

A. mangium unigenes

The 8963 sequences in the ESTs were assembled using PHRAP[13] into 6220 unigenes comprising 1614 contigs and 4606 singletons. The average length of the unigenes was 654 nt. BlastX searches of the unigenes against the TAIR9 peptide database[16] showed that 19% of the unigene set have E values larger than 10^{-10}, implying that these unigenes were not orthologous to *Arabidopsis* genes.

Number of orthologs in the unigene set by plant species

Unlike *A. mangium*, leguminous model plants and crops including *L. japonicus*, *M. truncatula*, and soybean are all herbaceous plants. Thus, the unigene set expressed in the tissue where wood is actively formed might be more closely related to the genome of model forest trees, such as poplar. To evaluate this possibility, we submitted the unigene set to BlastN searches against the Gene Index Databases of leguminous plant species and tree species.[15] As a result, approximately 70% of the unigenes are considered to be orthologs ($E < 10^{-10}$) of soybean genes, and the number of such unigenes was the largest among the species tested (Fig. 3). Interestingly, this result suggests that the transcriptome of wood-forming tissue of *A. mangium* is most similar to that of herbaceous soybean among the above plant species, and that the genomic information of soybean[22] might be helpful for the functional annotation of the unigenes.

Analysis of unigenes

The current unigene set was generated from ESTs from shoot and developing xylem of *A. mangium*. Therefore, it was expected that the genes involved in cell division and growth, shoot apical meristem development, and cell wall biosynthesis would be present. To confirm this possibility, we submitted the unigene set to BlastX searches against the TAIR9 peptide database,[16] assigned the best-matching *Arabidopsis* gene to each unigene if the E value was less than 10^{-10}, and narrowed down the set of putative unigenes involved in the cell cycle, cell growth, shoot apical meristem development, and cell wall biosynthesis.

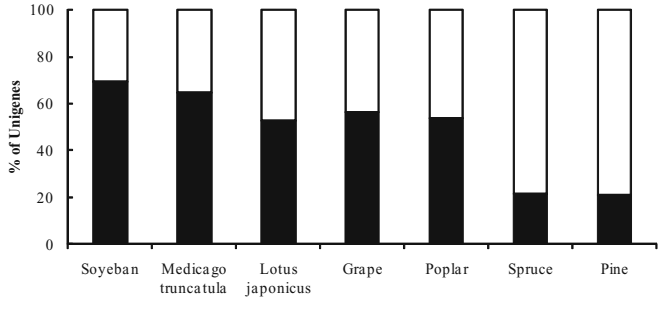

Fig. 3. The number of orthologs in the unigene set by plant species

Unigenes involved in the cell cycle

The cell cycle controls cell division and growth. The molecular mechanism of the cell cycle is highly conserved among higher organisms such as plants and animals.[23] The progress of the cell cycle is regulated by the activity change of cyclin-dependent kinase complex. The complex is composed of cyclins and cyclin-dependent kinases (CDK), which are activated by phosphorylation by a CDK-activating kinase (CAK) and inactivated by retinoblastoma protein (RB). The activity is also inhibited by the phosphorylation of cyclin-dependent kinase inhibitor (CKI).[24]

In the unigene set, two unigenes orthologous to *Arabidopsis cdc2a*, which encodes a CDK, were found. Eight unigenes were orthologous to *Arabidopsis* cyclin family genes[24] including C-, D-, T-, and U-type cyclins. Moreover, two unigenes orthologous to *Arabidopsis* CKI (ICK6) and a unigene orthologous to *Arabidopsis* CAK (CAK1) were found. These results suggest that the basic cell cycle machinery of eukaryotes appears to be present in *A. mangium*.

Unigenes involved in cell growth

After plant cells divide, cell growth, including cell enlargement or cell elongation, occurs. In this step, cell wall loosening is the direct key factor. Various enzymes and proteins are involved in cell wall loosening. Among them, xyloglucan *endo*-transglycosylase/hydrolase (XTH) and expansin are well-known proteins which loosen the cell wall.[25]

In *Arabidopsis*, *XTH* forms a gene family comprising 33 genes.[26] In our analysis, eleven different *A. mangium* unigenes were found to be similar to *Arabidopsis XTH*. Two unigenes shared homologies with *XTH9*, and the nine other unigenes were most similar to *XTH4*, *XTH5*, *XTH7*, *XTH10*, *XTH16*, *XTH23*, *XTH25*, *XTH28*, and *XTH32*. Since most *XTH* genes are distinctly expressed in terms of tissue specificity and response to hormones in *Arabidopsis*,[26] *A. mangium XTH* may also show tissue-specific expression.

