Molecular cloning and characterization of two differentially expressed cellulose synthase gene isoforms in *Leucaena leucocephala*: A pulp yielding tree species

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ABSTRACT

Leucaena leucocephala is fast growing leguminous tree species, acclimatized to variety of soil and climatic conditions. It is widely used for pulp production in India. Pulp mainly consists of cellulose, which is a simple polymer of unbranched β-1, 4-linked glucan chains. The polymerization of glucose residues into a β-1, 4-linked backbone is catalysed by the enzyme cellulose synthase (*CesA*). Here, cDNAs encoding *CesA* genes from *Leucaena* were isolated and characterized. The two complete cDNAs of 3.228 kb and 3.222 kb encoding *CesA* gene from *L. leucocephala* were designated as *Ll-7CesA* (FJ871987) and *Ll-8CesA* (GQ267555) respectively. *In-silico* studies showed that *Ll-7CesA* has 95.2% identities and *Ll-8CesA* has 95.8% identities with *Acacia mangium CesA*2. Phylogenetic analysis revealed significant similarity with known dicot *CesA* genes. The deduced amino acid sequence of both *CesA* genes contained the conserved D, D, D, QxxRW motif, eight membrane spanning regions and a putative zinc binding domain, which are characteristic of glycosyltransferases. DNA blot analysis suggested, *CesA* gene to be in multiple copies in *Leucaena* genome. Semi quantitative and quantitative real-time PCR expression analysis of *Ll-7CesA* gene showed more expression in stem than leaf and not detected in root where as *Ll-8CesA* gene was expressed more in stem than leaf and root. Overall *Ll-8CesA* was expressed in all tested tissues and could be involved in active cellulose biosynthesis.

Keywords: Cellulose Biosynthesis; Cellulose Synthase; Expression Analysis; *Leucaena leucocephala*; Tree Species

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1. INTRODUCTION

Trees are reservoirs of many economically and biotechnologically significant products. Wood is one such gift of nature, which has diverse applications for mankind. One of the most well known applications of wood is paper and paper products. World paper production was around 330×10^6 tons per annum in the year 2003 and is increasing on yearly basis to a great extent. In India paper and pulp industry uses Bamboo, *Eucalyptus* and *Leucaena* as a major source of pulp. *Leucaena* is fast growing, multipurpose, tropical tree species, acclimatized to variety of soil and climatic conditions and is extensively used in India as a source of pulp [1]. The genus is native of Central America and has a wide range of usage from forage for domestic animals to fuel wood to timber and pulpwood [2].

The major constituents of wood are cellulose, hemicelluloses, and lignin. Pulp is obtained after removal of lignin and hemicelluloses from wood. Pulp consists of cellulose, which contains apparently simple linear chains of β-1, 4-linked glucan residues, but these chains aggregate to form immensely strong microfibrils. The polymerization of glucose residues into a β-1, 4-linked backbone is catalyzed by the enzyme cellulose synthase (*CesA*), which utilizes UDP-glucose as the substrate [3]. Despite the importance of cellulose, only recently the studies have started to unravel details of its synthesis [4]. Genes encoding plant cellulose synthases were first identified in cellulose-enriched cotton fibres [5]. With the advent of molecular approaches coupled with genome sequence information, 10 *CesA* genes have been identified in *Arabidopsis* [6].

The *CesA* gene has also been isolated from various plant species such as maize [7], rice [8], barley [9], *Eucalyptus* [10] and aspen [11,12]. Based on mRNA localization, in situ localization and qRT-PCR expression studies *CesAs* have been classified as primary or secon-

dary wall-related *CesAs* [10,13,14]. The primary aim of above studies was to characterize the *CesA* isoform, responsible for cell wall development.

To obtain better quality cellulose, in terms of increased degree of polymerization and crystallinity, with special reference to wood production, it is essential to identify the *CesA* which is directly involved in cellulose biosynthesis. *CesA* genes from few tree species have been isolated and characterized [11,12]. Our knowledge about *CesA* isoforms and its activity in cellulose biosynthesis in tree species is very limited till date. With an aim to meet the increasing demand of high quality wood for paper industry, it was crucial to identify the *CesA* isoform involved in cellulose biosynthesis from *L. leucocephala*. Here we report two different cDNA clones for *CesA* gene from *L. leucocephala*. Expression pattern of these two genes was also studied which suggests that *Ll*-8*CesA* might be more active form of *CesA* than *Ll*-7*CesA*, expressed in all tested tissues.

2. MATERIALS AND METHODS

2.1. Plant Material

Approximately three months old cultured *Leucaena* plantlets were used for the analysis. To obtain cultured *Leucaena* plantlet, seeds of *L. leucocephala* were treated according to the protocol described by Shaik *et al*. (2009) [15].

