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Isolation and expression analysis of cellulose synthase 3 (*Ces*3) genes from sugarcane (*Saccharum officinarum* L.)

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Abstract

Plant cellulose synthase is one of the important glycosyltransferases, which catalyzes the synthesis of the paracrystalline of H-bonded-β-(1,4)-glucose chains. This study isolated the cellulose synthase 3 (*Ces3*) sequence from sugarcane (*Saccharum officinarum* L.) leaves. *SoCes3* (GenBank accession No. MG324347) has a full-length cDNA sequence of 3625 bp. It contains an open reading frame (3225 bp), encoding 1074 amino acids with a molecular weight of about 120.89 kDa and isoelectric point of 6.26. *SoCes3* protein showed high activity with other plant cellulose synthases. The recombinant protein contains plant cellulose synthase (*Ces*) protein conservative domains. In subcellular localization experiments, the fusion protein of *SoCes3* with green fluorescent protein (GFP) was specifically localized in the cell membrane. The gene expression of *SoCes3* was found in the leaf, leaf sheath, and internodes of the sugarcane stem. The highest expression level was found in the internode, especially with the highest expression level in the 5th internode and lowest in the leaves, and the gene expression level of *SoCes3* was upregulated by PP333 and not in gibberellic acid-treated plants. It was conducted in tobacco plants to understand the biotechnological potential of *SoCes3*. The contents of cellulose and lignin were increased in *SoCes3*-overexpressing tobacco. Transcriptomic analysis showed that the transgenic tobacco induced different genes associated with different biological regulatory processes. Differentially expressed genes (DEGs) mediated plant hormone signal transduction, starch and sucrose metabolism signaling pathways were widely induced and mostly upregulated. The transcription levels in *SoCes3*-overexpressing transgenic lines were higher than wild-type.

Keywords Gene expression, DEGs, *SoCes3*, Transgenic tobacco, *Saccharum officinarum* L.

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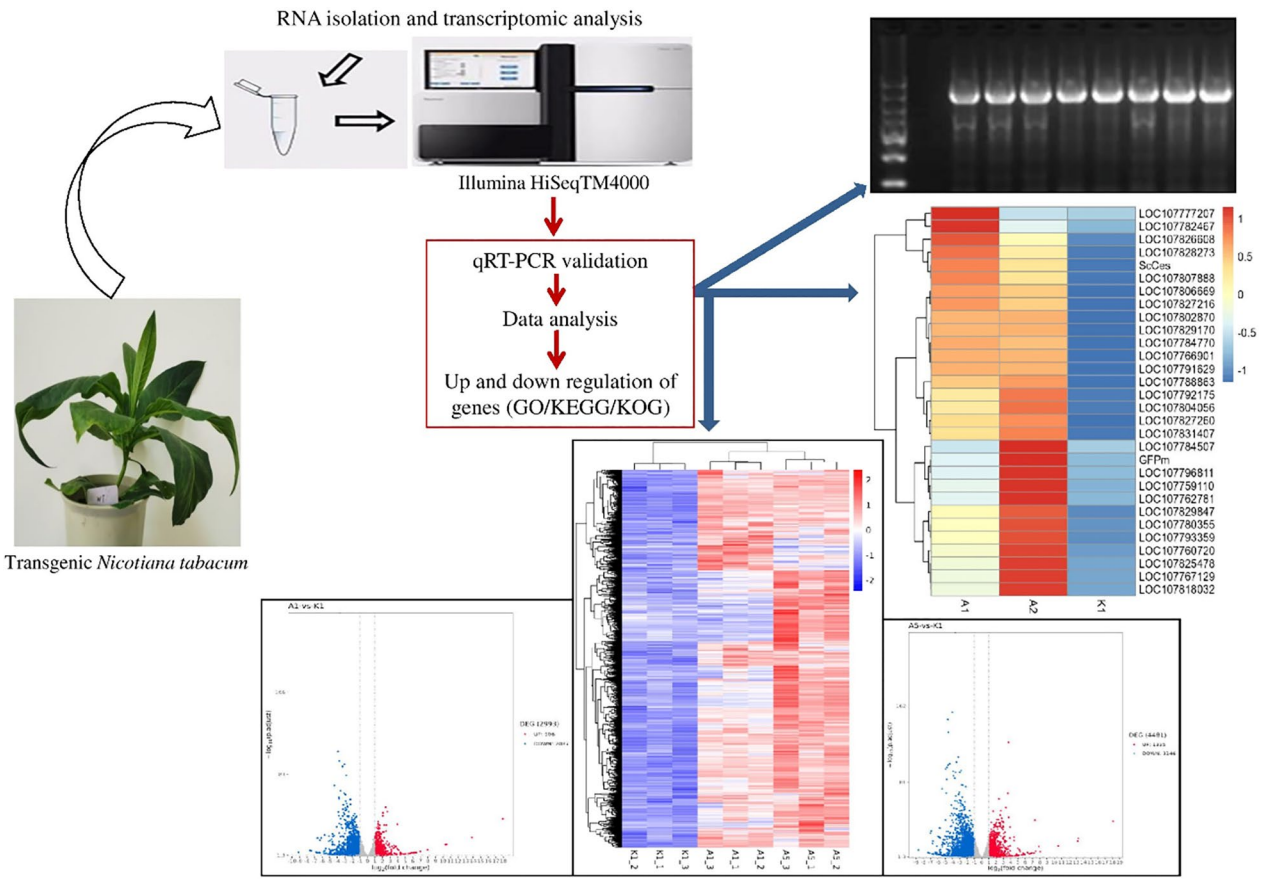
Graphical Abstract



Introduction

Sugarcane (*Saccharum* spp. Hybrid) is a major C_4 energy crop. It accounts for over 90% of China total sugar production with optimum biomass and fiber. Developing high yield, increased biomass, and stress resistance are the major goals of sugarcane breeding. Fiber is one of the main components in sugarcane stems with higher content (11.5–12.5%) [1, 2]. Cellulose synthase is the most important for sugarcane growth and cell development. Cellulose is the world's most abundant biopolymer and a major component of plant cell walls [3]. Cellulose consists of a β-1,4-linked glucose chain synthesized by cellulose synthase (*CesA*, EC2.1.4.12) [4]. The members of the *CesA* genes family in plants encode a glycosyltransferase that plays a key role in the process of cellulose synthesis [4–8].

Plant cellulose synthase A (*CesA*) belongs to a multigene family with complex expression patterns. The *CesA* gene was first cloned from the bacteria that produced extracellular cellulose [9, 10]. Due to the high cellulose content in cotton fiber cell walls, it is no surprise that plant homologs were identified from the cotton fibers undergoing active secondary cellulose synthesis walls (*GhCesA1/GhCesA2*) [11]. The *CesA* genes have also been isolated from other plant species, such as *Arabidopsis* [12, 13], *Oryza sativa* [14], *Zea mays* [15], *Populus* [16–19], *Boehmeria nivea* (Linn.) Gaud. [20]. These orthologous *CesA* proteins possess similar functions and exhibit high sequence similarity. The *CesA* gene family in monocot crop plants, such as *Zea mays*, *Hordeum vulgare*, and *Oryza sativa* were screened by the analysis of cDNA, expressed sequence tags, and genome sequencing [21–24].

In higher plants, the family of *CesA* genes showed interesting similarities and differences from the genes in the bacteria that predicted proteins in plants contain a plant-specific conserved and a hypervariable (HVR-2) domain that separates the domains containing these conserved residues [4]. The *CesA* proteins are inferred to be the components of cellulose synthase complexes

(CSCs) associated in the synthesis of the primary cell wall [3, 23, 25]. Several lines of evidence implicate the plant *CesA* genes in the process of cellulose synthesis. Genetic and biochemical responses shown that the three unique *CesA* isoforms are required for CSCs function that separate CSCs associated with cellulose biosynthesis of primary and secondary cell walls [15, 25–29]. In *Arabidopsis thaliana*, *CesA1, CesA3,* and *CesA6* are required for PCW cellulose biosynthesis, while *CesA4, CesA7,* and *CesA8* are required during the development of secondary cell wall. The remaining *CesA* (*CesA2, CesA5, CesA9,* and *CesA10*) are linked in tissue-specific processes and partially redundant with *CesA6* [13, 25, 27–29].

