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Genome-Wide Identification and Expression Analysis of CesA Gene Family in *Corymbia citriodora*

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Abstract: The CesA proteins are key players in cellulose synthesis. *Eucalyptus* is well-known for cellulose synthesis, although the mechanism of cellulose synthesis is largely unknown. In this study, 11 CcCesA genes were identified by comprehensive bioinformatics analysis in *Corymbia citriodora*, a eucalypt species. CcCesA genes were generally conserved in each subfamily. Among them, four genes lacked a transmembrane domain in the N-terminal. We showed that genes of CesA2, 4, 5, and 8 may regulate the synthesis of secondary cell walls (SCWs) through phylogenetic trees analysis. Two pairs of segmental duplication and one pair of tandem duplication were detected in CcCesAs. Analysis of Ka/Ks ratios revealed that the duplicated genes were under negative or purifying selection. Numerous cis-acting elements related to plant hormones and light reactions were identified in the promoters of CcCesAs. Expression analysis confirmed that genes of CesA2, 4, and 8 regulate the synthesis of SCWs, and CesA8, in particular, had the highest expression compared with other genes. The results will help us understand the complexity of the CcCesAs in different diameters at breast height and provide valuable information for future functional characterization of specific genes in *C. citriodora*.

Keywords: CesA; gene expression; *Corymbia citriodora*

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1. Introduction

Cellulose, the most important structural component of the plant cell wall, is a polysaccharide containing repeated unbranched β (1–4) D-glucose units synthesized on the plasma membrane by a cellulose synthase (CesA) complex (CSC) in bacteria and plants [1]. CSC is rosette-like structure and consists of six globular protein complexes, which are involved in the biosynthesis of cellulose microfibers containing multiple cellulose synthase proteins [2]. In addition to the CesA gene family, non-CesA genes such as radial swelling root 3 (RSW3) and cytochrome 1 (CYT1) are involved in cellulose biosynthesis [3]. Understanding the biosynthesis mechanism of cellulose is beneficial to improve the production of economically valuable forest wood, and is of great significance to plant breeding. However, cellulose biosynthesis is a complex system whose details are still largely unclear [4]. The properties of cellulose are determined by the co-synthesis of multiple glucan chains, whose collective properties confer higher strength and cell wall rigidity, as well as increased resistance to chemical attacks [5]. The functions of cellulose include controlling cell wall morphology and making certain plant cells specific to the cell wall [6]. Therefore, research related to cellulose synthesis has instructive significance.

Main components of cell walls are polysaccharides, such as cellulose, hemicellulose, and pectin, which determine the shape of plant cells and how they respond to their environment [7]. In higher plants, two types of plant cell wall are found [8]. The primary

cell wall (PCW) is created during cell division and expansion and determines the cell shape and volume. After cell expansion, certain types of plant cells produce a lignified cell wall called a secondary cell wall (SCW). Most of the plant biomass is secondary cell walls, which accumulate in xylem tissues and are largely difficult to biodegrade and glycosylate. In contrast, primary cell walls are chemically unique, flexible, and usually non-lignified, making them easier to break down [9]. In addition to cellulose, the main component of SCWs, lignin, is frequently deposited in the SCW and is a compound polymer of aromatic alcohols that provides mechanical strength and water resistance for SCWs of sclerenchyma cells such as vessel elements and fibers [10].

The CesA gene family belongs to the cellulose-synthase-like superfamily and is responsible for the synthesis of cellulose. This gene family belongs to the second family of glycosyltransferases (GT2) and contains three aspartyl residues, a QxxRW motif, and a zinc-finger domain, which catalyzes the linkage between glycosyltransferases [11]. In plants, CesA genes were first cloned from cotton (*Gossypium hirsutum*) fiber [12]. The CesA protein sequence is highly conserved, containing eight transmembrane domains, two at the N-terminus and six at the C-terminus [5]. In the advanced structure of CesA protein, these six transmembrane regions can form a channel to secrete glucose chains, while three aspartyl residues are adjacent to each other and close to another conserved structure, QXXRW, forming a functional domain with catalytic glycosyltransference activity, and the CesA protein transmembrane region forms adjacent to the glucose-chain secretion channels [13]. In *Arabidopsis thaliana*, there are at least 10 CesA genes, among which three genes (AtCesA1, 3, and 6) are involved in the formation of PCWs, and the other three genes (AtCesA4, 7, and 8) are involved in the formation of SCWs. The remaining genes (AtCesA2, 5, and 9) are partially redundant with AtCesA6, because they show non-overlapping expression patterns, and the null mutants of these genes show mild alterations in seed-coat development and root/hypocotyl elongation [11,14]. In recent years, the cellulose-biosynthesis gene family has been extensively studied in various plants, including identification, expression analysis, and function analysis of this gene family [15–18]. The CesA gene family is involved in more economic crops than trees. In forest trees, the CesA gene family has been evaluated in *Acacia mangium*, *Eucalyptus grandis*, *Poplar trichocarpa*, *Betula platyphylla*, etc. [19–22]. Therefore, it is necessary to identify new trees of economic value.

Corymbia citriodora maintains a high-growth rate under the conditions of drought, poor-quality soil, and biotic stresses. It is grown for timber, pulp and paper, and essential oils in Australia, South Africa, Asia, and Brazil [23]. A recent study found that *C. citriodora* could be used in construction, greatly increasing its economic value [24]. However, few studies have been done on the genes of *C. citriodora*. It has been reported that the expression of putative phosphate transporter 1 (Pht1) genes in *C. citriodora* does not increase with the increase of seedling age, suggesting that *C. citriodora* is not sensitive to phosphorus deficiency [25]. Wood is the most abundant biomass on earth and consists of secondary xylem derived from the vascular cambium [26]. Yet the underlying molecular mechanisms of wood formation remain largely unknown. In our field investigation, we found differences in diameter at breast height (DBH) among *C. citriodora* of the same age, which may be influenced by genes regulating xylem. In the past, xylem was used as experimental material in the studies of cellulose synthase. In view of previous studies, the CesA gene family may be involved in cambium activity and secondary xylem formation [27], and cambium was thus selected as the test material.

Nevertheless, no comprehensive study on CesA genes in *C. citriodora* has been reported so far. With the release of the *C. citriodora* genome [23], it is feasible to systematically study the putative function of the CesA genes in *C. citriodora*. In this study, 11 non-redundant members of the CcCesA gene family were identified from this species. Subsequently, we performed genome-wide comparative analysis of CcCesA sequences and constructed phylogenetic analyses of the CesA gene family in six plants. Domain organization, motif discovery, gene structure, gene duplication, and synteny analysis are all conducive to understanding the evolutionary relationships among members of the CesA gene family.

