RESEARCH ARTICLE

Isolation of developing secondary xylem specific cellulose synthase genes and their expression profiles during hormone signalling in *Eucalyptus tereticornis*

BALACHANDRAN KARPAGA RAJA SUNDARI and MODHUMITA GHOSH DASGUPTA*

Division of Plant Biotechnology, Institute of Forest Genetics and Tree Breeding, P.B. No. 1061, Forest Campus, R.S. Puram Coimbatore 641 002, India

Abstract

Cellulose synthases (*CesA*) represent a group of β-1, 4 glycosyl transferases involved in cellulose biosynthesis. Recent reports in higher plants have revealed that two groups of *CesA* gene families exist, which are associated with either primary or secondary cell wall deposition. The present study aimed at identifying developing secondary xylem specific cellulose synthase genes from *Eucalyptus tereticornis,* a species predominantly used in paper and pulp industries in the tropics. The differential expression analysis of the three *EtCesA* genes using qRT-PCR revealed 49 to 87 fold relative expression in developing secondary xylem tissues. Three full length gene sequences of *EtCesA1, EtCesA2* and *EtCesA3* were isolated with the size of 2940, 3114 and 3123 bp, respectively. Phytohormone regulation of all three *EtCesA* genes were studied by exogenous application of gibberellic acid, naphthalene acetic acid, indole acetic acid and 2, 4-epibrassinolide in internode tissues derived from three-month-old rooted cuttings. All three *EtCesA* transcripts were upregulated by indole acetic acid and gibberellic acid. This study demonstrates that the increased cellulose deposition in the secondary wood induced by hormones can be attributed to the upregulation of xylem specific *CesAs*.

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Introduction

The genus *Eucalyptus* is native to Australia and its neighbouring islands and comprises more than 800 species (Turnbull 1999). It is one of the widely planted hardwood crops in the world, occupying 19.61 Mha globally, because of its superior growth, adaptability and wood properties. India ranks second in eucalyptus plantations based on area size (3.943 Mha) after Brazil (4.259 Mha) (Iglesias Trabado and Wilstermann 2008). *Eucalyptus tereticornis* Sm. commonly known as forest red gum has an extensive natural distribution from southern Papua New Guinea (PNG) to southern Victoria in Australia (5°20′S – 38°08′S), and ranks among the most extensively planted *Eucalyptus* in the tropics and subtropics (Boer 1997). It has been introduced in several countries to provide wood for fuel, poles, construction and pulp. Its popularity, as that of other widely planted eucalypts, is attributed to its rapid growth and production of desirable wood when grown in a wide range of environmental and soil conditions (Orwa *et al.* 2009).

Wood in angiosperm trees generally comprises 42 to 50% cellulose, 25 to 30% hemicelluloses, 20 to 25% lignin and 5 to 8% extractives (Delmer and Haigler 2002). This implies that the major composition of wood by dry weight is cellulose and understanding its biosynthesis and complex gene expression pattern is of vital importance to improve the quality of wood.

Cellulose synthases (*CesA*) are the key enzymes which catalyse the synthesis of cellulose in plants and are assumed to be a multienzyme complex (Delmer and Amor 1995). All members of the cellulose synthase superfamily are integral membrane proteins, with three-to-six transmembrane domains in the carboxy-terminal region of the protein and one or two transmembrane domains in the amino terminal region (Doblin *et al.* 2002; Somerville 2006). Plant *CesA*s belong to a multigene family with a coordinated and complex gene expression pattern. Specialized plant cells (e.g. xylem and phloem fibres) sequentially deposit primary

*For correspondence. E-mail: ghoshm@icfre.org; modhumitaghosh@hotmail.com.

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and secondary cell walls that differ significantly in the quantity and quality of cellulose along with their biological function (Kalluri and Joshi 2004).

Recent reports in several higher plant species have revealed that two groups of a *CesA* gene family exist, which are associated with either primary or secondary cell wall deposition (Ranik and Myburg 2006). These studies indicate that *CesA*s are involved in two different modes of cell wall formation signifying that there are different roles for the individual catalytic subunits of *CesA*s participating in cell wall biosynthesis (Taylor *et al.* 2000; Endler and Persson 2010).

In woody plants, the development of secondary xylem cells encompasses the successive processes of cell division, expansion, secondary cell wall formation and cell death which contribute to the organization of secondary xylem cells. The secondary xylem in trees is induced and controlled by cascades of inductive hormonal signals which characterize wood quality and quantity (Aloni *et al.* 2000). The four major hormones that control xylogenesis and wood development include auxin, cytokinin, gibberellin and ethylene (Aloni 2001). Several specific genes and transcription factors regulate the different phases of secondary growth (Groover and Robischon 2006; Demura and Fukuda 2007). Further, cross talk between pathways regulated by different phytohormones also affects the secondary growth (Taiz and Zeiger 2006).

In recent years, advances have been made in understanding the transcriptional regulation during wood formation at the tissue and cellular levels (Demura and Fukuda 2007). A group of NAC (NAC was derived from the abbreviated first letters of three proteins, NAM (no apical meristem), ATAF1-2 (Arabidopsis transcription activation factor), and CUC2 (cup-shaped cotyledon)) domain transcription factors, *PtrWND*s that are preferentially expressed in developing wood were identified in *Populus trichocarpa* and qRT-PCR analysis revealed that the secondary wall biosynthetic genes, including cellulose synthases (*CesA4, CesA7* and *CesA*8), xylanbiosynthetic genes and lignin biosynthetic genes were induced by the overexpression of *PtrWND2B* and *PtrWND6B* in comparison with the wild type (Zhong and Ye 2010). In addition, studies in *Zinnia* and *Arabidopsis* have revealed that gibberellic acid (Digby and Wareing 1966), auxin (Koizumi *et al.* 2000), cytokinins (Mahonen *et al.* 2006), brassinosteroids (BRs) (Cano-Delgado *et al.* 2004) and tracheary element differentiation inhibitory factors (TDIFs) are major signalling molecules in wood formation (Ito *et al.* 2006). However, the specific role of hormones on expression of xylem specific cellulose synthases are not extensively studied in *Eucalyptus*. Hence, the present study was undertaken to isolate and characterize the differentially expressed cellulose synthase genes in developing secondary xylem tissues of *E. tereticornis* and to study their expression profiles during exogenous hormone signalling.