CesA genes are poorly identified and annotated in sugarcane plants. We identified a unigene highly homologous to the *Zea Mays Ces3* sequence isolated from the sugarcane full-length cDNA library in response to limited water supply (i.e., drought stress). This study utilized an innovative approach to isolate and characterize a full-length cellulose synthase gene (*SoCesA3*) from sugarcane plants (*Saccharum* spp.). The functions of the *SoCesA3* were inferred based on their phylogenetic relationships and differential expression patterns. This study not only lays a foundation for understanding the biosynthesis of cellulose and further functional analysis of *SoCesA3* in sugarcane, but also sheds light on future improvements in the production and properties of cellulosic biomass in sugarcane plants. In addition, a pair of stably expressed housekeeping genes were screened out and validated in sugarcane plants for the quantitative real-time reverse transcription PCR (qRT-PCR) experiment.

Materials and methods

Plant material and growth conditions

The main sugarcane cultivar (Taitang 22 from Guangxi Province, China) was used for gene cloning. Two genotypes (Guitang 46 and Guitang 49) were planted in the greenhouse of Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, Guangxi, China for gene expression analysis. Tobacco (*Nicotiana tabacum* L.cv. *Petit Havana*) plants were used in subcellular localization of *SoCes3* and gene overexpression.

The first leaf was collected from the Taitang22 cultivar for gene isolation at the pre-elongation stage, which contained 9–10 leaves [30]. Two genotypes (GT 46 and GT 49) at different leaves, such as 1, 3, and 5th leaf position (from top to bottom), leaf sheaths and leaf stalks were collected for gene expression analysis during elongation stage of plant. Gibberellic acid (GA, 100 mg/L) and paclobutrazol (PP333, 100 mg/L) were sprayed on GT 49 variety at seedling stage with contained 3–5 leaves (45 days). The plant leaf samples were collected at specific time intervals, i.e., 15, 30, 40, 50, and 60 days after treatment. During 60 days, different leaves, leaf sheaths and stalks (1, 3 and 5th leaf position from top to bottom) were collected. These plant materials were immediately frozen and stored in liquid nitrogen for RNA isolation.

Full-length gene cloning and sequence bioinformatics analysis of *SoCes3* genes

RNA was extracted from sugarcane leaves, sheaths, and stems using the TRIzol component (Invitrogen, USA). Then, first-strand cDNA was synthesized from 1 to 5 µg of total RNA using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Based on the unigene sequence highly homologous to the *Zea Mays Ces*3 sequence, it was isolated from the sugarcane full-length cDNA library in response to limited water supply (drought stress). A primer pair CES-F and CES-R was designed and used to amplify the cDNA of *SoCesA3* (Table 1). The PCR reaction was performed in a volume of 50 µl containing 1.0 µl of template, 25 µl of Premix Primes STAR HS (TaKaRa, Dalian, China), 1.0 µl of each 20 µmol/L upstream and downstream primer, 22 µl of double-distilled water (DDW), and using the following program: 1 cycle at 94 ℃ for 4 min, 35 cycles at 98 ℃ for 10 s, 55 ℃ for 15 s, 72 ℃ for 3 min and 72 ℃ for 10 min. After PCR amplification, the products were purified and cloned into the PMD19-T vector (TaKaRa, Dalian, China) and sequenced on both strands in Takara Bio Institute (TaKaRa, Dalian, China). Based on the sequence of partial *SoCes3* gene fragments, gene-specific primers for 5'-RACE and 3'-RACE was designed (Table 1). 5'-RACE was performed using SMARTer®RACE 5'/3' Kit (TaKaRa, Dalian, China), and 3'-RACE using 3'-Full RACE Core Set with Prime Script Rtase Kit (TaKaRa, Dalian, China). 5'- and 3'-end cDNA fragments of *SoCes3* gene were obtained.

Analysis of sequence alignment

The obtained DNA sequence and amino acids were analyzed using bioinformatics tools (www.ncbi.nlm.nih.gov, www.expasy.org). The multiple sequence alignment was conducted with Vector NTI 8.0. Phylogenetic analysis of *SoCes3* was aligned with DNAMAN 5.2.2 software, and evolutionary studies were constructed with the neighbor-joining (NJ) method using MEGA 6.0 software and the neighbor-joining way with 1000 bootstrap replicates [31, 32].

Table 1 Primers used for the *SoCes3* amplification and qRT-PCR

Primer name	Sequence (5′–3′)	Purpose
CES-F	ATGGAGGCGAACCGGGGGATGGTGGCTG	Gene cloning
CES-R	TCAGCAGTTTACGCCACACTGACCAC	Gene cloning
CES-5-F	TAGAGGTAGCCGTGCATCGTCAGC	5′-RACE amplification
CES-5-R	CCTTCCGCTGGATATTTATGAGTC	5′-RACE amplification
CES-3-F	TTGGTATAGTGGCTGGAGTATCGT	3′-RACE amplification
CES-5-R	ATCGTATGCTATCAACAGTGGCTA	3′-RACE amplification
CES-q-F	GGTTGGTATAGTGGCTGGAGTA	*SoCes3* qPCR
CES-q-R	ATGGAAGCAAGGAGGATGGA	*SoCes3* qPCR
GAPDH-F	TGGTGTCAACGAGAAGGAGTA	Sugarcane housekeeping genes
GAPDH-R	CAAGAGGAGCAAGGCAGTT	Sugarcane housekeeping genes
NtActin-F	ACCTCTATGGCAACATTGTGCTCAG	Tobacco housekeeping genes
NtActin-R	CTGGGAGCCAAAGCGGTGATT	Tobacco housekeeping genes
GFP-F	CAGTGGAGAGGGTGAAGGTG	GFP qPCR
GFP-R	CGAAAAGGCAGATTGTGTGG	GFP qPCR

Subcellular localization of *SoCes3*

To construct the *SoCes3*-GFP fusion fragment for the expression in tobacco epidermal cells, PCR products were digested with *NcoI* and *SpeI* and then ligated to the pCAMBIA1302 vector. The recombinant plasmids (pCAMBIA1302-*SoCes3*) were confirmed by double digestion with *Nco*I and *Spe*I and sequencing, then transferred to the TOP10 clone strain. The empty plasmid pCAMBIA1302 was used as the control. A positive monoclonal antibody containing recombinant plasmid pCAMBIA-*SoCes3* was amplified in YEP liquid culture (containing 50 mg/mL rifampicin and 25 mg/mL kanamycin) and cultivated to OD_{600} nm = 1.5–2.0 at 28 ℃. After centrifugation, *Agrobacterium* bacteria were collected. Then, 50 mL of *Agrobacterium* suspension was added, mixed, injected into the tobacco epidermis, and cultured for 72 h under normal illumination conditions. The tobacco epidermis was observed with a confocal laser scanning microscope (excitation wavelength 488 nm; emission wavelength 625–725 nm).

Quantitative real-time PCR analysis

Specific primers for qRT-PCR are shown in Table 1. qRT-PCR experiments were performed using HiScrip®II 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd, Nanjing, China) and Power qRT-PCR PreMix Kit (Generay Biotech Co., Ltd, Shanghai, China). The total volume of the reaction system was 7 µL SYBR Green I Master Mix (2×); 0.5 µL Forward-Primer (10 µmol/L); 0.5 µL Reverse-Primer (10 µmol/L); 8 µL cDNA template. Three biological replicates ($n = 3$) were performed for each sample. In order to draw a melting curve for the separation of the genes, the temperature cycle was as follows: 95 ℃ for 10 min, 40 cycles of 95 ℃ for 10 s, 60 ℃ for 34 s, and 95 ℃ for 15 s according to the manufacturer's protocol. Finally, melting curve profiles were assessed and analyzed using the ABI Step one software. The control was used as a reference sample and treatment as measuring samples. The method of $2^{-\triangle\triangle CT}$ was adopted to analyze the relative changes in gene expression from real-time quantitative PCR experiments [33]. Statistical analyses were performed using the software in Excel and ASCsee. The results were processed statistically with DPS v14.10 statistical software [34]. The analyzed data subjected to analysis of variance (ANOVA), and Duncan's multiple range test were employed to determine significant differences between treatments at $P < 0.05$ level. Mean values ± SD are calculated thrice with two independent biological replicates.