The results of our study will provide a basis for further functional identification of CesA genes in *C. citriodora*.

2. Methods

2.1. Identification of CesA Genes in the C. citriodora Genome

C. citriodora gene sequence files, gene annotation files, protein sequences, and coding DNA sequence files were downloaded from the Phytozome (https://phytozome-next.jgi.doe.gov/ (accessed on 13 February 2022)) website. The Phytozome ID is 507. Protein sequences of Arabidopsis CesA gene family were downloaded from Phytozome, and the protein sequence of Arabidopsis CesA1 (At4g32410) was compared with the *C. citriodora* genome to screen out candidate genes on the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 13 February 2022)) website. Putative CesA sequences were uploaded to Pfam (http://pfam.xfam.org/ (accessed on 15 February 2022)) and SMART (http://smart.embl-heidelberg.de/ (accessed on 15 February 2022)) databases to identify sequences containing protein domains (PF03552 and PF14569). Subsequently, the molecular weights (MWs), isoelectric points (pI) and grand average of hydropathicity (GRAVY) of CcCesA proteins were calculated in the ExPasy website (https://web.expasy.org/protparam/ (accessed on 18 February 2022)). The subcellular location of CcCesA proteins was predicted by WoLF PSORT (https://wolfpsort.hgc.jp/ (accessed on 22 February 2022)). TMHMM (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0 (accessed on 22 February 2022)) was used to predict the transmembrane helices (TMHs) for each putative peptide.

2.2. Sequence Alignment and Phylogenetic Analysis

In addition to the *Arabidopsis thaliana* and *C. citriodora* mentioned above, we downloaded the CesA protein sequences of *B. platyphylla*, *E. grandis*, *P. deltoides*, and *Oryza sativa* from the Phytozome website. ClustalW program (version 2.1; http://www.clustal.org/ (accessed on 25 February 2022)) was used for multiple sequence alignment, and the phylogenetic tree based on CesAs protein sequence was constructed by using the MEGA5.0 program neighbor-joining method and bootstrap analysis (1000 replicates).

2.3. Chromosome Localization, Genome Synteny, and Gene Duplication Analyses

Chromosomal locations of all CcCesA family genes were confirmed using the *C. citriodora* genome annotation file downloaded from Phytozome and mapped using the TBtools [28]. The NCBI BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 26 February 2022)) of the CcCesA genes against each other was conducted to identify duplicated genes based on the query coverage percentage and identity of each gene. Segment duplication genes were defined as the query coverage percentage and identity of the candidate genes was $\geq$80%, while tandem duplication genes were defined as the genes tightly linked within 200 kb, and the identity of the genes $\geq$ 70% [29]. Syntenic analysis of CesA genes between *C. citriodora* and two representative plant species (*A. thaliana* and *E. grandis*) used MCScanX [30] (default parameters) to examine duplication genes [31]. Homology relationships were visualized by Tbtools software.

2.4. Analysis of Ks and Ka of Homologous Genes in CcCesA Family

Tbtools software was used to calculate the synonymous replacement rate (Ks), nonsynonymous replacement rate (Ka), and Ka/Ks ratio between homologous gene pairs of CcCesAs, and to analyze the selection pressure during the evolution of CcCesA family genes. The formula T = Ks/2r Mya (millions of years) was used to calculate the divergence time (T) of each duplicated gene pair, where Ks was synonymous substitution at each site of dicotyledons, and r was 1.5×10^{-8} substitution at each site of dicotyledons per year [32].

2.5. Motif Identification and Gene Structure

The CcCesA family gene structures were displayed by comparing the coding and genomic sequences with the TBtools. The obtained conserved motifs of the CcCesA fam-

ily proteins were analyzed through the online website MEME (https://meme-suite.org/ (accessed on 2 March 2022)).

2.6. Protein Structure Analysis of CesA Family Proteins in C. citriodora

SOPMA online software (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html (accessed on 5 March 2022)) was used to predict the secondary structure of the CesA family proteins of *C. citriodora*, and different conformation such as spiral, fold, rotation, and random curl was obtained. The tertiary structure of proteins was predicted using the SWISS-MODEL (https://swissmodel.expasy.org/interactive (accessed on 5 March 2022)).

2.7. Cis-Element Analysis for CcCesA Gene Promoters

The promoter sequences of 1500 bp upstream of each CcCesA gene-coding region were retrieved from Phytozome. The PlantCARE online program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 7 March 2022)) was used to search for assumed cis-acting elements. The location of cis-elements was annotated and displayed in a figure by building a physical gene map using TBtools.

2.8. RNA Isolation and Quantitative Real-Time Polymerase Chain Eaction (qRT-PCR) Analysis

Cambium of large diameter (20.7–23.6 cm), medium diameter (14.8–15.8 cm), and small diameter (9.6–10.8 cm) 6.5-year-old *C. citriodora* was collected at breast height (DBH) from 9 a.m. to 12 a.m. The DBH of different sizes were treated as three groups with six samples per treatment. All samples were collected and frozen in liquid nitrogen, then stored at −80 °C until used.

Total RNA was isolated using the Trelief RNAprep Pure Plant Plus Kit (Tsingke Biotech, Beijing China) according to the manufacturer's instructions. Then, the RNA was used for first-strand cDNA synthesis with reverse transcriptase and the reverse transcription was performed using 1 mg of total RNA and PrimeScrip RT reagent Kit with gDNA Eraser (TaKaRa, Tokyo). Subsequently, cDNA was used as a template for qRT-PCR analysis using Premier 5.0 primers based on *C. citriodora* internal reference gene sequences (Table 1). The specificity of primers to their target genes was evaluated on the website EnsemblPlants (http://plants.ensembl.org/hordeum_vulgare/info/index (accessed on 1 February 2022)). All of the primers were synthesized by TSINGKE Biotechnology Co., Ltd. (Beijing, China). A CFX96 real-time PCR system (Bio-RAD, Laboratories, Hercules, CA, USA) was used for qRT-PCR analysis. Each reaction mixture contained TransStart Top Green qPCR SuperMix 10 μL, cDNA template 1.5 μL, upstream primer 0.4 μL, downstream primer 0.4 μL, and sterile distilled water 7.7 μL. The reaction conditions were as follows: ① 94 °C, 30 s; ② 94 °C, 5 s; ③ 60 °C, 15 s; ④ 72 °C, 10 s. The analysis was completed after 40 cycles of reactions ②–④.