2.2. RNA Isolation and cDNA Synthesis

Total RNA was isolated from *L. leucocephala* tissue samples (Three months old germinated seeds) according to the TRIZOL (Sigma) method. First-strand cDNA synthesis, primed with an oligo $(dT)_{15}$ primer, was performed with avian myeloblastosis virus reverse transcriptase (AMV RT) according to the manufacturer's protocol (Promega Corp., Madison, USA). One microgram of total RNA was used to prepare cDNA from stem, leaf and root tissue.

2.3. Amplification of Internal Region and RACE PCR for *CesA* Gene

A PCR based approach was followed to isolate the internal regions of *CesA* gene. Known *CesA* sequences at GenBank database were aligned using Clustal X software and primers were designed from the conserved regions. Four primer sets were designed, CesAF1, CesAF2, CesAR1, CesAR2 (**Table 1**). These primers were used to amplify internal regions of *CesA* gene. BLAST result of these amplicons showed a significant similarity to *Populus*, *Betula* and *Acacia CesA* genes. *CesA* genes from these three species were aligned and two different degenerate primer sets CesFullF1 and CesA3kR (**Table 1**) were designed. The primers were designed from the start codon and near to stop codon (3'end) of *CesA* gene. The expected size fragment was sub cloned and sequenced.

Table 1. List of primers.

S. No.	Primers Name	Primer sequence (5'→3')
1	*CesA*F1	GCA CCT GTT GAC ATA TTT GTC AGT AC
2	*CesA*F2	GAG CAA TGA AGA GAG AAT ATG AAG A
3	*CesA*R1	ACT CGA ACC ATT GCA TTC ATG GC
4	*CesA*R2	CAT ACA CTG GGC CTT GAA TTC CAT C
5	*CesA*FullF1	ATGGAGTCAGAAGGGGARRCYGKGGSRAAG
6	*CesA*3kR	CGGTTCTGGCGRCCCATSARACCT
7	Race *Ces* F	CCAAGGCGTCCGACGAAGACGGAGA
8	Race *Ces* NF	GGTGGGTGTTGTCGCTGGGATCTCC
9	*CesA*Full F2	ATGGAGTCAGAAGGGGAAGCTG
10	*CesA*Full R	CTAGCAGTTTATTCCACACTGCTC
11	Q*Ces*7 F1 (*Ll*-7*CesA*)	CTC TTC ATT TCC ATC TTC GCT ACG
12	Q*Ces*7 R1 (*Ll*-7*CesA*)	CAA AGT CTC CGT CTT CGT CG
13	Q*Ces*8 F1 (*Ll*-8*CesA*)	TTC ATT TCC ATC TTC GCG ACC
14	Q*Ces*8 R1(*Ll*-8*CesA*)	GCA GAG TCT CCA TCT TCA TCA
15	CT F(5.8S rRNA)	CTA AACGACTCTCGGCAAC
16	CT R(5.8S rRNA)	TTCAAAGACTCGATGGTTCAC

RACE PCR was attempted to isolate the full length *CesA* gene. Gene racer RACE kit (Invitrogen) was used according to the manufacturer's instruction. The information obtained from the amplification of internal regions of *CesA* gene was used to perform the RACE PCR reaction. In brief, total RNA was treated according to manufacturer's instruction and cDNA was prepared for 3' RACE reaction. 3' RACE PCR fragment was obtained after two step PCR reaction using RaceCesF and RaceCesNF primers (**Table 1**). The amplified fragment was purified and cloned in pGEM-T Easy cloning vector (Promega, USA). Plasmids were isolated from 10 clones and sequenced.

Sequence analysis of 3' RACE PCR clones, allowed us to design primers for full length *CesA* gene. The following primers were designed: *CesA*FullF2, *CesA*FullR (**Table 1**). PCR amplification was performed using above primers and *Leucaena* cDNA as a template. HiFi taq DNA polymerase (Stratagene, USA) was used for the PCR reaction. The amplified fragment was cloned in pGEM-T Easy cloning vector (Promega, USA) and sequenced.

2.4. Phylogenetic Analysis

CesA deduced amino acid sequences from GenBank database were used to construct a phylogenetic tree. The nucleotide sequences were conceptually translated into amino acid sequence (www.expasy.ch) and evolutionary history was inferred using the Neighbor-Joining method [16]. *CesA* sequence from prokaryotic system *Cronobacter sakazakii CesA* (Crsa*Ces*) and *Mesotaenium caldariorum* (Meca*CesA*) were chosen as an out-group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches [17]. The evolutionary distances were computed using the Poisson correction method [18] and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA4 [19].