Materials and methods

Plant materials

Plant tissues were harvested from 15-year-old *E. tereticornis* (south of Helenvale Provenance, Commonwealth Scientific and Industrial Research Organization, Australia, seedlot no. 12944) maintained at the seed orchard at Karunya Research Plot, Coimbatore, India. The tissues used for the study included leaves, internodes, developing xylem and mature xylem. Fresh tissues were harvested and immediately frozen in liquid nitrogen and stored at −80°C until RNA isolation. Additionally, the effect of hormones was conducted on three-month-old vegetatively propagated cuttings of *E. tereticornis* (IFGTB clone Et86), in an open environmental condition at vegetative multiplication garden of the Institute of Forest Genetics and Tree Breeding, Coimbatore, India. The hormone effect was studied by spraying the hormones exogenously at different concentration to the clonal cuttings.

RNA isolation and cDNA synthesis

Total RNA was isolated from different tissues (100 mg) of *E. tereticornis* using an in-house protocol (patent pending). The quality of RNA was checked on a 1% agarose gel and the concentration was determined spectrophotometrically using picodrop (Picodrop, Hinxton, UK). Subsequently, total RNA was treated with RNase-free DNase I (Fermentas, Pittsburgh, USA) according to the manufacturer's protocol.

First strand cDNA was synthesized from RNA using the First strand cDNA synthesis kit (Fermentas, Pittsburgh, USA). About 1 μg of total RNA was converted into cDNA in a 10 μL reaction containing 0.5 μM oligo $(dT)_{18}$ and 2 units of M-MuLV Reverse Transcriptase. The mixture was incubated at 37°C for 1 h and the reaction was terminated by incubation at 65°C for 5 min. The concentration of cDNA was also quantified using the picodrop (Picodrop).

Amplification of CSRII conserved region of EtCesA transcripts

The primer pairs targeting the conserved CSRII (class specific region) of cellulose synthase gene families (*CesA1, CesA2* and *CesA3*) from *E. grandis* were used to amplify the *CesA* transcripts in cDNA pools derived from different tissues (Ranik and Myburg 2006) (table 1). Ten μL reaction included 100 ng of cDNA, 10 μM forward and reverse gene specific primers, 1 μL of 10× *Taq* buffer, 1 unit of Prime *Taq* polymerase (Genetbio, Nonsan, Korea) and 0.4 μL of 2.5 mM dNTP mix (Sigma Aldrich, St Louis, USA). PCR was performed in Veriti 96-WellThermal Cycler (Applied Biosystems, Foster City, USA) using the following programme: initial denaturation at 94°C for 5 min, 94°C for 1min, 58°C for 30 s, 72°C for 2 min (30 cycles) and final extension at 72°C for 10 min. The amplified fragments were separated on a 1% agarose gel. A 100 bp DNA ladder (Chromous Biotech, Bangalore, India) was used to determine the size of the amplicons.

Table 1. Primer pairs and amplicon size of conserved class specific (CSRII) region of the *EtCesA* transcripts.

Primer ID	Sequence (5′–3′)	Amplicons size (bp)
*CesA1*CSRIIF	AATGCCGCCATCTTCAACCTTGGA	700
*CesA1*CSRIIR	GATCGCAGTGATCGATATG	
*CesA2*CSRIIF	ACATGTGATTGCTGGCCTTC	250
*CesA2*CSRIIR	TTCTGAGACATGAGCGATGA	
*CesA3*CSRIIF	CTGTGATTGCTGCCCATGCT	300
*CesA3*CSRIIR	CGCCACCCTGTTCCATCAAA	

The PCR fragments were cloned using InsTAclone™ PCR Cloning Kit (Fermentas) by following the manufacturer's procedure and sequenced. These amplicons were sequenced by capillary electrophoresis method using the Big Dye Terminator ver. 3.1 cycle sequencing kit (Applied Biosystems) in AB 3500 Genetic Analyser (Applied Biosystems). Sequence similarity was conducted using the BLASTn and BLASTx programs of NCBI (http://www.ncbi.nlm.nih.gov).

Expression analysis of EtCesA transcripts in different tissues of E. tereticornis

The specific expression of the *EtCesA* transcripts in different tissues of *E. tereticornis* was assessed using qRT-PCR. The tissues used for this study included leaves, internodes, developing xylem (after debarking 1.5 cm from the periphery) and mature xylem tissues (after debarking 1.5 to 3.0 cm). Primer pairs targeting the CSRII region of three classes of *EtCesA* genes (table 1) were used for the expression studies. *EtAct* and *EtSAND* were used as reference genes for normalization of the data based on our earlier report (Karpaga Raja Sundari and Ghosh Dasgupta 2012).

The qRT-PCR reactions were performed in fast optical reaction tube (Microamp, Applied Biosystems), comprising 25 μL reaction which included 11.5 μL Milli-q water, 200 ng of cDNA from different tissues, 12.5 μL SYBR Green PCR Master Mix (Applied Biosystems), 0.5 μL each of 200 nM forward and reverse primers. A negative control with water (instead of template cDNA) was also included. All reactions were conducted with three independent biological replicates in ABI PRISM 7500 Step one plus Sequence Detection System (Applied Biosystems) using the following programme: one cycle of 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min. The melting curve was determined for each primer pair to confirm the specificity of the amplified product. The relative expression analysis was obtained using the $\Delta\Delta$Ct method (Yuan *et al.* 2006). The complete qRT-PCR workflow was followed based on the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/) according to Bustin *et al.* (2009).