The relative expression levels of each treatment were calculated by the $2^{-\Delta\Delta CT}$ method, and the values represented the mean ± standard deviation (SD) of four independent biological replicates. The inner reference gene actin was used to normalize the data. CesA1 and the small DBH group were used as control. Differences in gene expression levels were analyzed via analysis of variance (ANOVA) followed by Student's t-test in R software and the Y-axis data were scaled by Log2.

Table 1. Primer sequences of CcCesA gene family for qRT-PCR.

Gene Number	Forward Primer Sequences	Reverse Primer Sequences
Cocit.F0728.1.v2.1	CCTATATGGTATGGTTACAGTGGGA	GTGCAATAAGCAATCAGAGGGA
Cocit.A0286.1.v2.1	CCAGGTTTATTTGGGAAGTGCT	GCCTTCTTGTGGTGCTGGTAA
Cocit.C1849.1.v2.1	TATGACGCAGATGCCAAACAA	TAATCAACCGAAGGATAATCACAAC
Cocit.C0102.1.v2.1	GATGCCTTCTTCCCTTCACAA	TCCACCCTCCCTCTTTCTTTT
Cocit.C0101.1.v2.1	TGGTATGGTTACAAAGCAGGGA	GTAGGCGATGAGTGGTAAAGAGG
Cocit.G2461.1.v2.1	GGATCAGTTTCCGAAGTGGTTT	CAGTATTTGCCGTCACAAGAGG
Cocit.H0683.1.v2.1	TGTGGTTTCTGTCGCTTTTCAT	CGAGACTCCTCCAATGACCC
Cocit.D0255.1.v2.1	CCGCTCTTTGGCAAAGTCTT	ACAATGGTCGGAGTCCTGTTCT
Cocit.J0893.1.v2.1	GCCAGAACCGCACTCCTACT	TCAATCCTCACCCACAACAAAG
Cocit.L2008.1.v2.1	AATACAGATGATTTGGAGCGTGAG	AAAGTTAAGGCGAGTGGACAGC
Cocit.L2008.2.v2.1	GGGTCTCATCTCACCTGTTTGC	TTGGACGTGACCATGAAGTTTG
Cocit.I0213.1.v2.1	GAAAGAAGCCATTCAAGTCATCAG	ATCCTCAGTCACCGAACCGTAT
Actin	TCAGGATAAGTCTTGGCGAGTG	TCCGTCCTTGTAGGCTCTGG

3. Results

3.1. Identification of CcCesA Genes

In this studies, amino acid sequences of *Arabidopsis* CesA1 genes were applied to identify homologs of CesA proteins in *C. citriodora* by using BLAST. After analyzing the conserved domain and removing the redundant sequences, a total of 11 putative CcCesA genes were identified in *C. citriodora*, including two copies of the CcCesA10 gene. For convenience, the 11 CcCesA genes were named as CcCesA1 to CcCesA11.

Detailed information of CcCesAs is listed in Table 2. The putative cellulose synthase genes in this analysis were predicted to range from 978 to 1096 amino acids in length and 110.02 kDa to 123.76 kDa in molecular weight. Furthermore, the protein isoelectric points (pIs) ranged from 6.18 to 7.41, the value of the instability index ranged from 37.21 (CcCesA2) to 42.75 (CcCesA3), the value of the aliphatic index ranged from 81.89 (CcCesA2) to 88.38 (CcCesA10.2), and the number of predicted TMHs ranged from 6 to 8. All genes had six transmembrane domains at the C-terminal, but CcCesA1, 3, 5, and 8 lacked two transmembrane domains at the N-terminal. In addition, the GRAVY of all CcCesAs was less than zero, indicating the hydrophilic nature of CcCesA proteins. The subcellular localization of the putative cellulose synthase genes was predicated to be located in the plasma membranes.

Table 2. Characteristics of CesA gene family in *C. citriodora*.

Gene Name	Accession Number	Protein (AA)	MW (kDa)	pI	Instability Index	Aliphatic Index	GRAVY	Loc	TMHs	Identity with AtCesA1 (%)
CcCesA1	Cocit.F0728.1.v2.1	1082	121.83	6.66	38.77	85.50	−0.225	plas	6	87.47
CcCesA2	Cocit.A0286.1.v2.1	1045	117.91	7.41	37.21	81.89	−0.238	plas	8	64.29
CcCesA3	Cocit.C1849.1.v2.1	1085	122.15	6.34	42.75	84.00	−0.247	plas	6	87.19
CcCesA4	Cocit.C0102.1.v2.1	1040	117.81	6.18	41.60	82.39	−0.218	plas	8	66.17
CcCesA5	Cocit.C0101.1.v2.1	1041	117.35	6.41	41.79	83.63	−0.224	plas	6	62.00
CcCesA6	Cocit.G2461.1.v2.1	1080	121.33	6.61	38.52	85.81	−0.198	plas	8	70.20
CcCesA7	Cocit.H0683.1.v2.1	1096	123.42	6.68	39.03	84.68	−0.201	plas	8	67.46
CcCesA8	Cocit.D0255.1.v2.1	978	110.02	6.33	39.01	85.72	−0.087	plas	6	63.43
CcCesA9	Cocit.J0893.1.v2.1	1079	121.05	7.00	41.02	85.26	−0.184	plas	8	69.89
CcCesA10.1	Cocit.L2008.1.v2.1	1092	123.76	6.50	40.24	87.72	−0.174	plas	8	64.50
CcCesA10.2	Cocit.L2008.2.v2.1	1021	115.46	6.38	41.29	88.38	−0.169	plas	8	67.30
CcCesA11	Cocit.I0213.1.v2.1	1092	123.51	6.52	40.70	85.12	−0.246	plas	8	64.65

Note: AA, MW, pI, GRAVY, Loc, Plas, and TMHs, respectively, indicate amino acid residues, molecular weight, theoretical isoelectric point, grand average of hydropathicity, subcellular location, plasma membrane, and transmembrane helices.

3.2. Phylogenetic Analysis of CesA Family

The protein sequences of CcCesAs and CesAs from five well-studied species *B. platyphylla*, *O. sativa*, *A. thaliana*, *P. deltoides*, and *E. grandis* were used to investigate the phylogenetic relationship of CesAs. An unrooted cladogram suggested that these CesAs were clearly clustered into five categories and these categories contained members from both monocot and dicot plants except group E (Figure 1). Each CesA gene contained the Cellulose_synt protein domain. According to previous studies on these species, the functions of CesAs are basically consistent with the phylogenetic relationships of other homologous higher plants. Except for *O. sativa*, the evolutionary relationships of CesAs in other species were consistent, indicating that CesAs between species was evolutionarily conserved. As a result, the CesAs (CcCesA2, 4, 5, and 8) in groups B and D may be involved in secondary cell wall biosynthesis, while others may participate in primary cell wall biosynthesis.