Construction of plant expression vector of transgenic tobacco

The coding region of *SoCes3* was inserted in the expression vector pCAMBIA1302 controlled by *CaMV 35S* promoter using *NcoI* and *SpeI* restriction enzyme cutting sites. This plasmid was used to transform *Agrobacterium tumefaciens* strain *EHA105* by triparental mating. The transgenic tobacco (*Nicotiana tabacum* L. cv. *Petit Havana*) was obtained via the *Agrobacterium*-mediated leaf disc method following the standard transformation protocol [35]. Plants rooted on hygromycin selection (50 mg/L) and positive (T_0) generation transformants were self-pollinated, and the seeds obtained from T_0 were analyzed for segregation by germinating on half-strength Murashige and Skoog

medium (MS medium) supplemented with hygromycin (25 mg/L). One-month-old plants were transferred to the greenhouse in pots containing the manure, sand, and soil (1:1:2 ratio). Genomic DNAs were extracted to identify the transgenic lines. The target gene was detected using these DNA as templates via qRT-PCR using GFP primers (GFP-F, R) and the *SoCes3* primers (CES-F, R). Applied primers are shown in Table 1. Total RNA was extracted from plant tissue; extracted RNA was used for single-strand cDNA synthesis. Real-time reverse transcription-polymerase chain reaction (qRT-PCR) was used to screen tobacco plants with exogenous *SoCes3* and GFP gene expression.

Seeds from a single tobacco plant positive for the overexpression of *SoCes3* gene were sown. When the seedlings reached the 4th-leaf stage, each plant was planted in plastic container (12X15 cm, diameter and height). The culture substrate uses a mixture of peat soil and vermiculite (1:1). Plants were grown at 26/25 ℃ (light/dark), light intensity of 400 μmol m^{-2} s^{-1}, photoperiod of 12 h, and relative humidity of approximately 75% in an artificial culture chamber. When the plants reached the 4th-leaf stage, qRT-PCR was applied to screen T_1 tobacco plants with exogenous *SoCes3* and GFP gene expression (designated as S). Non-transgenic '*Nicotiana tabacum* L.cv. *Petit Havana*' seeds were used as wild-type controls (designated as WT). Two lines (*SoCes3-40* and *SoCes3-45*) were selected for further experiments at 8th-leaf stage of plant. Tobacco plants were used to measure physiological and histological characteristics. Transcriptome sequencing was carried out for photosynthetically mature leaves of WT, *SoCes3-40*, and *SoCes3-45* seedlings.

Measurements of physiological and histological characteristics

When the tobacco plants grow, the top 3rd and 5th leaf and leaf veins were sampled from WT, *SoCes3-40,* and *SoCes3-45* at the eight-leaf stage. Cellulose was isolated and solubilized according to Updegraff [36]. Cellulose content was reduced from Glc concentration measured by the anthrone method as described by Scott and Melvin [37] and Turner and Somerville [38]. The lignin content was analyzed by detection kit from Soraibao Biotechnology Co., Ltd. Stems, leaves, and leaf veins from *SoCes3*-40 and *SoCes3*-45 plants together with the same-aged wild-type plants were sectioned and stained with fluorescence single-label staining of paraffin sections.

Transcriptome sequencing

High-quality RNA was extracted with RNAprep Pure kit (Tiangen, Beijing, China) according to the manufacturer's recommendation. Sequencing libraries were analyzed using the MGIEasy RNA Library Prep Kit, following the manufacturer's protocol. The sample library was sequenced on the Illumina high-throughput sequencing platform. Use Trimmomatic software to collect sequencing data from raw data and remove low-quality sequencing fragments. Finally, differentially expressed genes (DEGs) were screened using the R software package based on the criteria of log2 (fold change) > 1 or < 1 and $P < 0.05$. Using the GO (http://geneontology.org/) and KEGG database (https://www.genome.jp/kegg/), annotate differentially expressed genes (DEGs), and the annotated DEGs are used to analyze function and signaling pathways further.

Data analysis

Excel and DPSv14.10 analyzed all the data in this study. All column diagrams were drawn using DPSv14.10. Duncan's multiple comparison of three replications was represented. Significant differences between wild-type and transgenic tobacco were used ($^{*}P \leq 0.05$) and ($^{**}P \leq 0.01$).

Results

Isolation, characterization, and bioinformatics analysis of *SoCes3*

A full-length of *SoCes3* cDNA sequence of 3625 bp was isolated from the sugarcane plant leaves. The entire open reading frame (ORF) was 3225 bp in length, contained most 5'-end excluding 80 bp and the whole 3'-end with poly(A), named as *SoCes3* (GenBank accession No. MG324347). Ten other *Ces* protein sequences with high homology to sugarcane were selected for phylogenetic analysis using DNAMANv5.2.2 software. The un-rooted phylogenetic tree of the *SoCes3* gene and different *Ces3* sequences was constructed with MEGA 6.0 software. The results revealed that the *SoCes3* protein had the highest (99.00%) similarity with other *Ces3* proteins of *Miscanthus* x *giganteus* (cellulose synthase catalytic subunit 3) and *Zea mays* (*ZmCes3*), other *Ces3* proteins in 95–99% of *Saccharum* hybrid cultivar and *Phyllostachys edulis*, and other *CesA* proteins in 92–99% of *Sorghum bicolor*, *Setaria italica* and *Oryza brachyantha* (Fig. 1). The *SoCes3* sequence presented high level with the Gramineae plants, such as *Miscanthus* x *giganteus Ces3*, *Saccharum* hybrid cultivar *Ces*, *Sorghum bicolor CesA*, *Zea mays Ces*, etc. After that, most of *CesA* genes have been observed to occur in the form of gene families in the genome of other higher plants.

Subcellular localization analysis of *SoCes3*

According to PSORTb version 3.0.2, *SoCes3* has 92.6% possibility of locating in the cell membrane. To determine whether *SoCes3* found in the cell membrane, the

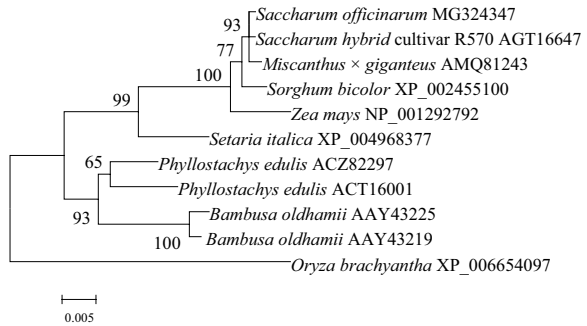


Fig. 1 The phylogenic tree induced amino acid sequence between sugarcane *SoCes3* and other *Ces3* proteins

vector containing *SoCes3*-GFP fusion genes was constructed. The fusion protein expression vector pCAMBIA1302 was constructed by fusing the C-terminus of *SoCes3* protein to the green fluorescent protein (GFP), driven by the 35S promoter of Cauliflower Mosaic Virus (CaMV). The fused protein was expressed transiently in *Nicotiana benthamiana* while the 35S:GFP functioned as a control. As shown in Fig. 2A, the GFP protein was distributed throughout the cell membrane and nucleus with the pCAMBIA1302 vector. However, green fluorescence was specifically detected in the cell membrane transiently with the pCAMBIA-*SoCes3* (Fig. 2B), demonstrating that the product of *SoCes3* is located in the cell membrane.

Expression patterns of *SoCes3* gene in different plant parts of sugarcane

Quantitative reverse transcriptase (qRT)-PCR expression analysis of *SoCes3* gene was performed from different plant parts, such as leaves, sheaths, and stalks of GT46 and GT49 sugarcane varieties and specific treatment intervals on GT42 variety with different treatments at early growth stage. The expression of *SoCes3* showed significant differences in various organs in sugarcane varieties, i.e., GT46 and GT49 (Fig. 3A). *SoCes3* was expressed in leaf, sheath, and internode of both sugarcane varieties. The highest expression level was found in the internode of stem, especially with the highest expression level in the fifth internode and lowest in the leaf. Compared with GA treatment, PP333 treatment can enhance the *SoCes3* gene transcription level of first leaf of sugarcane, reaching the highest level on the 40th day after treatment. After 60 days of GA treatment, transcription level of *SoCes3* gene was found lower as compared to control and PP333 treatments (Fig. 3B). The lowest transcription level of *SoCes3* were found in the leaf sheath (1, 3 and 5th leaf position) of control, GA and PP333 treatment conditions (Fig. 3C).