Isolation of full length EtCesA genes expressed in developing secondary xylem

Based on the qRT-PCR results, the transcripts which showed predominant expression in the developing secondary xylem tissues were subsequently taken up for isolation of full length coding domain sequence (CDS). Primers were designed based on the CSRII sequences and also from sequences derived from *E. grandis* and *E. urophylla* (table 1; tables 2, 3 and 4 in electronic supplementary material). Nine primer pairs were designed for *EtCesA1*, 16 primer pairs for *EtCesA2* and 12 primer pairs for *EtCesA3* and were amplified in cDNA pool derived from the developing xylem tissues (tables 2, 3 and 4 in electronic supplementary material). The amplicons were cloned and sequenced as mentioned earlier. Additionally, based on the sequences obtained in *E. tereticornis*, seven primer pairs from *EtCesA1*, nine primer pairs from *EtcesA2* and four primer pairs from *EtCesA3* were designed and synthesized (Sigma Aldrich) to span the complete genes. Subsequent to sequence assembly, primer pairs

Table 2. Primer pairs synthesized for isolation of full length CDS of *EtCesA* transcripts.

Primer ID	Sequence (5′–3′)	Product size (in bp)
*EtCesA1*FL	FP: ATGATGGAATCCGGGGTTCCCCTGTG RP: TCAACAATCTATAGAACTGCAGCTCT	2940
*EtCesA2*FL	FP: ATGTCCGGCTTCGCCGTGGGCTCTCAC RP: TCAGCACTCCACCCCACACGGTTTGAG	3114
*EtCesA3*FL	FP: ATGGAAGCCGGAGCTGGACTTGTCGCC RP: TCAGAGAATTCCGGCAACTACTCCAAC	3123

were designed to amplify the complete CDS of all three genes (table 2) and amplified in the cDNA pool obtained from developing xylem tissues.

Sequence analysis of full length CDS

Sequence analysis of all the three *EtCesA* genes were conducted using BLASTn program of NCBI to analyse the similarity of the *EtCesA* sequences with existing plant *CesA* sequences in the database. The comparative analysis of the deduced amino acid sequences were predicted using BLASTx program. All single sequences which harboured the coding domain were assembled using the CAP3 program to generate the overlapping contiguous sequences (contigs) (http://pbil.univ-lyon1.fr/cap3.php/). The coding domain for each of the transcripts was analysed with the conceptual translation of the nucleotide sequence using open reading frame finder program (http://www.ncbi.nlm.nih.gov/projects/gorf/). Multiple sequence alignments were generated by the ClustalW method (Thompson *et al.* 1994) using the EMBL server (http://www.ebi.ac.uk/clustalw/). Domain structure of the EtCESA was also predicted by multiple alignment of EtCESA with its orthologues from other tree species using ClustalW analysis and the conserved region and motifs in the protein were predicted using InterProScan ver. 4.8 (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and Pfam analysis (http://pfam.sanger.ac.uk/search). The transmembrane domain structures were analysed by the TMHMM Server, v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Phylogenetic analysis was done using Mega 5.05 (Tamura *et al.* 2011) using the translated sequences *EtCesA*1, *EtCesA*2 and *EtCesA*3 and their orthologues from other plant species.

Expression analysis of EtCesA transcripts during hormone signalling

Optimization of hormone concentration: The optimal concentration of hormones eliciting observable phenotypic variations was studied in three-month-old rooted cuttings of *E. tereticornis* (IFGTB clone Et86). The concentration range used for optimization of auxins (α-NAA, IAA) was 50, 100, 200, 400 and 500 μM; gibberellic acid (GA) was 10, 20, 50, 100 and 200 μM while the concentration of 2, 4-epibrassinolide (epiBL) tested ranged from 0.25, 1.25, 2.5, 12.5 and 25 μM. The hormones were prepared as 200 mL solution and sprayed weekly twice as foliar spray by mixing with 0.01% teepol solution. The phenotypic data including height of the rooted cutting, girth of the stem, leaf area and number of leaves were measured and recorded weekly twice for up to eight weeks. After eight weeks, the variation in phenotypic data was compared across different concentration of hormone. The data were examined by analysis of variance and subjected to Duncan's multiple range test using the SPSS 16.0 software package (SPSS, Chicago, USA). The optimum hormone concentration was further used in treatment of the cuttings for the expression analysis of the *EtCesA* genes.

Expression analysis during hormone signaling using qRT-PCR: Total RNA was isolated and first strand cDNA was synthesized from the internode tissues of control and hormone-treated samples as described earlier. qRT-PCR was performed using the control and hormone-treated cDNA in three biological replicates for *EtCesA1, EtCesA2* and *EtCesA3* transcripts following the procedure described earlier. *EtAct* was used as the reference gene for normalization of data based on the earlier report (Karpaga Raja Sundari and Ghosh Dasgupta 2012). The relative quantification value was obtained through $\Delta\Delta$ Ct method to determine the fold expression variation of *EtCesA* genes during different hormone treatment.

Results

Amplification of the EtCesA transcripts

Amplification of primer pairs targeting three classes of CSRII region in the cDNA pools derived from different tissues produced amplicons in the size ranging from 700 bp for *EtCesA1*, 250 bp for *EtCesA2* and 300 bp for *EtCesA3* (figure 1 in electronic supplementary material). The amplicons were cloned and sequenced, and the sequences showed significant similarity to the sequences of *CesA*s from *E. grandis*.

Expression analysis of EtCesA transcripts in different tissues of E. tereticornis

The relative expression of all three *EtCesA* genes were analysed with the most stable *EtAct2* reference gene in combination with *EtSAND* (calibrator). The relative expression profile of *EtCesA1* was 55-fold higher in developing xylem and 25-fold in mature xylem tissues in comparison to 5.56-fold in leaf and 20-fold in internodes. Similarly, the *EtCesA2* expression was upregulated by 49-fold in developing xylem tissue while the transcript was less abundant with only 2-fold expression in leaf, 27-fold in internodes and 24-fold in mature xylem tissues. Further, the expression pattern of *EtCesA3* transcript was predominant in developing xylem tissue with 87-fold in comparison to 3-fold in leaf, 29-fold in internodes and 20-fold in mature xylem tissues. These observations indicated that all these three *EtCesA* genes were predominantly expressed in developing xylem tissues of *E. tereticornis* (figure 1).