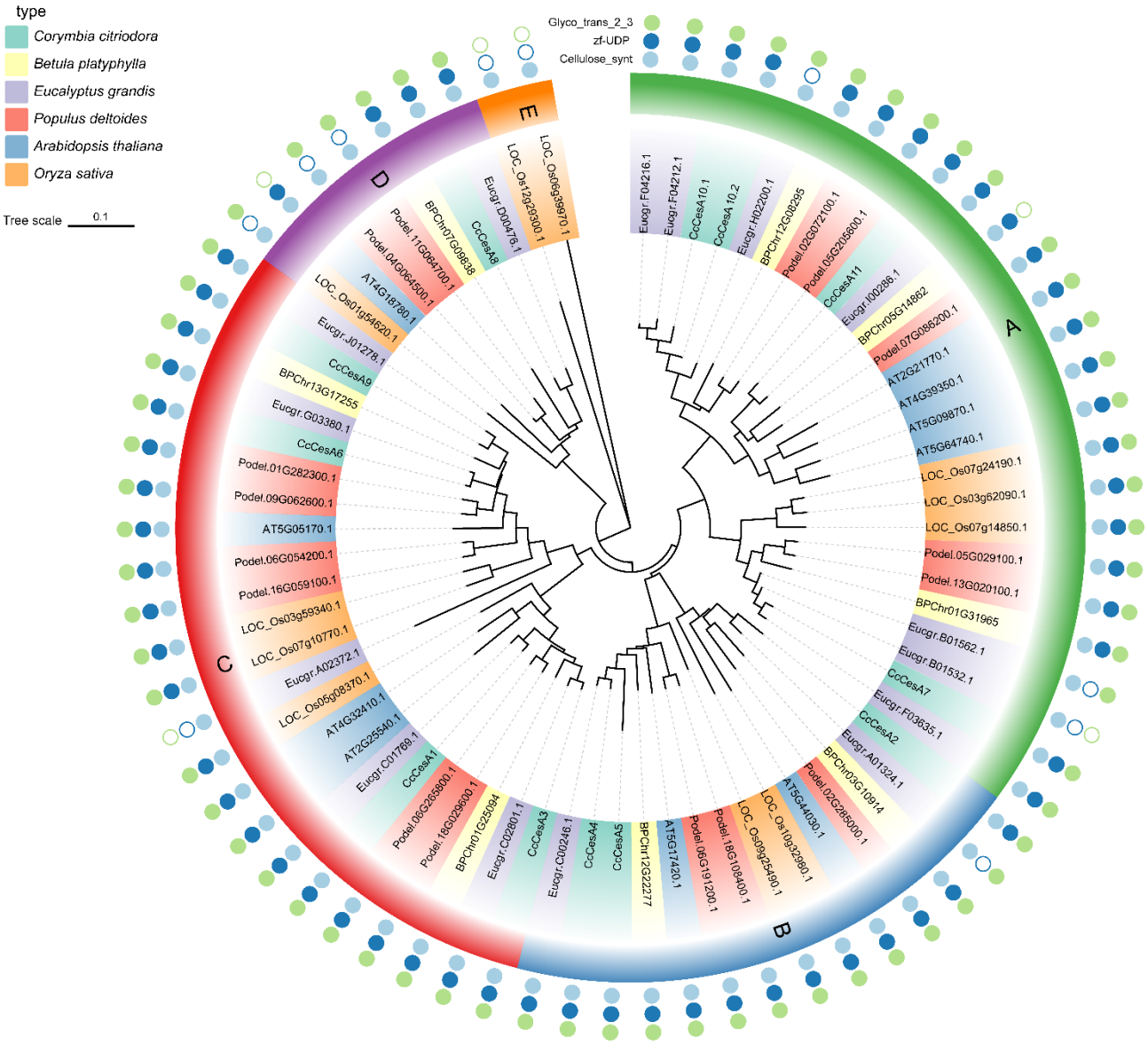

Figure 1. Phylogenetic tree of CesAs. Different species are shown in different colors. Groups A–E represent the classification of genes in the evolutionary tree. Solid and hollow circles correspond to the presence or absence of protein domains.

3.3. Chromosomal Localization, Gene Duplication, and Genome Synteny of CcCesAs

To generate the graphics of chromosomal positions of CcCesA genes, the physical locations of CcCesA genes were investigated by analysis of genomic distribution in chromosomes. The result revealed that all CcCesA genes, except for CcCesA10, were unevenly distributed on all 11 chromosomes in *C. citriodora*. Due to the important effect on functional differentiation and gene expansion, gene duplication events among CcCesA genes were also investigated. To determine the segmental duplication events between the genes, the following criterion was used [32]: when the query coverage percentage and identity of the candidate genes was ≥80%, they were considered to be duplicated genes. According to the previous studies, chromosomal regions within the 200 Kb range, and including two or more genes, were designated as tandem duplication [33]. In this study, two pairs of segmental duplicated genes (CcCesA1/3 and CcCesA6/7) and one pair of tandem duplicated genes (CcCesA4/5) were revealed and distributed (Figure 2). To determine the selection constraints on the duplicated CcCesA genes, we estimated the Ka/Ks ratio of each pair of paralogous genes using the Tbtools and found that the value of the Ka/Ks ratio of these paralogous genes was less than 1. These results indicated that these duplicated genes underwent strong purification/negative selection pressure, and almost no mutation occurred after duplication. The duplication of paralogous gene pairs is estimated to have occurred 10.93 to 30.98 million years ago (Mya) (Table S1). Gene density distribution showed that most genes are concentrated at two ends of the chromosome.