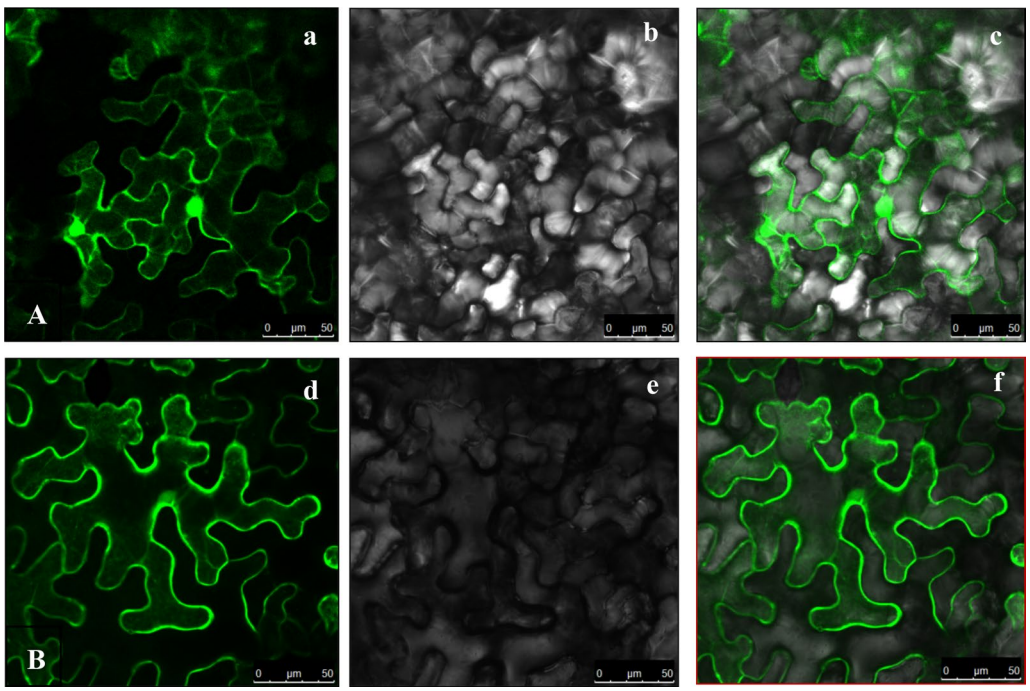


Fig. 2 Subcellular localization of pCAMBIA1302-*SoCes3*. **A** Histochemical analysis of transactivation activity of pCAMBIA1302 plasmids in *N. benthamiana* leaves. excitation: 488 nm, emission: 625 nm. (a) GFP images, (b) dark-field images and (c) overlapping GFP fluorescence and dark-field images. **B** Histochemical analysis of transactivation activity of pCAMBIA1302-*SoCes3* plasmids in leaves of *N. benthamiana*. excitation: 488 nm, emission: 625 nm. (d) GFP images, (e) dark-field images and (f) overlap of GFP fluorescence and dark-field images

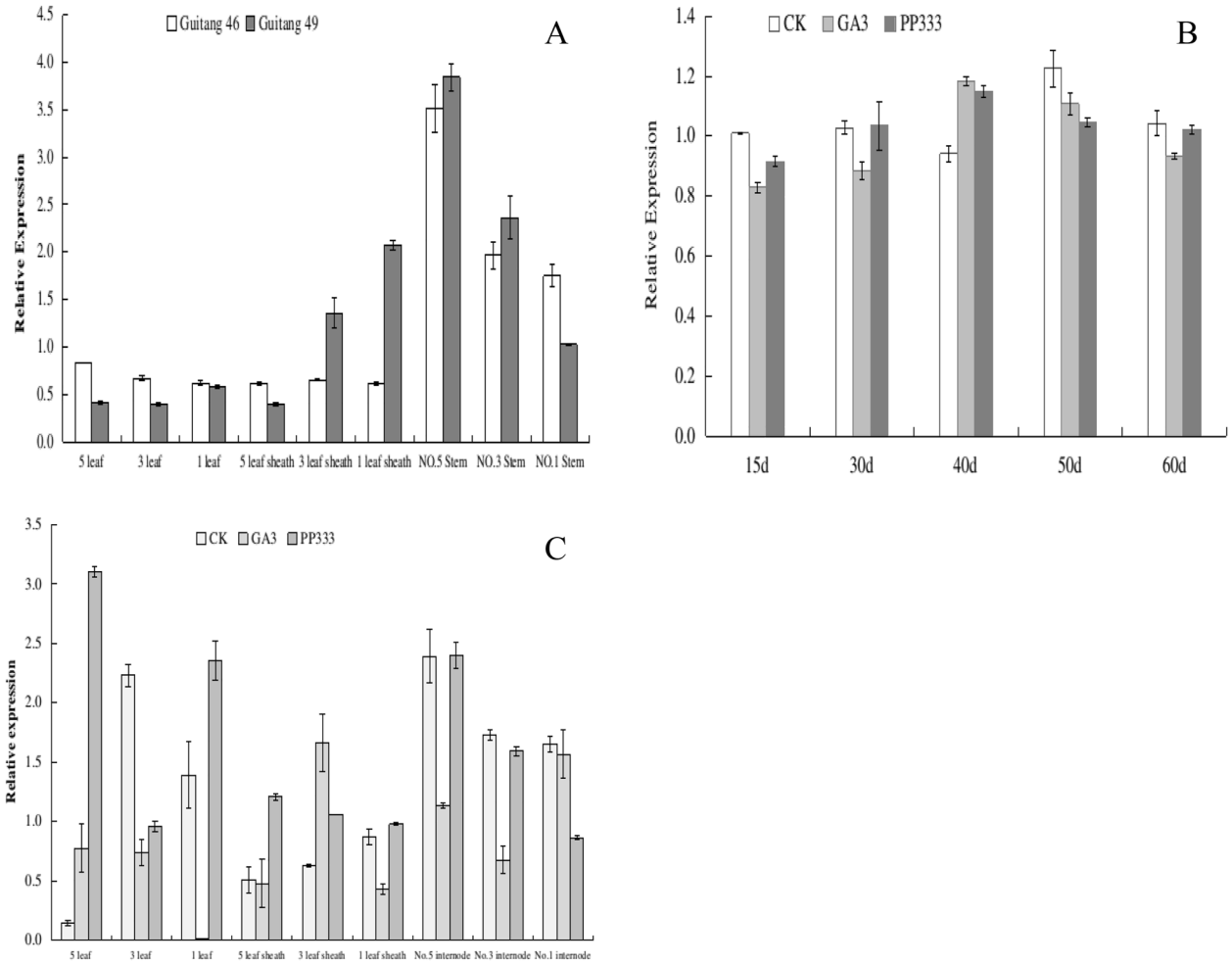


Fig. 3 Average expression pattern values of *SoCes3* gene in sugarcane ($n = 3$). **A** Impact of gene expression levels in leaf sheath and stem internode of GT46 and 49 varieties, **B** gene expression levels on 1st leaf position of sugarcane during GA and PP_{333} treatments of GT49 variety on early growth stage at specific time interval and **C** gene expression levels in different plant organs during GA and PP333 treatments of GT49 variety at 60 days of growth stage

Development and screening assay of transgenic tobacco plants

Identified eight hygromycin-resistant transgenic tobacco lines integrated with sugarcane gene (Fig. 4A, B), and their mRNA levels of *SoCes3* by qRT-PCR (Fig. 4C). qRT-PCR was used to analyze the transcript expression of *SoCes3* in transgenic plants with a single copy of transgenic. The constitutively expressed *NtActin* was used as the internal standard in expression analysis. Compared with the wild-type tobacco, these transgenic lines showed the increasing expression pattern of *SoCes3* gene at different levels. When tobacco plants reached the 4th-leaf stage, *SoCes3*-33 showed a relatively lower expression level of the *SoCes3* gene, the same as WT, while *SoCes3*-2, 3, 40, 45, 47, 55, 58 showed the highest expression level. For GFP, *SoCes3*-2, 3, 47, 55, 58 showed a relatively low expression level of *SoCes3* gene as same as WT. In contrast, *SoCes3*-40 showed the highest expression level

(See figure on next page.)