2.5. *In-Silico* Analysis of Two *CesA* Sequences

Ll-CesA gene sequences were characterized with the Genscan software and homology was verified by database searching at the National Center for Biotechnology Information server using BLAST algorithm (http://www.ncbi.nlm.nih.gov). The deduction of the amino acid sequences, calculation of the theoretical molecular mass and pI, was performed with ExPASy Proteomic tools provided at http://www.expasy.ch/tools/. Global alignment of two nucleotide or amino acid sequences and percentages of identity were calculated using the EM-BOSS Pairwise Alignment Algorithms (http://www.ebi.ac.uk/emboss/). Multiple alignments of the amino acid sequences were carried out with the Clustal W1.8 program (http://www.ebi.ac.uk/clustalw/). Conserved domains in amino acid sequence were defined by searching the Pfam protein families database (http://pfam.janelia.org/) and with RPS-BLAST (Search the conserved domain database) on NCBI server. Transmembrane regions were identified using following programme TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.6. Semi Quantitative and Quantitative (Real-Time) PCR

Total RNA was extracted individually from shoot, leaf and root of three month old cultured *L. leucocephala* on MS media. Total RNA (1 μg) was used for making cDNA. Brilliant SYBRGreen QPCR kit (Stratagene, USA) and Stratagene Mx3000P Real-Time PCR machine were used for all reactions. The primer sequences that were designed for *Ll-CesAs* and 5.8S rRNA is given in **Table 1**. Semi quantitative PCR conditions are as follows: 5 min at 94°C, followed by 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C (25 cycles for 5.8S rRNA and for *Ll-CesAs* 30, 31 and 32 cycles). Last step was for 5min at 72°C. qRT-PCR reactions were performed under the following conditions: 2 min at 50°C, followed by 10 min at 94°C, 40 cycles of 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C. Prior to start of qRT-PCR and semi quantitative PCR reaction, equal quantity of cDNA was normalised for each reaction. Optimal numbers of PCR cycles for visible amplification for each gene were determined in preliminary experiments. Fold of expression was calculated by ΔΔCt method ($2^{-\Delta\Delta Ct}$) [20,21].

2.7. Genomic DNA Isolation, Restriction Enzyme Digestion and DNA Blotting

Good quality genomic DNA was isolated using modified protocol as described by Lodhi *et al.* (1994) [22]. Four different restriction enzymes namely *EcoR*I, *BamH*I, *Dra*I and *Xho*I were used for identifying as the multigene family of *CesA* gene. Standard protocol as described by Sambrook *et al.* (1989) [23], was followed for the experiment. In brief, Digested DNA was electrophoretically separated on 0.8% agarose gel and transfered to a Nylon Hybond-N+ membrane (Amersham, USA). Membrane was prehybridized for 8 h at 62°C in hybridization buffer (1% BSA; 0.5 M $Na_2HPO_4 \cdot 2H_2O$, pH-7.2; 7% SDS and 1 mM EDTA, pH-8.0) (Sambrook *et al.*, 1989). Hybridization was performed at 62°C for 18 h in same buffer containing radiolabeled probe (ά-P^{32}). Common region of ~800 bp fragment was used to prepare radiolabeled probe using Amersham's random labelling kit. The blot was washed with moderate wash buffer (2X

SSC and 0.1% SDS) before exposing to Storage Phosphor Screen (GE, Healthcare) for 4 h. The Phosphor Screen was scanned at 200 micron resolution in Typhoon Trio+ (GE).

3. RESULTS

3.1. Isolation and Cloning of Two *CesA* Genes from *L. leucocephala*

Amplification of internal regions of *CesA* gene using primer combination *CesA*F1-*CesA*R1 and *CesA*F1-*CesA*R2 gave amplification of approximately 600 and 800 bp respectively (**Table 1**, **Figure 1**). *In silico* study of 800 bp clones showed 88% identity with *Acacia mangium* and *Betuala platyphylla*, and 86% with *Populus trichocarpa* nucleotide sequences. Above three sequences were aligned and degenerate primers were designed from start codon (*CesA*FullF1) and near 3' end (*CesA*3kR) of the *CesA* sequence (**Table 1, Figure 1**). Approximately 3.0 kb amplicon was amplified and cloned in pGEM-T Easy vector and sequenced. Sequence analysis of the clones led us to conclude the possibility of two different *CesA* genes. Now as 5' end was known, 3' RACE was performed to reach the 3' end of the *CesA* coding sequence. Sequencing of 10 3' RACE clones again suggested presence of two *CesA* isoform in *Leucaena* and allowed us to design primers (*CesA*Full F2 and *CesA*Full R) for amplification of full length *CesA* coding region (**Table 1**, **Figure 1**). An approximately 3.2 kb fragment was amplified, cloned and sequenced. Two different types of cDNA sequences were designated as *Ll*-7*CesA* and *Ll*-8*CesA* (GenBank Accession No. FJ871987 and GQ267555) respectively. Identities and similarities between both *Ll-CesAs* were 96.3% and 97.6% respectively.