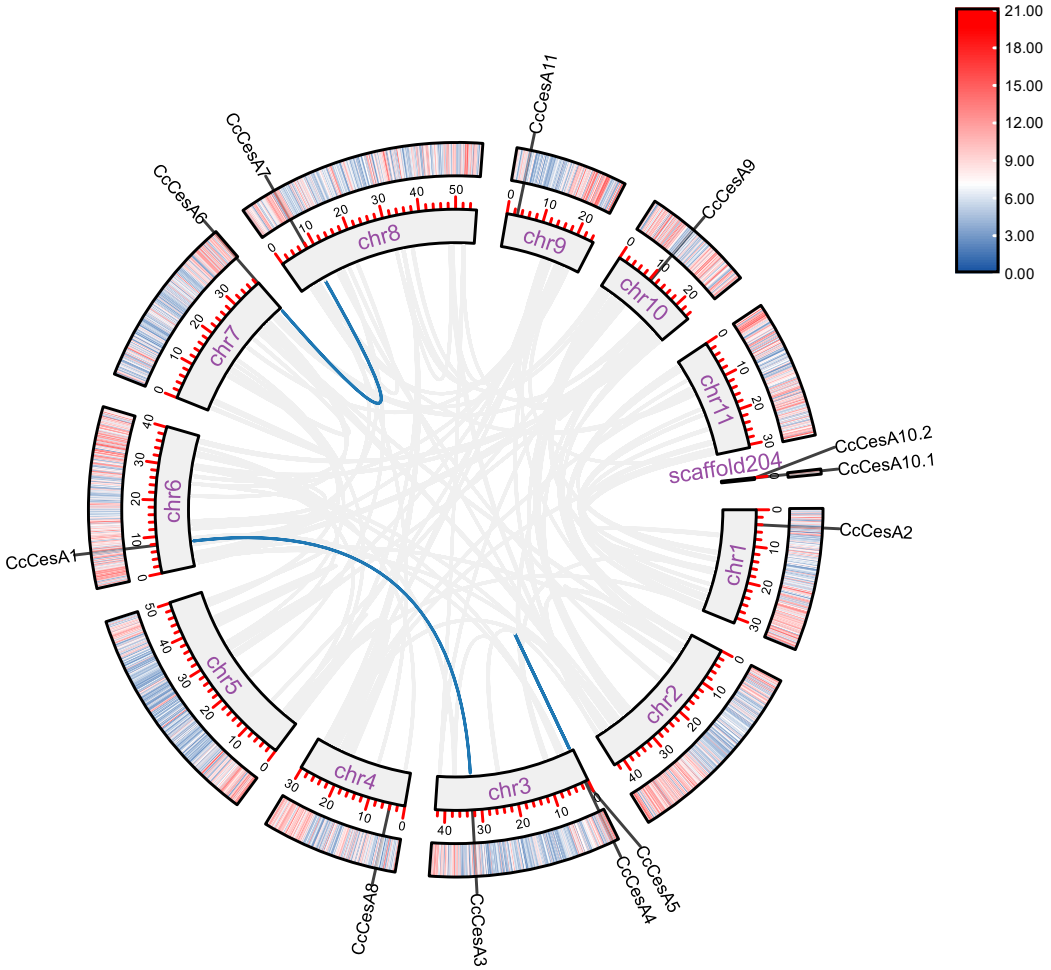

Figure 2. CcCesA chromosomal localization and tandem duplication events in the *C. citriodora* genome. The 11 chromosomes are plotted along with an Mb (million base pair) scale. A heat map shows the gene density of chromosomes. The blue line shows the homologous genes that are collinear. Gray lines indicate collinear blocks in the whole *C. citriodora* genome.

Based on a comparative micro-syntenic map of *C. citriodora* versus two representative plant species (including *A. thaliana* and *E. grandis*), we found two pairs of CesA orthologous genes between *C. citriodora* and *A. thaliana*, and nine pairs between *C. citriodora* and *E. grandis* (Figure 3). Detailed genetic pairs are shown in Table S2.

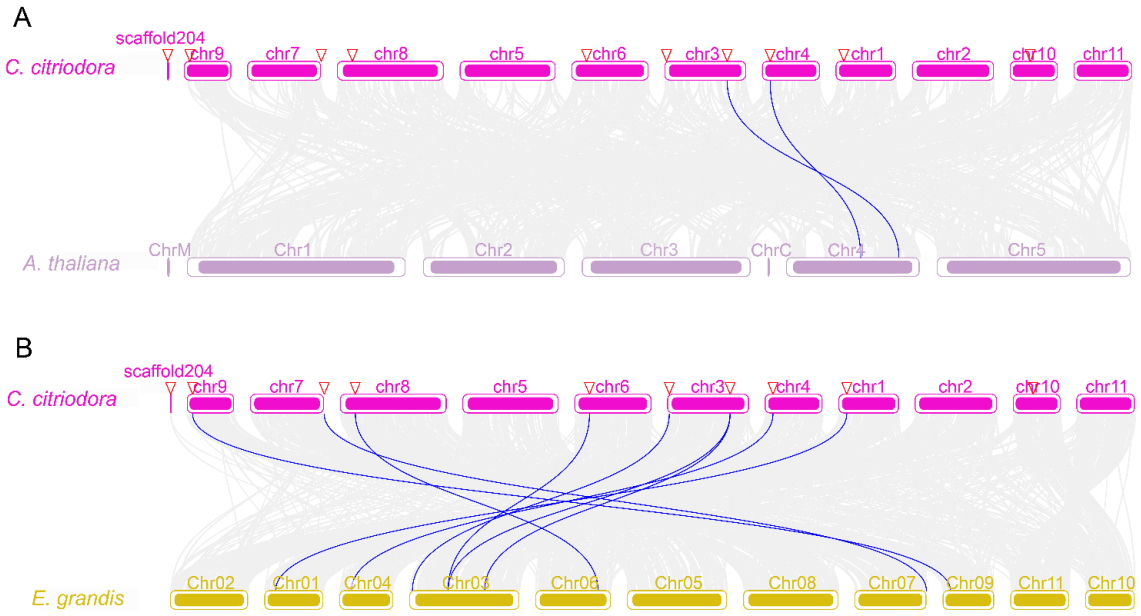

Figure 3. Syntenic analysis of CesA genes between *C. citriodora* and two representative plant species. (**A**) Syntenic relationship between *C. citriodora* and *Arabidopsis thaliana*. (**B**) Syntenic relationship between *C. citriodora* and *Eucalyptus grandis*. Gray lines at the bottom indicate the collinear blocks within the *C. citriodora* and other plant genomes. The blue lines indicate the pairs of CesA genes.

3.4. Motif Identification

To study the evolutionary relationship of CcCesAs, the phylogenetic tree of CcCesA proteins was built by using their amino acid sequences. The CcCesA proteins were classified into three subfamilies in this phylogenetic tree, which shows little difference to the results shown in Figure 4A based on phylogenetic analysis of the six plant species.