Isolation of developing xylem specific EtCesA full length coding domain sequence

The full length CDS of *EtCesA1*, *EtCesA2* and *EtCesA3* were isolated from cDNA pool derived from developing xylem tissues. Screening of primer pairs with all three *EtCesA* genes

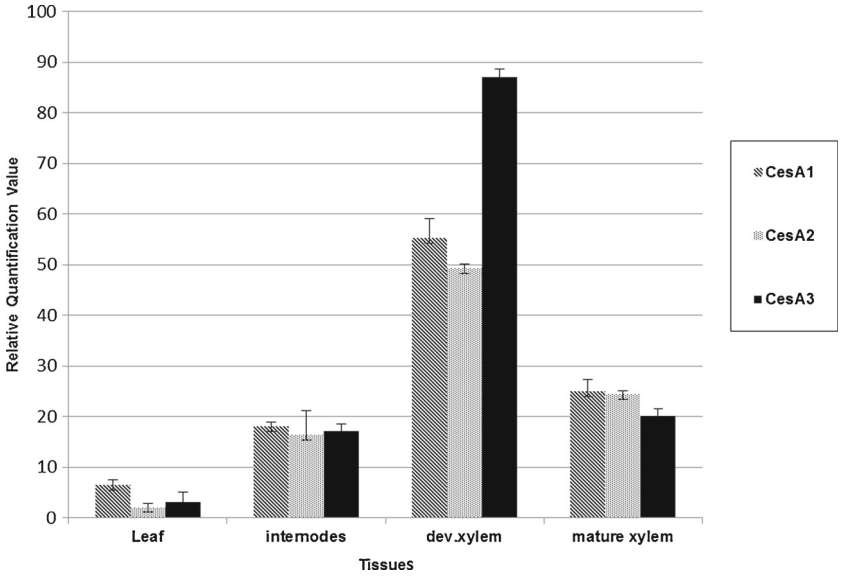

Figure 1. Relative expression of cellulose synthase transcripts in different tissues of *E. tereticornis*. Normalized expression levels of the three *EtCesA* gene transcripts in four different tissues are indicated. The relative quantification values (RQ) represent the difference in fold expression of *EtCesA* transcripts in different tissues. Error bars represent standard error derived from three replicates.

resulted in amplicons with size ranging from 500 bp to 1.2 kb for *EtCesA1*, 300 bp to 1.4 kb for *EtCesA2* and 350 bp to 1.4 kb for *EtCesA3*. These products were sequenced and showed significant similarity to cellulose synthase gene from *E. globulus, E. camaldulensis* and *E. grandis* genes (table 2, 3 and 4 in electronic supplementary material).

The sequences showing significant similarity to the *EtCesA* genes were assembled, and contiguous sequences were generated for all three genes using the CAP3 program. The complete CDS of the three *EtCesA* genes were isolated and the size of *EtCesA1* was 2940 bp (GenBank: JX276651) with the predicted translated product having 979 amino acid showing significant similarity to *E. globulus EglCesA1* gene (94% identity). Similarly, *EtCesA2* had a CDS of 3114 bp in length with 1037 amino acids (GenBank: JX276652) and documented 95% similarity to *CesA2* from *E. grandis*. The full-length CDS of *EtCesA3* was 3123 bp in length comprising of 1040 amino acids (GenBank: JX276653) having 99% significant similarity to *E. grandis, E. camaldulensis* and *E. globulus* orthologues. All the three *EtCesAs* harboured the glycosyl transferase superfamily conserved domain (figure 2).

ETCESA1 with predicted protein size of 979 amino acid residues showed 99% similarity to its orthologue from *E. grandis* (EgrCESA1) and 80% similarity with *Populus trichocarpa Ptr*CESA1 and *P. tomentosa Pto*CESA1. Similarly, EtCESA2 showed 98% similarity to its orthologue from *E. grandis* while about 80% sequence similarity was observed with *P. tomentosa Pto*CESA1, *Pinus taeda Pt*CESA2 and *Betula platyphylla Bepl*CESA2. Likewise, EtCESA3 showed 99% similarity with *Egr*CESA3 and 81% homology with *P. tomentosa Pto*CESA3 and *Betula luminifera BeluCESA3*. In all the species, the CESA families were reported to be expressed in tissues undergoing secondary cell wall deposition (Kalluri and Joshi 2004; Nairn and Haselkorn 2005; Ranik and Myburg 2006; Lu *et al.* 2008; Liu *et al.* 2012).

The three predicted EtCESA proteins were analysed by comparing the identical conserved residues to determine the presence of amino acid motifs distinctive to functional cellulose synthases using InterProScan and Pfam analyses. The analyses predicted the presence of a single conserved Zn-binding RING finger domain at the N-terminus containing four 'CXXC' motifs in all the three EtCESA proteins (figure 2). This was followed by two class specific region (CSR I and CSR II) comprising of conserved region and transmembrane domain. The CSR II region consisted of QXXRW motif, which is the glycosyl transferase signature sequence is essential for enzyme activity in all CESA proteins. The C-terminal region consisted of conserved region II, which is found to be highly conserved across different orthologous groups (Saxena *et al.* 1995). Eight transmembrane (TM) helices were predicted in all three classes of EtCESA1, EtCESA2 and EtCESA3 protein using TMHMM Server (figure 2; figure 2 in electronic supplementary material).