The expansin superfamily is made up of four families: α-expansin, β-expansin, expansin-like A, and expansin-like B. α-Expansin and some β-expansin proteins are known to exhibit cell wall loosening activity.[27] Eight *A. mangium* unigenes were homologous to *Arabidopsis EXPA6*, *EXPA8*, *EXPA10*, which belong to the α-expansin family. Only one unigene was homologous to *EXPB3*, a member of β-expansin.

Unigenes involved in shoot apical meristem development

The aerial part of higher plants, such as leaves, stems, and flowers, is ultimately generated from shoot apical meristem.

To date, several genes responsible for the normal function of meristem have been identified. The genes are classified into three groups.[28] The first group is responsible for stem cell activity in the central zone and includes genes such as *STM*, *KNAT1*, and *WUS* in *Arabidopsis*. The second group is responsible for differentiation at primordia and includes genes such as *CLV1* and *CLV3*. The third group controls the local cell proliferation at the developing organ and contains genes such as snapdragon *PHAN*.

In the *A. mangium* unigene set, two putative *KNAT1* and *STM* orthologs were identified. These are possibly responsible for the maintenance of stem cell activity in shoot apical meristem of *A. mangium*.

Unigenes involved in cell wall biosynthesis

Biosyntheses of secondary cell wall components such as cellulose, hemicelluloses, and lignin are the important metabolic events in wood formation, and they are coordinately regulated at the transcriptional level.[2,29] Thus, identifying genes putatively involved in the biosynthesis of cellulose, hemicelluloses, and lignin, and the related transcription factors, is the first step in studying the molecular mechanism of wood formation.

Lignin biosynthesis genes

Lignin is generated by the oxidative coupling of hydroxycinnamyl alcohols (monolignols) by laccases and peroxidases.[30,31] Monolignols are biosynthesized via the cinnamate/monolignol pathway. To date, all gene families involved in the pathway have been identified in *Arabidopsis*[32–34] and several laccases and peroxidases involved in lignin biosynthesis have been reported.[35,36]

Twenty-four *A. mangium* unigenes were orthologous to the *Arabidopsis* genes involved in the cinnamate/monolignol pathway reported by Raes et al.[32] (Table 1). Only the gene encoding coumarate 3-hydroxylase (C3H)[37,38] was not found.

The *A. mangium* unigene set contained each one of orthologs of laccase genes *LAC4/IRX12* and *LAC15/TT10*,[35,36] and two and three genes orthologous to peroxidase genes *AtPER12* and *AtPER64*, respectively (Table 1).[39] Because it has been suggested that these *Arabidopsis*

Table 1. The number of unigenes orthologous to *Arabidopsis* genes involved in cell wall biosynthesis

Function	*Arabidopsis* gene name	AGI code	Number of unigenes
Lignin biosynthesis	*PAL1*	At2g37040	3
	C4H/CYP73A5	At2g30490	5
	4CL-like8	At5g63380	1
	HCT	At5g48930	1
	CCoAOMT1	At4g26220	1
	CCoAOMT7	At4g34050	3
	CCR1/IRX4	At1g15950	1
	CCR-like3	At2g33590	2
	CCR-like5	At5g58490	1
	F5H1/CYP84A1	At4g36220	1
	COMT	At5g54160	3
	CAD2	At3g19450	1
	CAD4	At4g37980	1
	LAC4/IRX12	At2g38080	1
	LAC15/TT10	At5g48100	1
	PER12	At1g71695	2
	PER64	At5g42180	3
Cellulose biosynthesis	*CesA1/RSW1*	At4g32410	1
	CesA4 /IRX5	At5g44030	2
	CesA7/IRX3	At5g17420	1
	CesA8/IRX1	At4g18780	3
	SUS1	At5g20830	2
	SUS3	At4g02280	1
	SUS4	At3g43190	1
	KOR1	At5g49720	1
Xylan biosynthesis	*IRX8/GAUT12*	At5g54690	2
	IRX9	At2g37090	1
	IRX10-L/GUT1	At5g61840	1
	IRX14	At4g36890	1
NAC transcription factors	*SND3/ANAC010*	At1g28470	2
	NST1/ANAC043	At2g46770	2
	XND1/ANAC104	At5g64530	1
MYB transcription factors	*MYB52*	At1g17950	2
	MYB58	At1g16490	1
	MYB61	At1g09540	1
	MYB85	At4g22680	1

AGI, Arabidopsis Genome Initiative

genes are involved in lignin biosynthesis, these *A. mangium* orthologs might be involved in lignification.