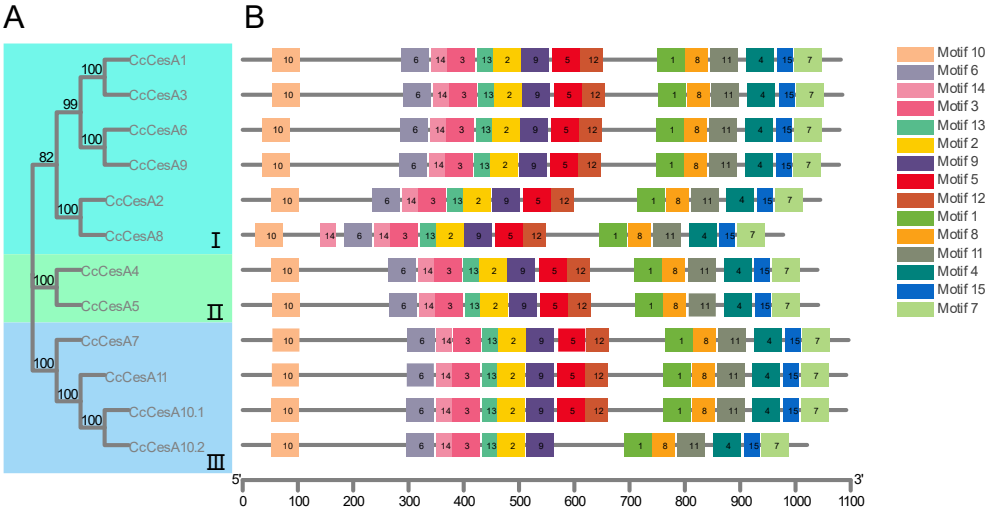

Figure 4. Phylogenetic and motif structural analysis of CcCesA genes and encoded proteins in *C. citriodora*. (**A**) The phylogenetic tree is constructed with the amino acid sequences of CcCesAs. Clades I–III are in different colors. (**B**) CcCesA protein motifs were identified using the online MEME program. Different colored boxes represent different motifs, where the number in center of each box indicates their name (Motifs 1 to 15).

To further investigate the diversity of the CcCesA genes, we analyzed CcCesA protein motifs using the MEME online server. Fifteen conserved motifs were identified, i.e., motifs 1 to 15 (Figure 4B). An overview of these protein motifs is presented in Figure S1. Among the 11 gene products, CcCesA10.2 lacked motif 5 and motif 12. The remaining CcCesA genes shared the same motifs.

3.5. Conserved Protein and Gene Structure Analysis of Cellulose Synthase Gene in C. citriodora

The structure of CcCesA proteins was investigated. All of CcCesA proteins had a zf-UDP domain in their 5′-terminus and a Glyco_trans_2_3 domain between two Cellulose_synt protein domains in their 3′-terminus (Figure 5A). The alignment of CcCesAs showed that the Glyco_trans_2_3 domain and the Cellulose_synt domain close to 3′-terminus were more conserved than the zf-UDP domains and Cellulose_synt domain close to 5′-terminus (Figure S2).

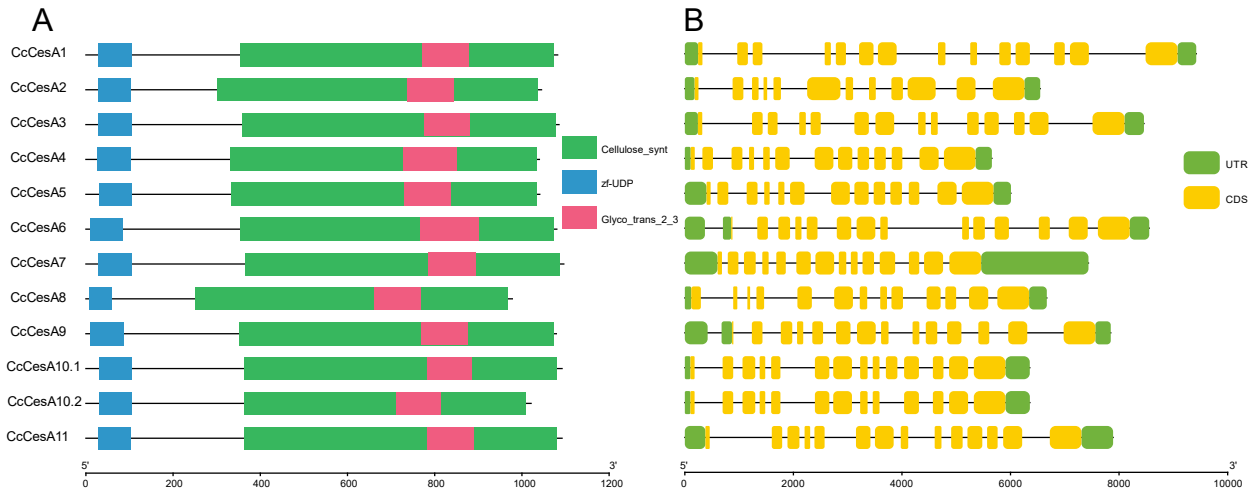

Figure 5. Conserved protein and exon–intron structure of the CcCesA genes. (**A**) Conserved protein structure. The blue boxes represent zf-UDP. The green boxes represent Cellulose_synt. The pink boxes represent Glyco_trans_2_3. (**B**) Exon–intron structures. Yellow boxes indicate exons. Solid lines indicate introns (connecting two exons). Green boxes indicate UTRs.

To provide more valuable information on CcCesA gene repetition events and evolution patterns, the gene structure is shown in Figure 5B. The number of exons varied from 12 to 14. Most of CcCesAs contained the highest numbers of exons (14), but CcCesA2 only had 12 exons among the CcCesAs. There was little difference in the length of different genes, among which CcCesA4 (5248 bp) was the shortest and CcCesA1 (9423 bp) the longest. Additionally, highly similar gene structure was identified in the same group of the CcCesA genes. For example, all CcCesAs (except CcCesA2 and 8) in group I contained 14 exons. The gene structure revealed that the CcCesA gene family was relatively conserved during evolution.

3.6. Modeling of Secondary and Tertiary Structural Homology of CcCesAs

The secondary structures of CcCesA proteins were compared (Table 3). The largest proportion of the secondary structures (12 proteins) was random coil, followed by α-helix, and the smallest proportion was β-turn. It is worth noting that there was little difference in protein secondary structure among genes. Online software SWISS-MODEL was used for homology modeling to predict the CcCesA gene family, and the results showed that the tertiary structure of the protein conserved region of the CcCesA gene family was very consistent (Figure 6). This particular structure is often related to its function, suggesting that their functions are likely to be similar. The transmembrane regions of the tertiary structure of CesA proteins were mainly composed of α-helix, and several transmembrane regions were connected and intersected with each other to form pore channels in the middle.

Table 3. Secondary protein structure of CcCesAs.