Phylogenetic analysis

A phylogenetic tree was constructed for all three translated sequences of *EtCesA*s and different classes of CESA orthologues from various plant species. The phloem filament protein from *Cucurbita maxima* was introduced as an

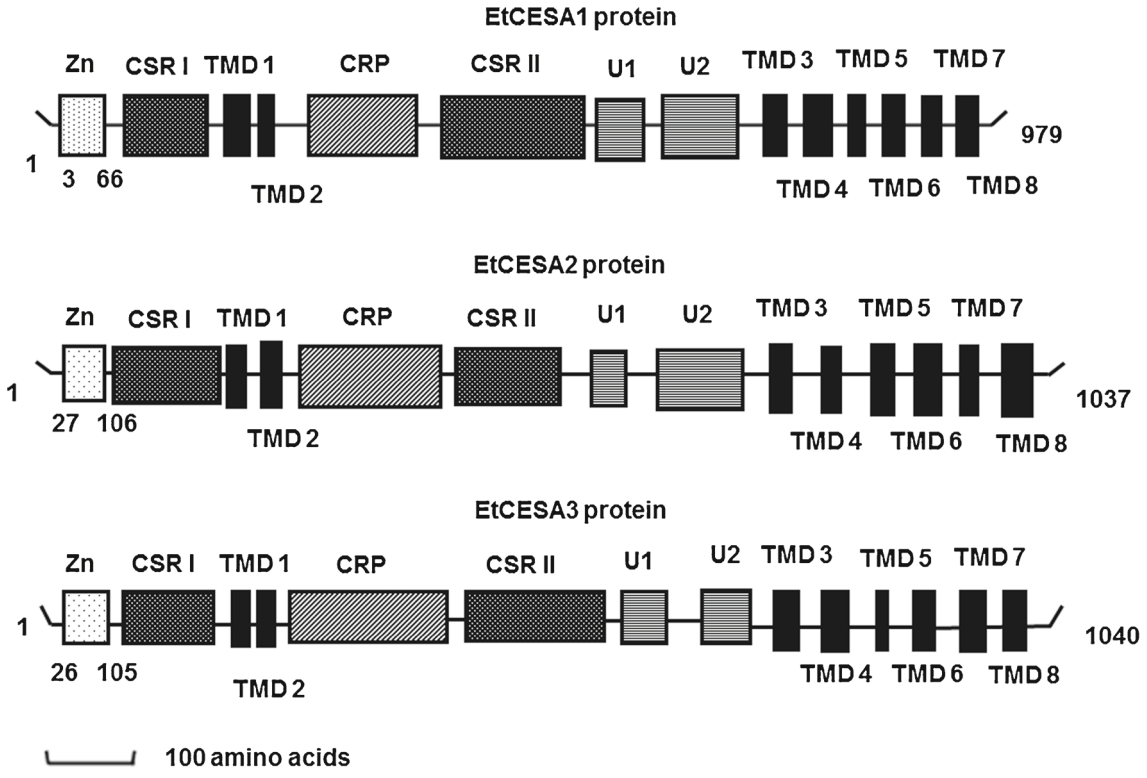

Figure 2. Schematic representation of the deduced CESA amino acid sequences of *Eucalyptus tereticornis*. Diagrammatic representation of protein model and conserved motifs for cellulose synthase EtCESA proteins consisting of zinc-finger region (Zn); class specific region (CSR) domains; eight transmembrane domains (TM 1–8); plant conserved region (CRP) domain; conserved 'U' motifs necessary for glycosyl transferase processivity (U1–U2)

out-group in the phylogeny as it is distinct from the xylem specific CESA protein. Phylogenetic analysis of 67 full-length CESA protein sequence belonging to different classes of subunits from both monocot and dicot plants including tree species is presented in figure 3. The phylogenetic tree clearly separated the sequences into three major clades with EtCESA1, EtCESA2 and EtCESA3 members in each clade and the EtCESAs grouped in the respective clusters along with their orthologues from *E. camaldulensis*, *E. grandis* and *E. globulus* revealing their phylogenetic closeness. In addition, the three CESA proteins grouped with their orthologues from different cellulose synthase catalytic subunits where EtCESA1 grouped with their orthologues from *Arabidopsis thaliana At*CESA8, *Zea mays Zm*CESA11, *Populus tremuloides Ptr*CESA1 and *Pinus taeda Pita*CESA1, while *Et*CESA2 grouped with their orthologues *At*CESA4, *Zm*CESA10, *Pto*CESA2, *Pita*CESA2 and *Et*CESA3 grouped with *At*CESA7, *Zm*CESA12, *Pto*CESA3 (figure 3).

Expression analysis of EtCesA transcripts during hormone signalling

The effect of hormones on rooted cuttings was studied using different concentrations of auxin (α-NAA, IAA), GA and 2, 4-epiBL for eight weeks. The phenotypic data were recorded twice a week, and the mean difference between the initial and the final data was calculated along with their standard deviation. Based on Duncan's multiple range test, the optimum concentration of hormone which had the significant effect on phenotypic data was chosen for treatment (table 5 in electronic supplementary material). The optimum hormone concentration was determined to be 50 μM for NAA and IAA, 10 μM for GA and 25 μM for 2, 4-epiBL which was further used for hormone treatment to determine the expression pattern of *EtCesA* genes.

The relative expression of all three *EtCesA* transcripts was analysed using *EtAct2* as the stable reference gene. The relative expression of *EtCesA1* transcript was significantly upregulated by 6-fold in GA treated internode tissues in comparison to control and by 4-fold in IAA treated tissues. On the contrary, cDNA derived from the NAA and epiBL treated internodes showed only limited expression of 1 and 2-fold respectively in comparison to control untreated samples. The relative expression of *EtCesA2* transcript was 3-fold upregulated in GA and IAA treated samples and only 1.45 and 1-fold expression in epiBL and NAA treated tissues. The expression level of *EtCesA3* transcript was 8.9-fold and 4.8-fold higher on IAA and GA treatment, whereas only 1.9-fold expression was observed during epiBL and NAA treatment (figure 4).