Fig. 4 Growth and development of transgenic tobacco plants. **A** Morphological development of transgenic with wild-type tobacco plant (WT) under normal conditions. **B** The expected fragment of *SoCes3* was observed from all transgenic lines except WT. **C** The expression levels of different transgenic tobacco lines were detected via qRT-PCR. **D** The expression levels of other leaves and lines 40, 45, and WT leaf veins were detected via qRT-PCR

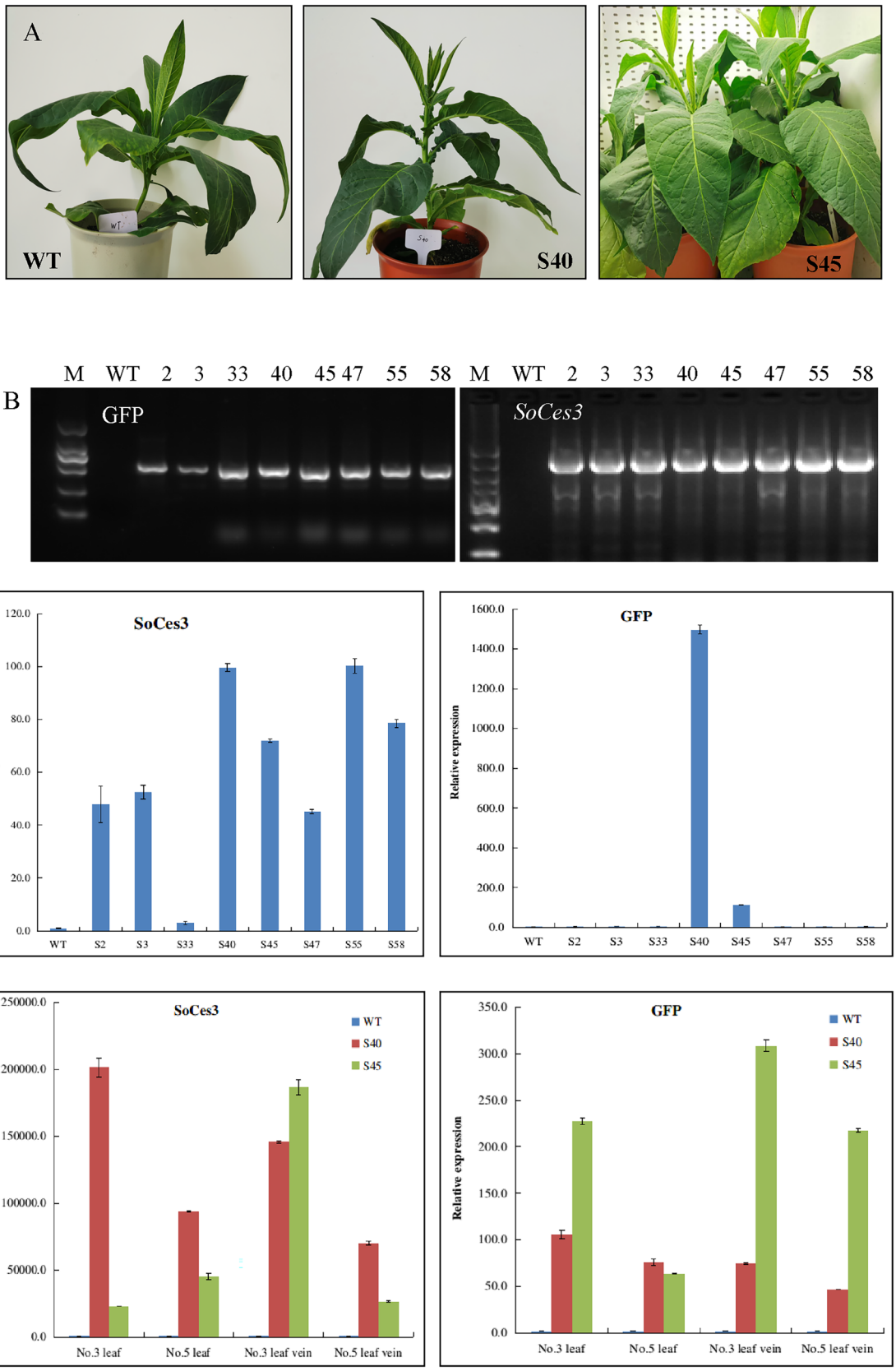


Fig. 4 (See legend on previous page.)

(Fig. 4C). Based on the transcripts of *SoCes3*, two lines (*SoCes3*-40 and *SoCes3*-45) were selected for the further experiments at 8th-leaf growth stage from top to bottom (Fig. 4A). Third and fifth leaf of plant, *SoCes3*-40 showed a relatively high expression level of *SoCes3* gene than those of *SoCes3*-45 and WT. Third leaf of *SoCes3*-45 showed a relatively higher expression level. On the third leaf and vein, and fifth leaf vein of plants, *SoCes3*-45 showed a relatively higher expression level than *SoCes3*-40 and WT plants.

SoCes3-overexpression of tobacco increases cellulose and lignin contents

The cellulose and lignin contents were found higher in transgenic tobacco leaves and leaf veins. As shown in Fig. 5A, B, the cellulose content increased in the transgenic plant *SoCes3*-40 of third and fifth leaf, and third leaf, but not in the transgenic plant *SoCes3*-45. The cellulose content was found higher in 5th leaf vein of WT as compared to *SoCes3*-40 and *SoCes3*-45. Lignin content increased in the transgenic plants *SoCes3*-40 and *SoCes3*-45 as compared to WT plants (Fig. 5B).

Histological observation of *SoCes3*-overexpression

Figure 6 shows that the histological differences between *SoCes3* transgenic tobacco plants and WT plants. It can be seen from the transverse stem sections stained with fluorescence single-label staining of paraffin sections in transgenic tobacco plants. *SoCes3* transgenic tobacco has stronger fluorescence signals than the WT and mainly primary cell wall tissues. Cell wall thickening suggests that the cellulose content in transgenic materials can upregulate (Fig. 6A). The expression pattern of *SoCes3* in heterologous plant tobacco is similar to that of Arabidopsis *AtCESA*3 [25, 27, 39], and related to the biosynthesis of primary cell wall.

Regulation of *SoCes3* gene overexpression in tobacco plants

Here, the WT and *SoCes3* transgenic lines *SoCes3-40* and *SoCes3-45* were compared for further analyses. Performed a comparative transcriptomic analysis between WT (K1) and *SoCes3* transgenic lines, i.e., *SoCes3-40* (A1) and *SoCes3-45* (A5) 3rd leaves using high-throughput RNA sequencing (RNA-seq). After background correction and normalization, a total of 2993 and 4481 differentially expressed genes (DEGs) were identified from the leaves of *SoCes3*-overexpressing transgenic tobacco plants, respectively (Fig. 7A, B).

GO annotation is mainly divided into three categories, such as biological processes, molecular functions, and cellular composition, and the results of A1 vs K1 and A5 vs K1 showed that the 22 and 17 most significantly enriched subcategories among the three major categories revealed that the cell wall biogenesis and metabolic, and cellulose biosynthetic processes (Fig. 8). Cell wall biogenesis, xyloglucan metabolic process, xyloglucan: xyloglucosyl transferase activity was the most significant biological process of differential gene enrichment. However, all the upregulated genes were selected to elucidate the cell wall biogenesis mechanism (Fig. 9).