Gene	Alpha Helix (%)	Beta Turn (%)	Random Coil (%)	Extended Strand (%)
CcCesA1	31.89	4.53	47.78	15.80
CcCesA2	32.44	4.88	47.65	14.93
CcCesA3	32.26	4.61	47.47	15.67
CcCesA4	33.46	5.00	45.77	15.77
CcCesA5	32.56	5.00	47.07	15.37
CcCesA6	31.57	4.35	49.54	14.54
CcCesA7	31.57	6.20	44.71	17.52
CcCesA8	33.54	3.89	48.06	14.52
CcCesA9	31.70	4.36	49.77	14.18
CcCesA10.1	32.14	5.04	47.53	15.29
CcCesA10.2	33.69	3.72	46.23	16.36
CcCesA11	31.68	4.03	49.73	14.56

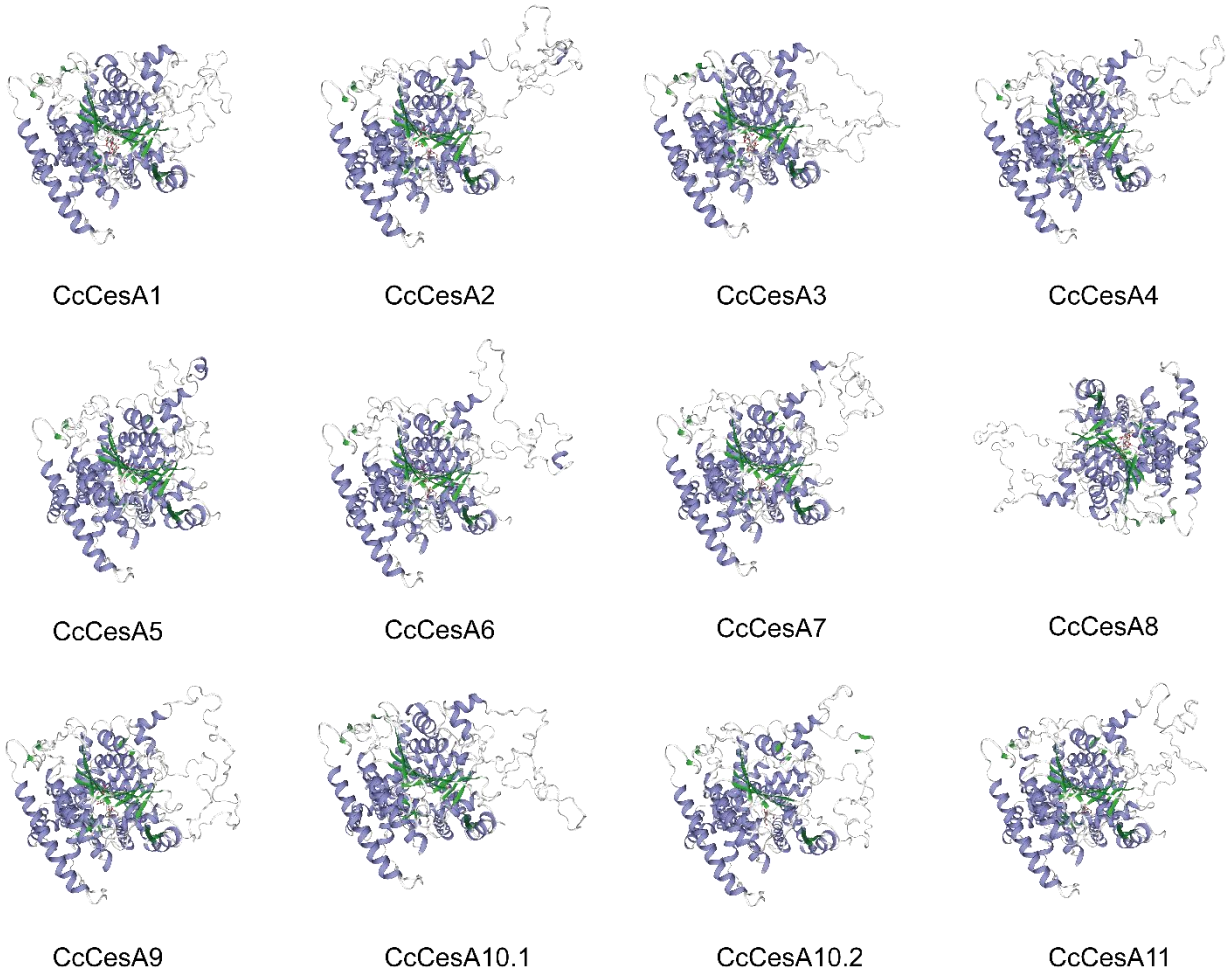

Figure 6. Protein tertiary structure prediction of CcCesAs.

3.7. Cis-Element Analysis of CcCesA Genes in C. citriodora

To understand the genetic functions and regulatory mechanisms of CcCesAs, the majority of cis-elements in their promoter regions were analyzed. The 1500 bp sequence upstream of CcCesAs was obtained as the hypothesized promoter. PlantCARE was used to scan the CcCesAs promoter region for potential common cis-elements. Then, in order to more clearly express the specificity of some gene, we classified cis-elements (except for CAAT-box and TATA-box) by their function into plant growth and development, phytohormone response, stress response, and light response.

The number of cis-elements of different genes is detailed in Table S3. A total of 249 cis-elements were identified, of which up to 104 were involved in regulating plant hormones. According to the different functions of each cis-element, we screened them and made drawings with TBtools. The results showed that the cis-elements regulating the light response were distributed in all genes (Figure 7), with the largest number of 11 in CcCesA1. However, it cannot be seen from the figure that there were 11 light-response cis-elements in CcCesA1, because several cis-elements overlapped with each other, and the same situation occurred in other functional cis-elements. CcCesA1, 4, and 7 are mainly involved in regulating plant growth and development, with three cis-elements each, but CcCesA3 does not have this function. There were at most 21 regulatory plant hormone cis-elements in CcCesA1 and at least 1 in CcCesA7. The most stress-response cis-elements were distributed in CcCesA10.1 and CcCesA10.2, with seven each, but not in CcCesA3. It can be seen that there were fewer functional cis-elements distributed by CcCesA3.