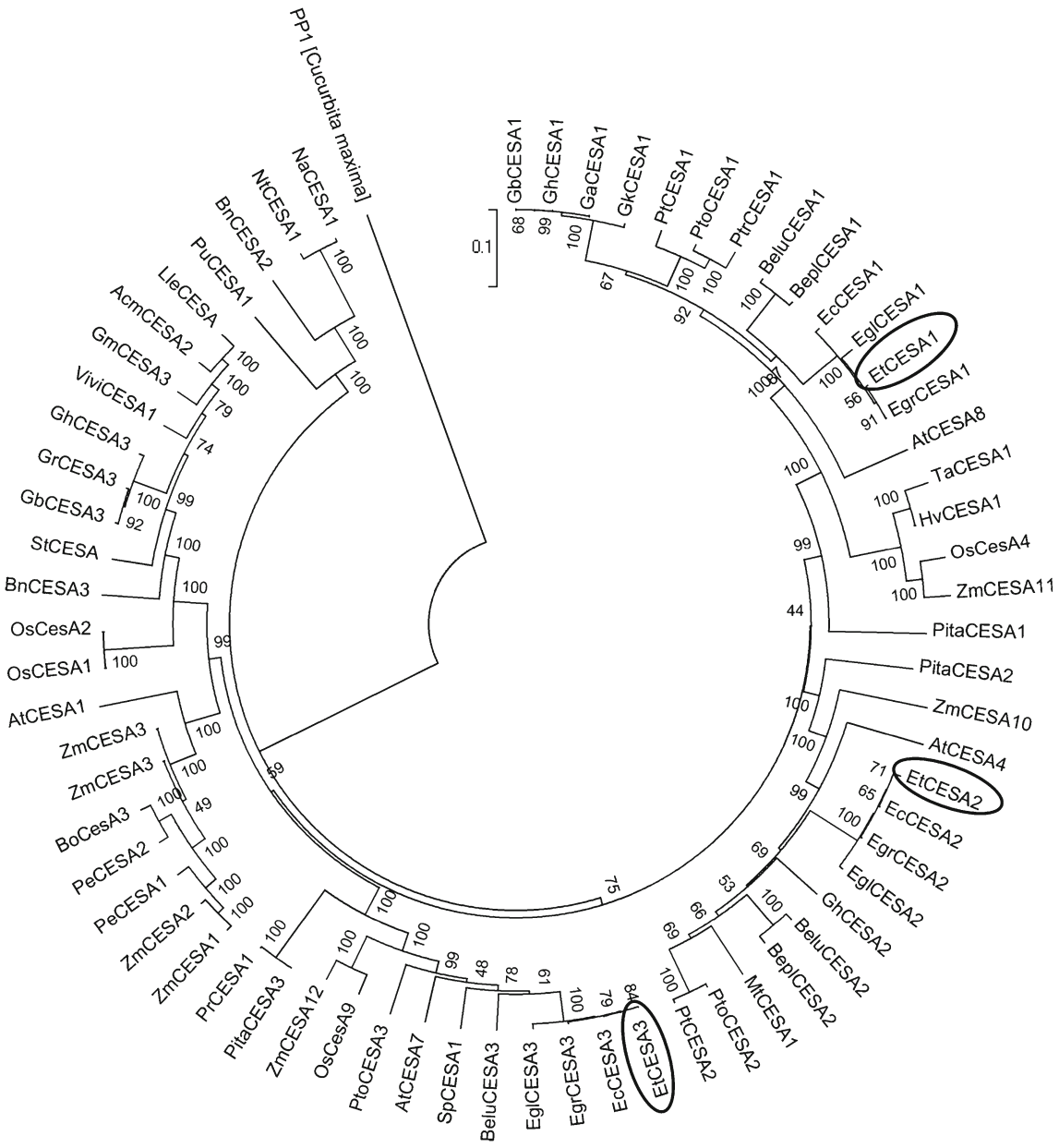

Figure 3. Unrooted neighbour joining tree derived from the alignment of the deduced amino acid sequences of EtCESA1, EtCESA2 and EtCESA3 with 67 CESA protein sequences where EtCESA, *Eucalyptus tereticornis*; EgrCESA, *Eucalyptus grandis;* EcCESA, *Eucalyptus camaldulensis*; EglCESA, *Eucalyptus globulus*; BeluCESA, *Betula luminifera*; BeplCESA: *Betula platyphylla*; ViviCESA*: Vitis vinifera;* PtoCESA*: Populus tomentosa;* PtCESA*, Populus* tremula × *Populus tremuloides*; AcmCESA*, Acacia mangium*; PtrCESA, *Populus tremuloides*; PuCESA, *Populus ussuriensis*; LleCESA, *Leucaena leucocephala*; PeCESA, *Phyllostachys edulis*; TaCESA, *Triticum aestivum*; HvCESA, *Hordeum vulgare*; PitaCESA, *Pinus taeda*; PrCESA, *Pinus radiata*; GbCESA, *Gossypium barbadense*; GhCESA, *Gossypium hirsutum*; GaCESA, *Gossypium arboretum*; GkCESA, *Gossypioides kirkii*; GrCESA, *Gossypium raimondii*; GmCESA, *Glycine max*; MtCESA, *Medicago truncatula*; AtCESA, *Arabidopsis thaliana*; NtCESA, *Nicotiana tabacum*; BnCESA, *Brassica napus*; ZmCESA, *Zea mays*; BoCesA, *Bambusa oldhamii*; NaCESA, *Nicotiana alata*; StCESA, *Solanum tuberosum*:; SpCESA, *Shorea parvifolia*; OsCESA, *Oryza sativa*. The bootstrap values are derived from 1000 replicates.

Discussion

Majority of the biomass produced in trees is the secondary xylem or wood and cellulose and hemicellulose represent the entire polysaccharide components in the walls of the secondary xylem cells. The carbohydrate component in the cell wall varies in structure and composition among plants and even within same tissues (Lee *et al.* 2011), which determines the functional diversity of the cell wall (Pauly and Keegstra 2010).