Discussion

Cellulose is a main component of plant biomass and represents 42–46% (w/w) of sugarcane cell wall components, is responsible for the rigidity of the cell

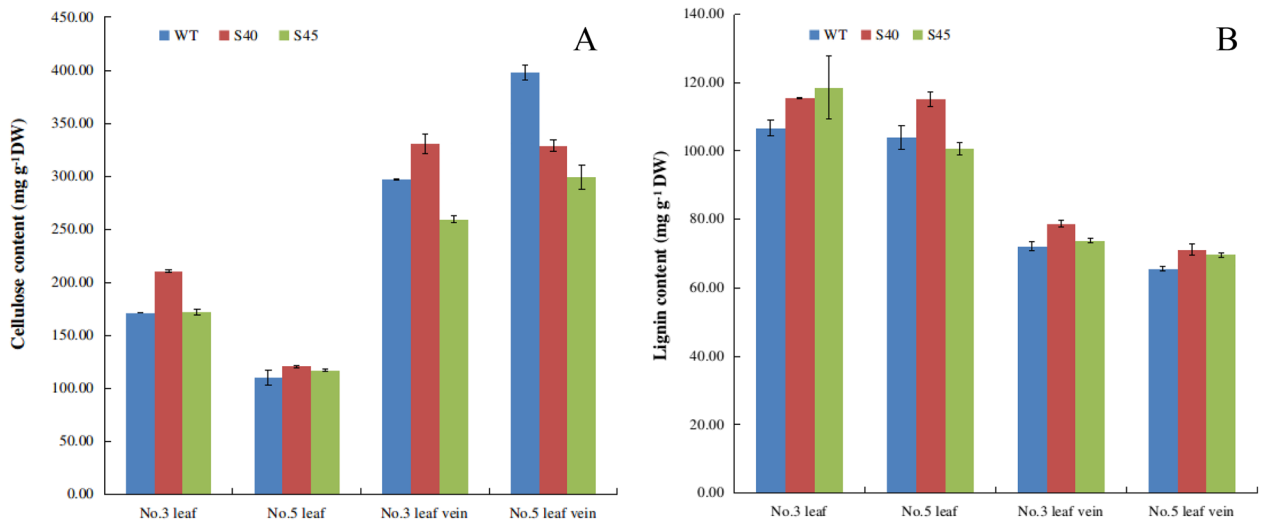


Fig. 5 Measurements of cellulose contents (**A**) and lignin contents (**B**). Error bars indicate standard error of three biological replicates ($n = 3$)

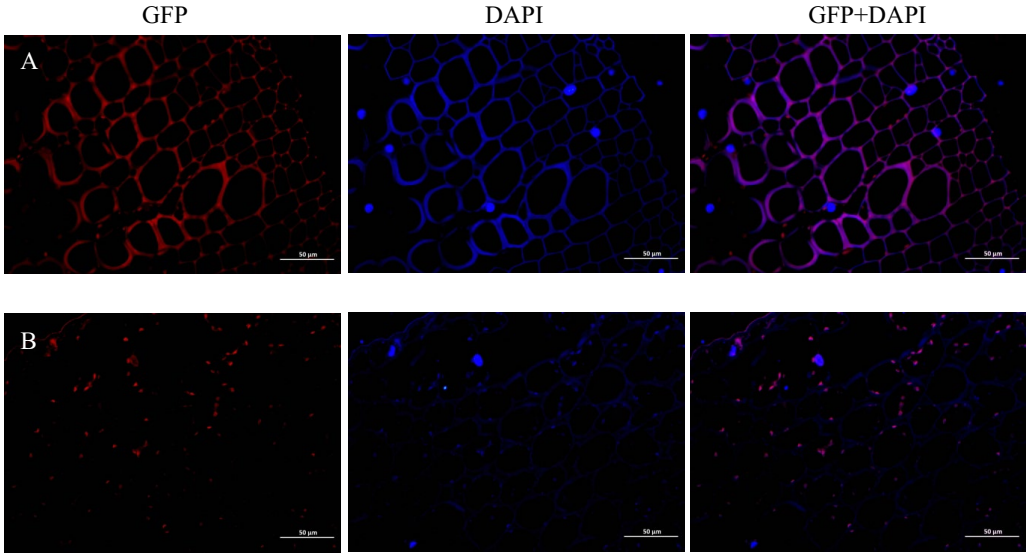


Fig. 6 Immunofluorescence test using GFP antibodies of transgenic tobacco in 3rd leaf vein. **A** *SoCes3* tobacco, **B** WT tobacco. Red fluorescent signals indicate *SoCes3* foci, cell wall, and nucleus indicated by DAPI staining. Bars = 50 μm

wall, providing the backbone to determine cell structure and maintaining the resistance of turgor pressure. The predominant polysaccharide component in culm cell walls is cellulose [39, 40]. Cellulose accounts for 28–30% of the above-ground dry matter in typical forage grasses [41], 42–45% in wood [42], and in sugarcane and energy cane cultivars [43]. It is composed of UDP-α-D-glucose through the catalysis by cellulose synthase, and degraded by cellulase. Cellulose biosynthesis in various species varies on specific members of the *CesA* family [44]. As the most abundant reservoir of C in nature, cellulose and other polymers can be prominent competing sinks for C in sugarcane [45]. Cellulose synthesis is catalyzed by enzyme complexes of cellulose synthase (*CesA*), arranged in rosette formations at the bases of growing cellulose fibrils. The *CesA* reactions are coordinated with those of SuSy that operate in the degradative direction in supplying UDP-Glu substrates at cellulose synthesis sites [46].

CesA gene families, which belong to the cellulose synthase gene superfamily consisting of more than eight membranes in higher plants [47, 48]. The *CesA* gene that synthesizes the β-1,4-linked glycan backbone of cellulose and hemicellulose polysaccharides encodes enzymes related to cellulose and hemicellulose polysaccharides [49]. Cellulose in plants are synthesized by multimeric protein complexes, which consist of hexameric, rosette-like structures in the plasma membrane [50, 51]. Cellulose is made of glucose linear polymer obtained from activated sugar donor UDP-glucose which is available due to sucrose cleavage by sucrose synthase (SuSy) [51–53]. The use of glucose by cleaving the stored sucrose in mature internodes may not occur in sugarcane, because other sources, including the depolymerization of the cell wall, improved photosynthate manufacture, or other metabolic pathways are available to provide glucose, so sucrose content is not affected, particularly in internodes of high sucrose sugarcane [54, 55].

All members of the *CesA* family contain a putative LIM-like Zn-binding domain/RING finger domain in the N-terminal region, which is similar to several putative plant Leucine zipper transcription factors [6, 7, 46]. All *CesA* gene products contain a D, D, D, QxxRW motif [21]. The amino-terminal domains are two predicted transmembrane domains near positions 270 and 300 in the Arabidopsis *CesA* proteins. The carboxy-terminal portion of the protein, extending from nearly 850 amino acid positions, contains six additional predicted transmembrane domains [56]. The various members of the plant *CesA* family range between 985 and 1088 amino acids and can vary in sequence identity from 53 to 98% [56]. The present study identified sugarcane *CesA3* of 1074 amino acids in length. The predicted protein contains random coils, α-helices, extended strands, and non-signal peptide structure.

There are more than 1,250 *CesA* and *Csl* sequences from 29 different plant species in GenBank. The genes are responsible for three distinct brittle mutations of rice, induced by the insertion of the endogenous retrotransposon *Tos17*, which correspond to *CesA* (cellulose synthase catalytic subunit) genes, *OsCesA4*, *OsCesA7*, and *OsCesA9*. Three *CesA* genes were