3.2. Molecular Characterization of *Ll*-7*CesA* and *Ll*-8*CesA* Gene from *L. leucocephala*

Like other plant cellulose synthase both *Ll*7-*CesA* and *Ll*-8*CesA* proteins contains the D, D, D, QxxRW (QVLRW) motifs respectively (**Figure 2(a)**), which is a characteristics of beta-glycosyltransferases [11,24]. The regions surrounding the D, D, D, QxxRW residues shows similarity between other cellulose synthases and is

known as U1, U2, U3 and U4 domains [24,25]. There are eight highly conserved cysteine residues in four pairs of CxxC in N-terminal region of both *Ll-CesA* proteins (CxxC motif underlined) were observed. These residues form the putative LIM like zinc-binding domain [26]. Like other cellulose synthases these two *CesAs* from *Leucaena* also have six transmembrane domains in carboxy terminal region of the protein and two transmembrane domains (T1-T8) in the amino terminal region. The amino terminal region of *Ll-CesAs* protein also contains SPXX motifs (**Figures 2(a)** and **(b)**) that are characteristics of nucleic acid binding proteins [27].

3.3. *Ll-CesA*: A Multigene Family

To investigate the multigene nature of *CesA* genes in *L. leucocephala*, DNA blot analysis was performed using genomic DNA. Good quality genomic DNA was isolated and digested with following restriction enzymes: *Eco*RI, *Dra*I, *Xho*I and *Bam*HI. Out of these restriction enzymes *Bam H*I and *Dra*I cuts inside the *Ll-CesA* gene. Radioactive probe of approximately 800 bp was prepared from a common region of both *Ll-CesAs* cDNA sequence. Multiple bands were seen on blot in all four lanes; which clearly suggest that existence of multiple copies of *CesA* gene (**Figure 3**). The presence of multiple copies of *CesA* in *Leucaena* was in coherence with other known plant species.

3.4. *Ll-CesA* Genes: Its Similarity to *CesAs* Involved in Cellulose Biosynthesis

An unrooted phylogenetic tree was drawn to investigate the evolutionary and similarity index of Ll-*CesAs* with homologous *CesA* sequences by using deduced amino acid sequences. *Ll*-7*CesA* and *Ll*-8*CesA* from *Leucaena* share a 95.8% and 95.2% identity and 97.8% and 96.9% similarity with *Acacia*; 90.8% and 89.7% identity and 95.3% and 94.2% similarity with *Populus* and 89.4% and 88.4% identity and 93.3% and 92.4% similarity with *Eucalyptus* respectively. The tree was divided into four clusters. Cluster I subdivided into two groups. The group I comprises monocotyledonous plant species and group II comprises dicotyledonous plant species. Both Ll-7*CesA* and *Ll*-8*CesA* were grouped with *CesAs* of dicotyledonous
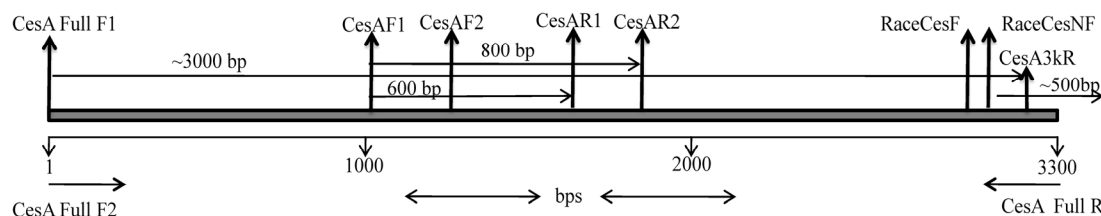