Cellulose being the major component of cell wall and a potential source of renewable biofuel has captured the interest of several research groups and has been widely reviewed (Delmer 1999; Williamson *et al.* 2002; Saxena and

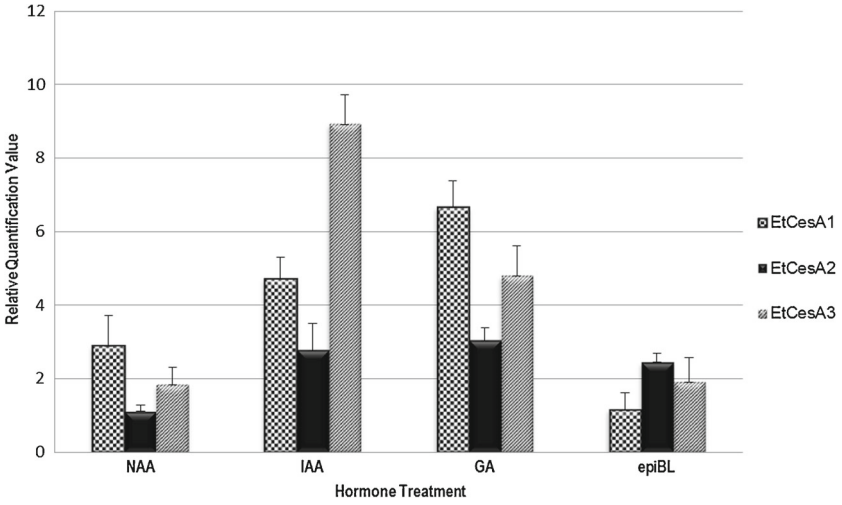

Figure 4. Relative expression analysis of *EtCesA* transcripts during hormone signalling by qRT-PCR. The relative quantification value represents the variation in fold expression of the transcripts in hormone treated sample in comparison to the untreated control. The mRNA levels were normalized by two reference genes *EtAct* and *EtSAND* as calibrator. Error bars represent standard errors derived from three replicates.

Brown 2005; Somerville 2006; Crowell *et al.* 2010; Lei *et al.* 2012). Cellulose synthase, a multienzyme complex is the key enzyme in cellulose biosynthesis and the first *CesA* gene in plants was reported from cotton fibre (Pear *et al.* 1996). Subsequently, they were isolated and characterized from several plant species including *A. thaliana* (Richmond and Somerville 2000), *Zea mays* (Holland *et al.* 2000), *Hordeum vulgare* (Burton *et al.* 2004), *Populus tremuloides* (Kalluri and Joshi 2004), *Pinus taeda* (Nairn *et al.* 2008), *Shorea parvifolia* (Lau *et al.* 2009), *Oryza sativa* (Wang *et al.* 2010), *Betula platyphylla* (Liu *et al.* 2012) and *Leucaena leucocephala* (Vishwakarma *et al.* 2012).

Recent reports in several higher plant species have revealed that two groups of *CesA* gene families exist, which are associated with either primary or secondary cell wall deposition as reported in *A. thaliana* (Fagard *et al.* 2000), *Oryza sativa* (Tanaka *et al.* 2003), *Populus tremuloides* (Kalluri and Joshi 2004; Liang and Joshi 2004), *Pinus taeda* (Nairn and Haselkorn 2005), *Hordeum vulgare* (Burton *et al.* 2004), *E. grandis* (Ranik and Myburg 2006; Lu *et al.* 2008), *Solanum tuberosum* (Obembe *et al.* 2009), *Bambusa oldhamii* (Chen *et al.* 2010) and *Gossypium hirsutum* (Kim *et al.* 2012). The presence of *CesA* orthologues in both short-lived plants and perennial tree species indicate that the underlying mechanisms of cellulose biosynthesis are conserved in plants (Joshi and Mansfield 2007). However, in *Populus trichocarpa*, 48 *CesA* super family genes were identified where seven genes were reported to be xylem specific suggesting that more than three *CesAs* or a set of *CesAs* that are different from *Arabidopsis* are required for the biosynthesis of cellulose in xylem cell walls of *P. trichocarpa*, or of trees in general (Suzuki *et al.* 2006).

In the present study, the relative expression of three *EtCesA* (*EtCesA1*, *EtCesA2* and *EtCesA3*) transcripts studied using qRT-PCR indicated their predominant expression in the developing xylem tissues. The expression of the *EtCesA3* transcripts was 87-fold higher in developing secondary xylem tissues, in comparison to the primary tissues like leaf and internodes, which was analogous to the 50-fold abundant expression of *EgCesA3* in the secondary xylem tissues of *E. grandis* (Ranik and Myburg 2006). Correspondingly, the expression of *EtCesA1*, *EtCesA*2 and *EtCesA*3 in developing xylem tissues was similar to the secondary xylem tissue specific predominant expression of *PtCesA1*, *PtCesA2* and *PtCesA3* in *Pinus taeda* (Nairn and Haselkorn 2005), *PtrCesA3* in *Populus tremuloides* (Kalluri and Joshi 2004), *BplCesA8, BplCesA7 and BplCesA4* in *Betula platyphylla* (Liu *et al.* 2012), *Ll-7CesA* and *Ll-8CesA* in *Leucaena leucocephala* (Vishwakarma *et al.* 2012) and *SpCesA1* in *Shorea parvifolia* (Lau *et al.* 2009), which reveals the expression of specific groups of the *CesA* genes during the secondary cell wall formation in woody perennials.

In the present study the full-length genes were isolated from the developing xylem tissues of *E. tereticornis* with size ranging from 2940 bp for *EtCesA1*, 3114 bp for *EtCesA2* and 2406 bp for *EtCesA3*. The sizes of the full length CDS are comparable to their orthologues from other tree species like *Betula platyphylla* (Liu *et al.* 2012), *Leuceana* (Vishwakarma *et al.* 2012), *Populus* (Wu *et al.* 2000), *Eucalyptus grandis* (Ranik and Myburg 2006) and *Shorea parvifolia* (Lau *et al.* 2009).

The deduced proteins from members of this *CesA* gene family are characterized by the presence of domains that share significant sequence homology with other glycosyl transferase family and characterized by having conserved motifs surrounding three conserved D residues and a QXXRW motif. A conserved, extended N-terminal region containing zinc fingers resembling LIM/RING domains

followed by the CSR-I region also characterize the plant CESA proteins (Kawagoe and Delmer 1997). The translated amino acid sequences of all three EtCESAs harboured the signature domains including zinc binding, transmembrane, D, D, D, QxxRW, CxxC motifs and glycosyl transferase catalytic domain as reported from other CESA proteins of plants. The prediction of a zinc-finger domain at the N-terminus in all the three EtCESA proteins is consistent with the predicted CESA protein structural characteristics of *Arabidopsis* (Richmond and Somerville 2000) and poplar (Joshi *et al*. 2004) which suggest their role in protein–protein interactions between cellulose synthase subunits (Richmond and Somerville 2000).