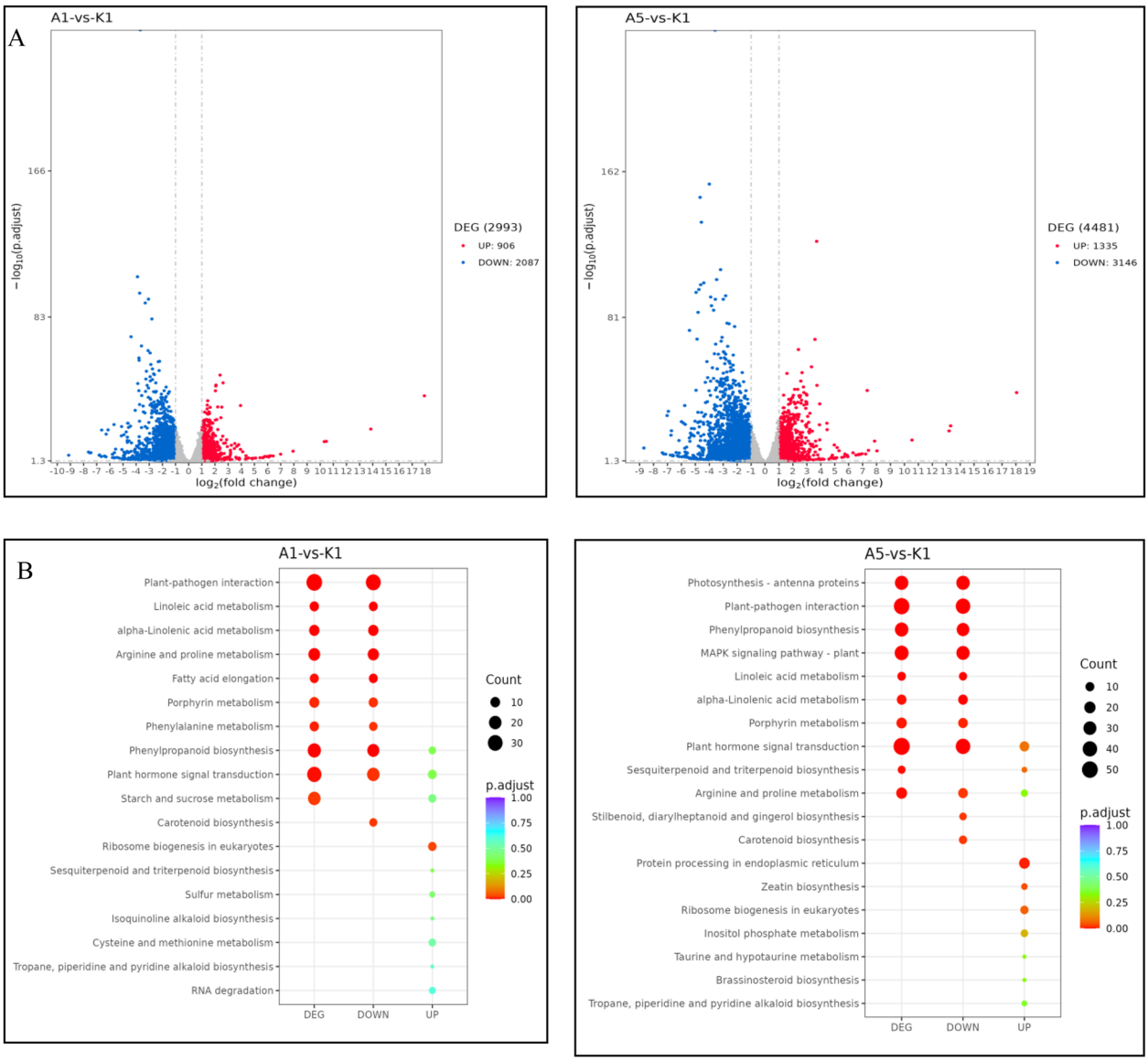


Fig. 7 Numbers of DEGs in various comparisons. **A** The bar graph presents the number of up- and down-regulated DEGs. **B** KEGG pathway enrichment analysis. K1: wild type; A1: *SoCes3*-overexpressing transgenic tobacco lines *SoCes3*-40; A5: *SoCes3*-overexpressing transgenic tobacco lines *SoCes3*-45

expressed in seedlings, culms, premature panicles, and roots but not in mature leaves, and the expression profiles were almost identical among the three genes [23]. The *Eucalyptus Cellulose Synthase* 3 (*EgCesA*3) gene had its expression pattern evaluated in leaf and xylem tissues among the three most economically important *Eucalyptus* species, such as *E. grandis*, *E. globulus*, and *E. urophylla* in Brazil [57]. Three of the maize genes, *ZmCesA10-12*, are associated with secondary wall formation [15].

Among 25 annotated sorghum cellulose synthase and 28 cellulase genes, 5 (20%) and 7 (25%) of them, were upregulated in optimum sugarcane biomass and no down-regulation was monitored [43, 58, 59]. In *Arabidopsis thaliana CesA*4, 7, and 8 are associated with the deposition of the secondary cell wall [13, 60], while *CesA*10 and *CesA*12 are responsible for the same process in *Zea mays* plants [15], and *CesA*7 and *CesA*8 in *Oryza sativa*. *CesA*3 is related to the primary cell wall biosynthesis in *Zea mays* plants [15, 61]. The upregulation

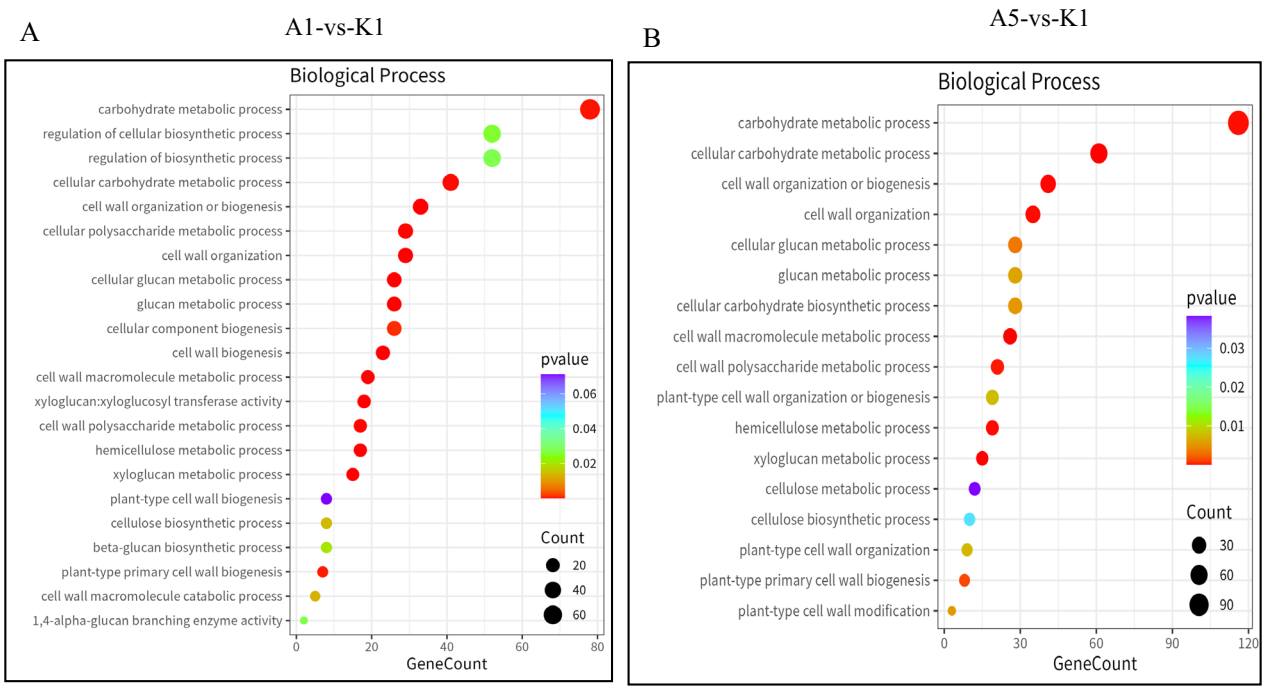


Fig. 8 Assessment of cellular biosynthetic-related genes induced by *SoCes3* gene. Comparison of GO annotation of DEGs between A1-vs-K1 and A5-vs-K1 biological processes (**A**, **B**). K1: wild type; A1: *SoCes3*-overexpressing transgenic tobacco lines *SoCes3*-40; A5: *SoCes3*-overexpressing transgenic tobacco lines *SoCes3*-45

of *CesA*10, 11, 12 in the rind, as compared to vascular parenchyma and vascular bundles of mature internodes of sugarcane [62]. Primary and secondary cell wall biosynthesis is a dynamic process and is active in immature and mature culms of sugarcane [63]. Poplar is a model tree with better genome annotation and contains 18 *CesA* genes. It is reported that several *CesA* genes in poplar are involved in cellulose synthesis [64].