Cellulose and xylan biosynthesis genes

The cellulose synthase catalytic subunit (CesA) is believed to transfer a glucosyl moiety to an elongating glucan chain in the rosette complex on the plasma membrane.[40,41] In the *Arabidopsis* genome, *CesA* forms a multigene family, and ten genes were annotated as *CesA*. At least three different CesA proteins are required to form a functional rosette complex. In *Arabidopsis*, CesA1 (RSW1), CesA3 (IXR1), and CesA6 (IXR2) are required for cellulose biosynthesis in primary wall formation, and CesA4 (IRX5), CesA7 (IRX3), and CesA8 (IRX1) are required for cellulose biosynthesis in secondary wall formation.[41] Of the *A. mangium* unigenes, seven unigenes were annotated as *CesA* (Table 1). These are homologous to *CesA1*, *CesA4*, *CesA7*, or *CesA8* in *Arabidopsis*.

Apart from CesA proteins, cellulose biosynthesis requires the participation of other proteins. The *KORRIGAN1* (*KOR1*) gene, which encodes a putative membrane-localized β-1,4-glucanase,[41] is required for cellulose biosynthesis. Sucrose synthases (SUS) are also required for the production of UDP-glucose, a substrate for cellulose biosynthesis.[42] The *A. mangium* unigene set contains one *KOR1* and four *SUS* orthologs of *Arabidopsis* (Table 1).

Glucuronoxylan is a major hemicellulose (14% wood weight) in *A. mangium*.[43] The genes involved in the biosynthesis of glucuronoxylan have been identified in *Arabidopsis*, and yet none of them has been biochemically characterized.[44] Thus far, *FRA8/IRX7*, *IRX8*, *IRX9*, *IRX10*, *IRX10-like*, *IRX14*, and *PARVUS* have been identified as genes directly involved in xylan biosynthesis.[44–46] Of the *A. mangium* unigenes, five unigenes were annotated as *IRX8*, *IRX9*, *IRX10-like*, and *IRX14* (Table 1).

Transcription factors

Recent studies have revealed that transcription regulation is a key step in wood formation.[2,29] BlastX searches against the Database of *Arabidopsis* Transcription Factor[47] classified 302 of the *A. mangium* unigenes as genes that encode transcription factors from 51 gene families (data not shown). Among them, MYB and NAC transcription factors are known to play pivotal roles in transcriptional regulation in wood formation in *Arabidopsis*.[2,29] In the *A. mangium* unigene set, ten orthologs of NAC and MYB transcription factors involved in wood formation in *Arabidopsis* were identified (Table 1). Two unigenes are orthologous to *NST1*, a NAC transcription factor gene that causes ectopic secondary wall formation,[48,49] and two unigenes are orthologous to SND3, which causes secondary wall thickening in fibers when overexpressed.[50] An ortholog of *XND1*, a negative regulator of secondary wall thickening formation,[51] was also found. As for MYB transcription factors, five unigenes were found as putative orthologs of *MYB52*, *MYB58*, *MYB61*, and *MYB85*, which induce secondary wall biosynthetic genes in *Arabidopsis*.[50,52,53]

Conclusions

We isolated 8963 ESTs prepared from a normalized cDNA library of developing secondary xylem and shoot of *A. mangium*. The ESTs were assembled into 6220 unigenes comprising 1614 contigs and 4606 singletons; this is the first comprehensive catalogue of the genes involved in wood formation and should enable us to design a microarray for gene expression analysis in *A. mangium*. Approximately 70% of the unigenes show homology with soybean Gene Indices, suggesting that the genomic information from soybean is also helpful for the functional annotation of the unigenes. Finally, unigenes putatively involved in the cell cycle, cell growth, shoot apical meristem development, and cell wall biosynthesis were predicted. These results will accelerate the functional genomics of wood formation and molecular breeding to improve the wood properties of *A. mangium*.

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