All members of the cellulose synthase super family also possess conserved motifs surrounding three conserved Asp (D) residues with eight predicted transmembrane helices, which contain one or two TM domains in the amino terminal region and three to six putative TM domains in the carboxy-terminal region (Doblin *et al.* 2002; Somerville 2006). This was also predicted in our study, where eight TM domains were identified in the EtCESA1, EtCESA2 and EtCESA3 proteins similar to other CESA proteins, which also possess eight TM regions (with two TM helices in the N-terminus region and six TM helices in the carboxy-terminal region) as in AtCESAs from *A. thaliana* (Taylor *et al.* 1999), *Zm*CESA proteins from *Zea mays* (Holland *et al.* 2000), *Eg*CESA from *E. grandis* (Ranik and Myburg 2006), *Populus tremuloides* (Wu *et al.* 2000), *Leucaena leucocephala* LlCESAs (Vishwakarma *et al.* 2012), *Bpl*CESAs from *Betula platyphylla* (Liu *et al.* 2012).

Phylogenetic analysis also revealed that these EtCESA protein sequences grouped with their orthologues from the corresponding three classes CESAs from both shortlived species and woody perennials (figure 4). The dendrogram revealed that the orthologues grouped together and were more similar than the paralogues, a characteristic feature of plant CESA protein (Vergara and Carpita 2001). This also supports the view that the divergences of subclasses of CESA protein may have arisen relatively early in the evolution of these genes (Holland *et al.* 2000; Appenzeller *et al.* 2004).

Wood formation is under strict developmental-stage-specific-transcriptional control. Hormones including auxin, ethylene, gibberellins and cytokinins are major players in signalling during xylogenesis. A complex interplay between hormones is also reported for optimal growth in plants (Bjorklund *et al.* 2007; Nieminen *et al.* 2008). Auxins are historically known to regulate diverse developmental processes, which involve changes in cellular auxin content, sensitivity of cells to the hormone and changes in polar auxin transport (Muday and DeLong 2001). During wood formation, it affects cambial activity and xylem development (Sundberg *et al.* 2000). In pine and hybrid aspen, presence of auxin concentration gradient suggests its role in providing positional information to cambial cells and their derivatives (Uggla *et al.* 1996; 1998). The specific effect of auxin on expression of cellulose synthase was demonstrated in cotton by Kim *et al.* (2011) where it was reported that auxins not only regulate the expression levels of cellulose synthase but also control a transition between cotton fibre elongation stages to fibre cellulose thickening.

In *Arabidopsis*, the upregulation of specific *CesA* during auxin signalling was reported (Goda *et al.* 2004). However, no direct evidence of *CesA* expression during exogenous application of auxins is reported in woody perennials. The present study elucidates a significant upregulation of xylem specific *CesA*s by the IAA confirming the results from cotton. Further, Singh *et al.* (2009) reported that synthetic auxin like NAA had a lesser effect on the secondary wall cellulose biosynthesis in cotton ovule/fibre culture, when compared to the natural auxin, IAA and the expression patterns of genes like *GhCesA1* and *GhCesA2* showed significant upregulation on exogenous application of IAA, while limited expression was reported on NAA application. A similar expression pattern was observed for all the three *EtCesA* transcripts in the present study.

The physiological and cellular difference in response to both the natural and synthetic auxins can be attributed to the difference in their mechanisms of uptake (Delbarre *et al.* 1996), rate of metabolism (Beyer and Morgan, 1970) and interaction with the auxin receptor *TIR1* (Tan *et al.* 2007). Further, it was reported that the response of *CesA*s to the naturally abundant IAA could be attributed to the presence of auxin response elements (*AuxRE*) in the promoter region of *GhCesA4* in cotton involved in the secondary wall synthesis (Wu *et al.* 2009).

Gibberellins regulate the radial and longitudinal growth and differentiation of secondary xylem cells in trees (Aloni *et al.* 2000; Eriksson *et al.* 2000; Bjorklund *et al.* 2007). GAs are reported to play tissue-specific roles in the wood-forming zone as demonstrated in hybrid poplar by Mauriat and Moritz (2009), where they stimulate xylogenesis in the cambial zone and fibre elongation in the developing xylem. Funada *et al.* (2008) reported that exogenous application of GA induced tension wood formation in four tree species, *Fraxinus mandshurica* var. *japonica, Quercus mongolica* var. *grosseserrata, Kalopanax pictus* and *Populus sieboldii*. Further, they also documented that GA signalling caused increase in cellulose content and changes in the orientation of cellulose microfibrils. In transgenic hybrid aspen lines, increased GA levels showed significant increase in number of xylem fibres and fibre length (Eriksson *et al.* 2000). In the present study, significant induction of xylem specific *EtCesA1, EtCesA2* and *EtCesA3* by exogenous application of GA was observed in the early formed xylem tissues (6, 3 and 4.8-fold, respectively) supporting the observation made by Funada and coworkers (2008). The effect of GA on expression of *EtCesA1* and *EtCesA2* was more predominant than that of IAA. The increased cellulose deposition reported in earlier studies could be explained through the upregulation of the xylem specific *CesA* genes as observed in the present study.

Brassinosteroids (BRs) include plant steroid hormones that regulate different aspects of plant development including

vascular differentiation. The presence of BRs in the cambial region of *Pinus sylvestris* revealed the involvement of endogenous BRs in xylogenesis (Kim *et al.* 1990). In *Arabidopsis* mutant, BR was reported to upregulate the expression of secondary xylem specific cellulose synthases (*CesA4*, *CesA7* and *CesA8*) (Oh *et al.* 2011). However, in the present study the expression of the xylem specific *CesA*s in *E. tereticornis* was not significantly induced by EpiBL, which may be due to the differential response of secondary xylem tissue in woody perennials like eucalypts and short-lived species like *Arabidopsis*. The present work provides a basis for exploring the molecular regulatory mechanism of cellulose biosynthesis in this species during hormone signalling.

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