Figure 1. Schematic representation of designing of primers and expected amplicon sizes: Start sites of primers used for isolation of *Ll-CesA* genes.
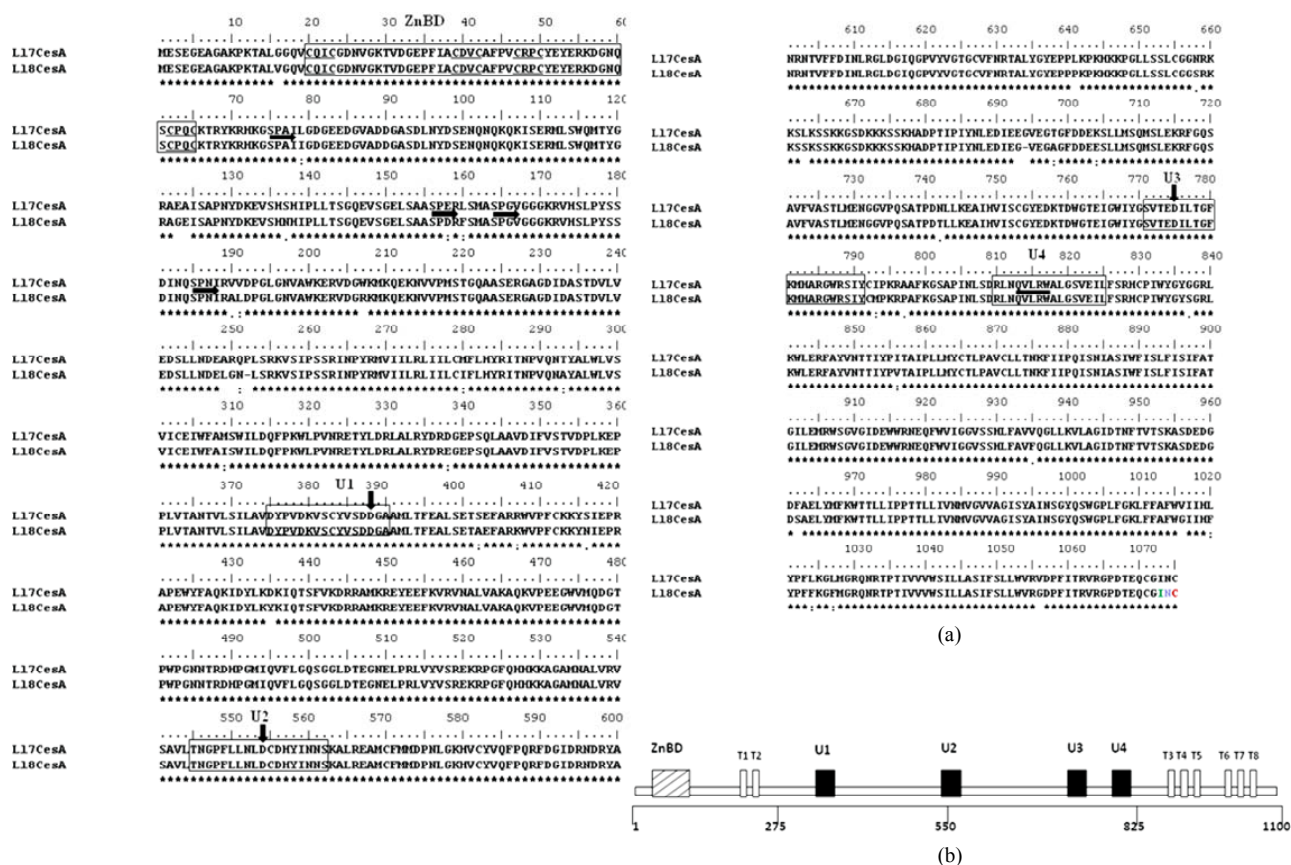
(a)

(b)

Figure 2. (a) Alignment of deduced amino acid sequences of Ll-7*CesA* and Ll-8*CesA*: Alignment was performed by Clustal analysis. Conserved residues are boxed. ZnBD is LIM-like zinc binding domain containing eight highly conserved cysteine residues in four pairs, underlined with thin horizontal lines. SPXX, motifs characteristic of Nucleic Acid Binding Proteins are highlighted with thick horizontal arrows. Ubiquitous (U) sequences U1, U2, U3 and U4 are boxed in thin rectangle. The positions of three aspartic acid (D) and QxxRW motifs are indicated by thick vertical arrows and horizontal line respectively; (b) Schematic representation of protein model and conserved motifs for cellulose synthase (*CesA*): Eight predicted transmembrane regions are indicated as T1-T8. Conserved 'U' motifs originally identified in bacterial cellulose synthase, also present in higher plants cellulose synthase [24].
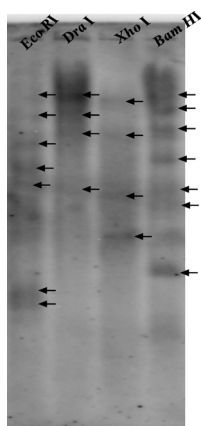

Figure 3. Genomic DNA blot analysis for *Ll-CesA* gene in *L. leucocephala*: *Leucaena* genomic DNA (20 µg) was digested with restriction enzymes *EcoR*I (lane 1), *Dra*I (lane 2), *Xho*I (lane 3) and *BamH*I (lane 4). Blot was hybridized with approximately 800 bp randomly ^{32}P-labeled probe. Dark arrows represents band in the lanes.

species *Eucalyptus*, *Populus* and *Acacia* in group II of cluster I (**Figure 4**).