Stalk lodging in sugarcane results in significant yield losses. In maize, an increase in cellulose concentration in the wall might permit simultaneous improvements in stalk strength and harvest index [15]. Isolation of the expressed *CesA* genes from sugarcane and their association with primary or secondary wall formation has allowed testing of their respective roles in cellulose synthesis through mutational genetics or transgenic approaches. This information would be useful in improving stalk strength. Isolation of the expressed *CesA* genes from maize and their association with primary or secondary wall formation, possible to test their respective roles in cellulose synthesis in different cell types through association genetics, mutational genetics, or a transgenic approach. It would be useful in improving stalk strength in cereals. *AtCesA3*(*IXR1)* was highly expressed in stems, flowers, roots, and shoots. *AtCesA3*(*IXR1*) appeared to be expressed more in roots than shoots [65]. The expression of 10 *Arabidopsis CesA* genes in the embryo at three postembryonic stages by semiquantitative reverse transcription (RT)-PCR. All 10 *CesA* genes were represented at the young plant, stem, and flowering stage, whereas only *CesA1*, *CesA2*, *CesA3*, and *CesA9* were significantly expressed in the embryo [66].

Auxin has been shown to promote the expression of a putative cellulose synthase gene (*At2g32610*) while

(See figure on next page.)

Fig. 9 Characterization of upregulated DEGs in transgenic tobacco. **A** The heatmap of DEGs between A1-vs-K1 and A5-vs-K1. **B** Top 20 GO functional annotations in biological process corresponding to **A**. **C** The heatmap of DEGs between A1-vs-K1 and A5-vs-K1 related to cellular biosynthesis. **D** Top 20 GO functional annotations in biological process corresponding to **C**. **A**, **C** Heatmap of selected DEGs related to cellular biosynthesis based on TPM or log2FC values. In **A**, **C**, the TPM values of DEGs were standardized by log2 (TPM + 1). The dark color, the higher gene expression level. In **B**, **D**, the gene ratio represents the percentage of selected genes, and the circle size represents gene numbers. The bigger circle means more gene numbers. The color of circle represents the p-value. The dark color smaller the p-value with higher significant difference. The darker color, the larger gene fold change. K1: wild type; A1: *SoCes3*-overexpressing transgenic tobacco lines *SoCes3-40; A5: SoCes3*-overexpressing transgenic tobacco lines *SoCes3-45*

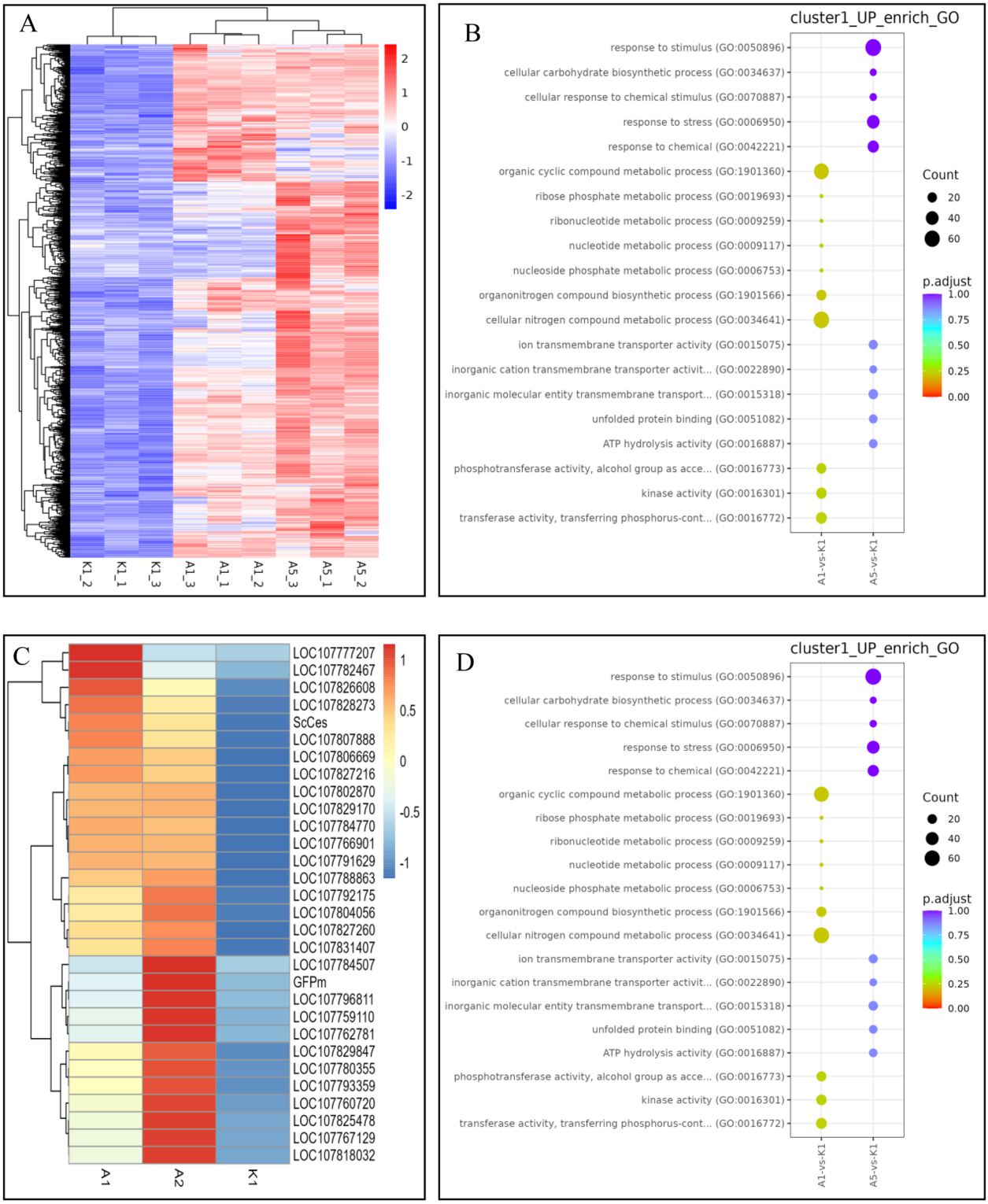


Fig. 9 (See legend on previous page.)

inhibiting another putative cellulose synthase gene (*At2g32530*) in *Arabidopsis* [67]. In response to auxin treatment, the *GhCesA4* promoter was inducible in transgenic tobacco plants [68]. Such results were similar to the expression profile of *GhCesA4* in the fibers where IAA levels were low in the stage of rapid cell elongation and then accumulated after the fiber entered the secondary thickening phase [69]. The upregulation of multi-CeS complex DEGs in immature internodes is a sign of the abundant requirement of cellulose in actively growing internodes. Some members of the *CesA* complex were upregulated in the maturing internodes of the mutant clones, suggesting that cellulose synthesis is still turned on, driving the internodes to final maturity [49, 51]. During culm maturation in sugarcane, the cellulose synthesis is regulated through coordinated expression of diverse genes and gene families including those encoding cellulose synthases, cellulose synthase-likes, enzymes for lignin biosynthesis, and a range of other genes identified in clusters from expression profiles of different tissues [63, 70]. *CesA*s are responsible for cellulose biosynthesis in secondary walls, and in most cases, the genotypes showed increasing expression levels across time points, this likely indicates ample synthesis of secondary cell walls in these young sugarcane culms [61].

In conclusion, transgenic *N. tabacum* overexpressing the *SoCes3* gene was successfully obtained, and transgenic plant analyses are being performed. Although we are unable to define whether the genetic differences in response to aberrant cell walls exist in sugarcane, it is evident that the process of how *CesA* works in higher plants is very complicated. Various questions regarding the *CesA* function remain to be answered. However, it is already possible to notice, by empirical view, growth improvement in the transgenic plants compared to the wild type.

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Author contributions
Conceptualization: C.M.H., K.C.W., Y.W.W., and L.X.; writing—original draft: C.M.H., K.C.W, and K.K.V.; resources: H.B.L., Z.N.D., G.Y.S., H.Q.C., X.J.W., and L.P.Y.; writing—review and editing: Y.W.W., and L.X.; supervision: Y.W.W., and L.X.; project administration: K.C.W., Y.W.W., and L.X.; funding acquisition: K.C.W., Y.W.W., and L.X. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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