3.5. Tissue Specific Differential Expression of Two *Ll-CesA* Genes

The expressions of both *Ll-CesAs* were determined using semi quantitative PCR and quantitative real-time PCR (qRT-PCR). The expression patterns of *Ll-CesAs* genes were examined in shoots leaves and roots. Semi quantitative PCR suggested that both the genes are differentially expressed. Equal quantity of cDNA was determined by 5.8S rRNA primers (**Figure 5**, lane A). The expression of *Ll-7CesA* was observed in stem and leaf only. However expression of *Ll-7CesA* in shoots was higher than leaves (**Figure 5**, lane B). In case of *Ll-8CesA*, expression level was observed highest in shoot than leaves and roots (**Figure 5**, lane C). Moreover the expression level of *Ll-8CesA* was comparatively higher than *Ll-7CesA*. qRT-PCR was used for determining fold
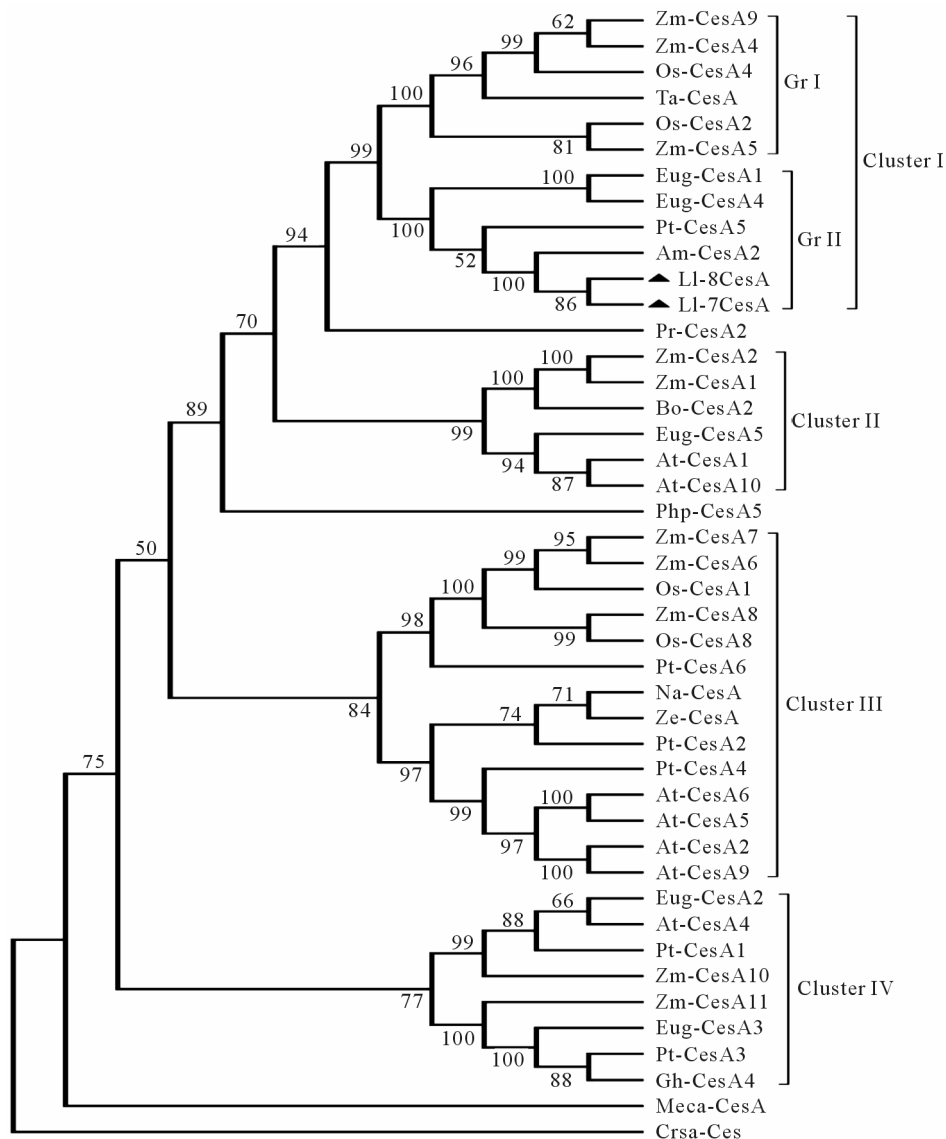
Figure 4. Evolutionary Relationship of *Ll-CesAs* among various *CesA* proteins: Unrooted Neighbor-joining Phylogenetic tree of *Ll-CesAs*: The phylogenetic tree was constructed on the basis of ClutalX2 multiple sequence alignment of deduced amino acids of *Ll-CesAs* with 42 *CesA* protein sequences. For development of tree 500 bootstrap replicates and only branches with 50% or greater support were considered. Species were abbreviated as follows and accession numbers as well as type of *CesAs* are given in brackets. *Arabidopsis thaliana* (At*CesA*1-NP_194967; At*CesA*2-NP_195645; At*CesA*4-NP_199216; At*CesA*5-NP_196549; At*CesA*6-NP_201279; At*CesA*9-NP_179768; At*CesA*10-NP_180124); *Zea mays* (Zm*CesA*1-NP_001104954; Zm*CesA*2-NP_001105574; Zm*CesA*4-NP_001105621; Zm*CesA*5-NP_001104955; Zm*CesA*6-NP_00110-4956; Zm*CesA*7-NP_001104957; Zm*CesA*8-NP_001104958; Zm*CesA*9-NP_001104959; Zm*CesA*10-NP_001105672; Zm*CesA*11-NP_001105236); *Oryza sativa* (Os*CesA*1-NP_001051830; Os*CesA*2-NP_001051648; Os*CesA*4-NP_001059162; Os*CesA*8-NP_001059303); *Populus tremula* (Pt*CesA*1-AAT09894; Pt*CesA*2-AAT09895; Pt*CesA*3-AAT09897; Pt*CesA*4-AAT09898; Pt*CesA*5-AAL-23710; Pt*CesA*6-AAP40636); *Eucalyptus grandis* (Eug*CesA*1-ABY25277; Eug*CesA*2-ABY25278; Eug*CesA*3-ABY25279; Eug*CesA*4-AAY60846; Eug*CesA*5-AAY60847); *Triticum aestivum* (Ta*CesA*-BAD06322); *Mesotaenium caldariorum* (Meca*CesA*-AAT48369); *Nicotiana alata* (Na*CesA*-AAK49454); Ze-*Zinnia elegans* (Ze*CesA*-BAG06272); *Gossypium hirsutum* (Gh*CesA*4-AAL37718); *Leucaena leucocephala* (*Ll-7CesA*-ACU87559; *Ll-8CesA*-ACU80553); *Acacia mangium* (Am*CesA*2-AAT66941); *Pinus radiata* (Pr*CesA*2-AAQ63936); *Bambusa oldhamii* (Bo*CesA*2-AAY43218); *Physcomitrella patens* (Php*CesA*5-ABI78958); *Cronobacter sakazakii* (Crsa-*Ces*-YP_001440220).
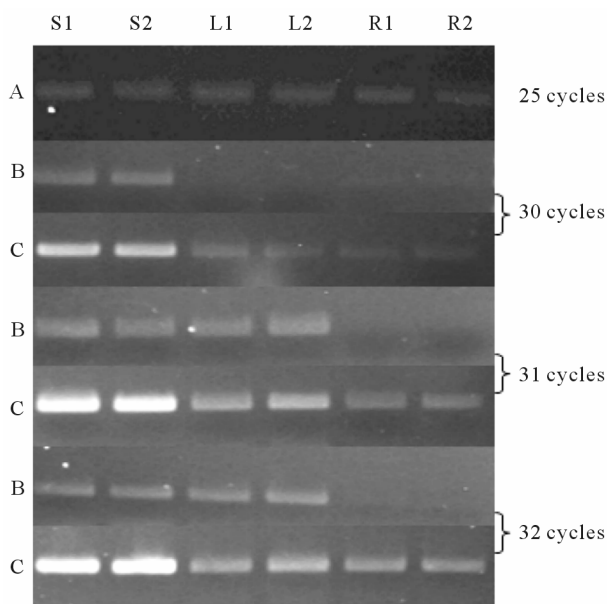
Figure 5. Semi quantitative PCR analysis: PCR was done at three different cycle numbers (30, 31 and 32 cycles) with cDNA from shoot (In duplicate, S1 and S2), Leaf (L1 and L2) and Root (R1 and R2); observed on 2% agarose gel. Lane B and Lane C: *Ll*-7*CesA* and *Ll*-8*CesA* expression respectively. Lane A: 5.8S rRNA (25 Cycles).

expression of both the *Ll-CesAs* genes in different tissues. No Ct value was recorded for *Ll*-7*CesA* in case of root. When compared to leaves, shoots have 5.35 fold more expression than leaves (**Figure 6(a)**). However In case of *Ll*-8*CesA*, least expression was recorded in roots. The expression was 15.03 fold higher in shoots and 1.51 fold more in leaves when compare to roots (**Figure 6(a)**). Moreover when expression of individual genes were compared in individual tissues then it was found that *Ll*-8*CesA* was 10 fold higher in case of shoot and 5.3 fold higher in case of leave tissue when compared to *Ll*-7*CesA* expression (**Figure 6(b)**).

4. DISCUSSION

It has been established in *Arabidopsis*, *Zea mays* and *Oryza sativa* that *CesA* is a multigene family [6,8,14,28]. Reports have also described that these genes perform individual function such as development of primary and secondary cell wall. On the contrary, presence of more than two sequences in the genome of *Eucalyptus*, *Populus*, *Arabidopsis*, maize and barley for *CesA* suggests that *CesA* genes might be involved in performing redundant functions inside the cell [7,26,29-31]. Compared to primary walls, secondary walls contain higher amounts of cellulose with a higher degree of polymerization and crystallinity [25,32]. Therefore, characterization of *CesA* gene, primarily responsible for development of secondary cell wall is of paramount importance in gener-
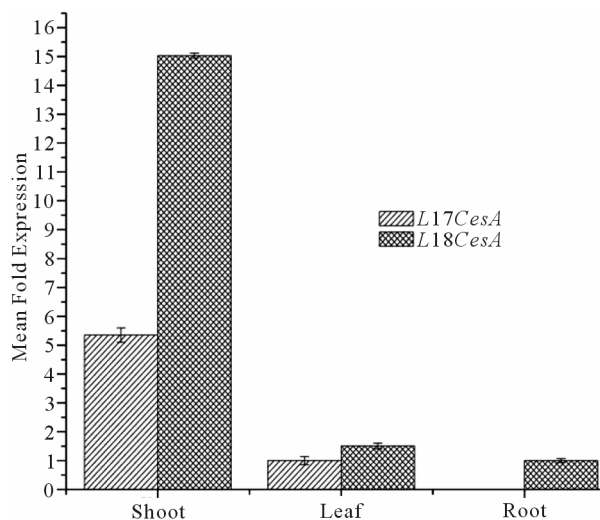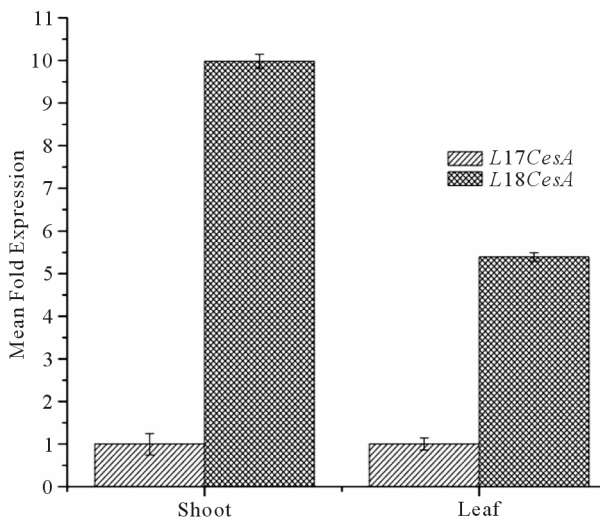
Figure 6. (a) Tissue specific normalized expression of *Ll*-7*CesA* and *Ll*-8*CesA* using qRT-PCR: Light grey shaded histogram represents mean fold expression of *Ll*-7*CesA* and dark grey histogram represents mean fold expression of *Ll*-8*CesA*; (b) Comparison of mean fold expression level of *Ll*-7*CesA* and *Ll*-8*CesA* transcripts in individual tissues: Light grey histogram: *Ll*-7*CesA*, Dark grey histogram: *Ll*-7*CesA*. Error bars are showing standard deviations (SD) for triplicate analysis.

ating elite tree species for paper industry.

With an aim to characterize *CesA* gene responsible for development of secondary wall, two full length *CesA* genes of approximately 3.2 kb were isolated from *L. leucocephala*. They possess 96.3% identity and 97.6% similarity with each other. Size of the cDNA was comparable to known sequence of *CesA* from other plant species. Primary *in-silico* analysis showed that both the cDNA sequences are similar to other known homologous *CesA* sequences. DNA binding, transmembrane, D, D, D,

QxxRW, SPXX, CxxC motifs were identified in both the cDNA sequences. All these data classifies them to be member of family glycosyltransferase (**Figure 2**). Phylogenetic analysis also revealed that both the cDNAs share a significant similarity with *CesA* sequence from other dicot tree species. *Ll-7CesA* and *Ll-8CesA* were grouped with *Eucalyptus* and *Populus* which have dual nature and are involved in primary as well as secondary cell wall biosynthesis which suggest that these sequences might be involved in such functions in *L. leucocephala* (**Figure 4**).

Multigene nature of *CesAs* has been well established in various research articles, Our DNA blot results also suggest that *CesAs* are present as a multigene family in *Leucaena*. Presence of multiple bands in the blot suggests that the *CesA* is present in multiple copies in *Leucaena* genome (**Figure 3**). Similar results have also been cited in model plants such as *Arabidopsis*, rice and poplar [8,33-38].

Now the question was where these transcripts are being expressed. We performed a semi quantitative PCR paralleled with qRT-PCR to investigate the presence of *Ll-7CesA* and *Ll-8CesA* transcript in various tissues. Root, stem and leaves were investigated for this experiment. The expression level of *Ll-8CesA* was higher than *Ll-7CesA* in all tissues tested. However the overall expression of individual transcript was highest in stem than leaves and roots in both the case. Differential expression pattern of *CesAs* in *Leucaena* corroborated with the expression pattern of other homologous *CesA* transcript. When compared to CesAs of *Eucalyptus*, *Arabidopsis* and *Populus*, it was found that the expression pattern of both *Ll-CesAs* is similar to primary/secondary cell wall synthesizing CesAs. This suggests that they might have dual role and involved in redundant functions in cell wall biosynthesis in *Leucaena*. As the semi quantitative and qRT-PCR data suggests that the expression of *Ll-8CesA* was higher than *Ll-7CesA* in case of all tissues tested, it can be assumed that this isoform might be involved in active cellulose biosynthesis.

5. ACKNOWLEDGEMENTS

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Abbreviations

AMV-RT: Avian Myeloblastosis Virus Reverse Transcriptase.

cDNA: Complementary DNA.

CesA: *Cellulose Synthase A.*

Ll-CesA: *Leucaen*a *leucocephala Cellulose Synthase A.*

RACE: Rapid Amplification of cDNA Ends.

qRT-PCR: Quantitative real-time PCR.