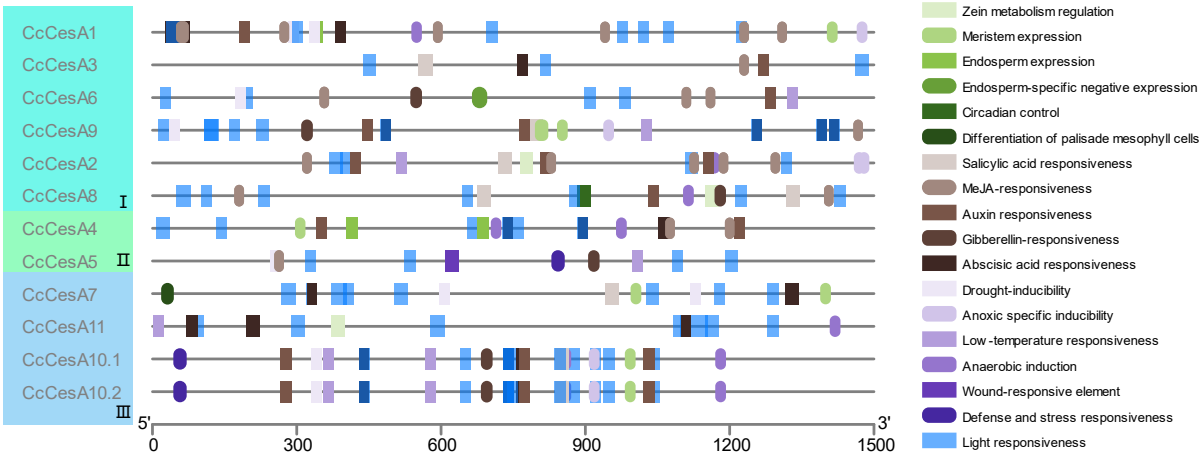

Figure 7. Prediction of cis-responsive elements in the 1500 bp upstream regulatory regions of the putative CcCesA genes. Cis-elements involved in regulating plant growth and development, phytohormone response, stress response, and light response are represented by different shades of green, brown, purple, and blue, respectively.

To understand the specificity of a gene, we found that only CcCesA8 had circadian-control functions and only CcCesA5 had wound-response cis-elements.

3.8. Expression Patterns of CcCesA Genes in Different DBH

In order to explore why there are differences in DBH among *C. citriodora* individuals of the same age, the Log2-standardized relative expressions of the 11 CcCesAs were determined in small DBH, medium DBH, and large DBH (Figure 8). All the 11 CcCesAs were detected in all different DBH. The expression of CcCesA1, 7, 8, 9, and 10.1 increased from short trees to tall trees, while the expression of other genes was the highest in medium DBH. The expression levels of CcCesA10.1 were not significant in all DBH, suggesting that other genes in addition to the CesA gene family may be responsible for different DBH sizes.

To obtain a clearer picture of which genes are most expressed in which DBH, the Log2-standardized relative expression data of three kinds of DBH were analyzed (Figure 9). Results showed that CcCesA8 was the most expressed in any DBH. Except for CcCesA8, CcCesA2, 4, 7, and 9 were significantly upregulated in any DBH and their expression levels were all larger than other genes. Except for CcCes6, 10.2, and 11, all the other genes were differentially expressed genes. The expression of CcCes3, 5, and 10.1 was significantly downregulated in large and small DBH, while only CcCes5 and 10.1 was significantly downregulated in medium DBH. This means that in plants, gene expression changes dynamically from time to time and does not necessarily follow uniform distribution. For example, gene expression will change under different developmental degrees and environmental stimuli. Moreover, it can be seen that the rank of gene expression of different

DBH was basically the same. Since the expression of each DBH is calculated relative to CcCesA1, the genes of different DBH are not comparable.

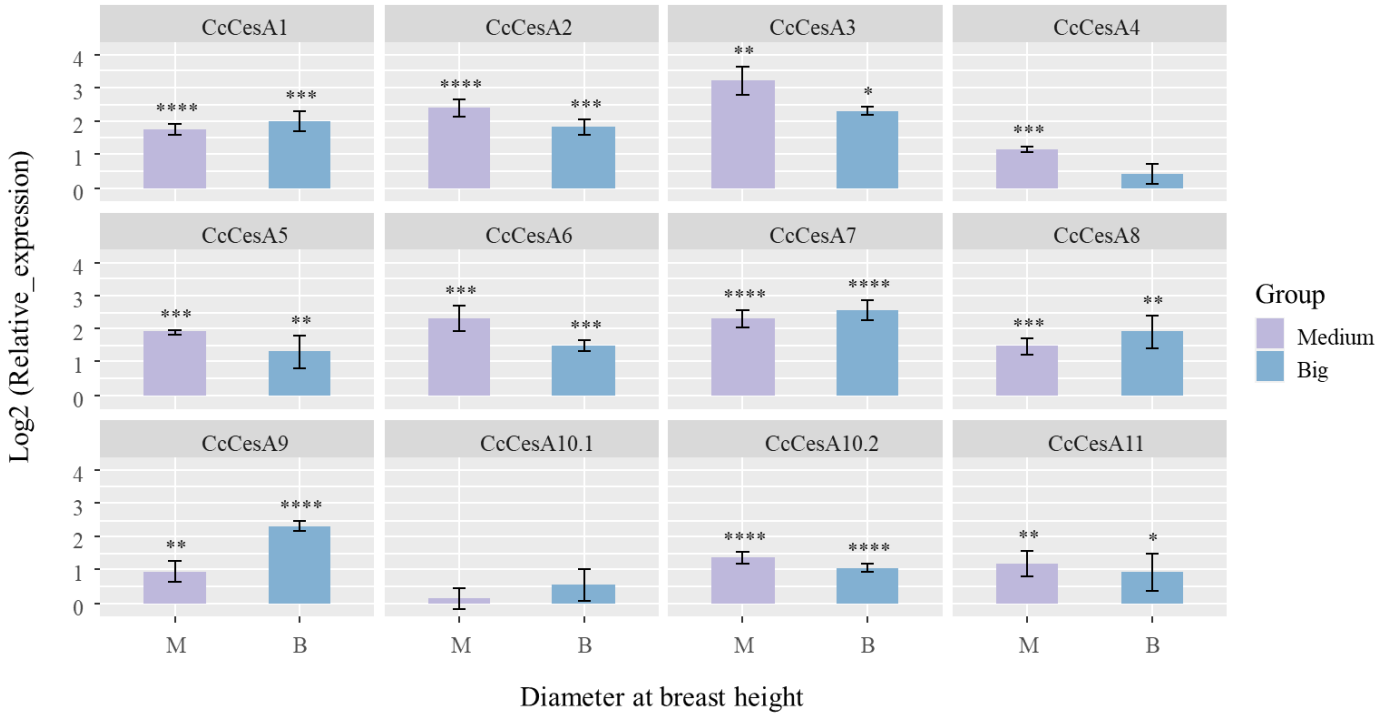

Figure 8. The relative expression levels of CcCesAs in different DBH of *C. citriodora*. Each gene was controlled by a small DBH (relative expression level close to 1). Error bars indicate standard error of four biological replicates. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

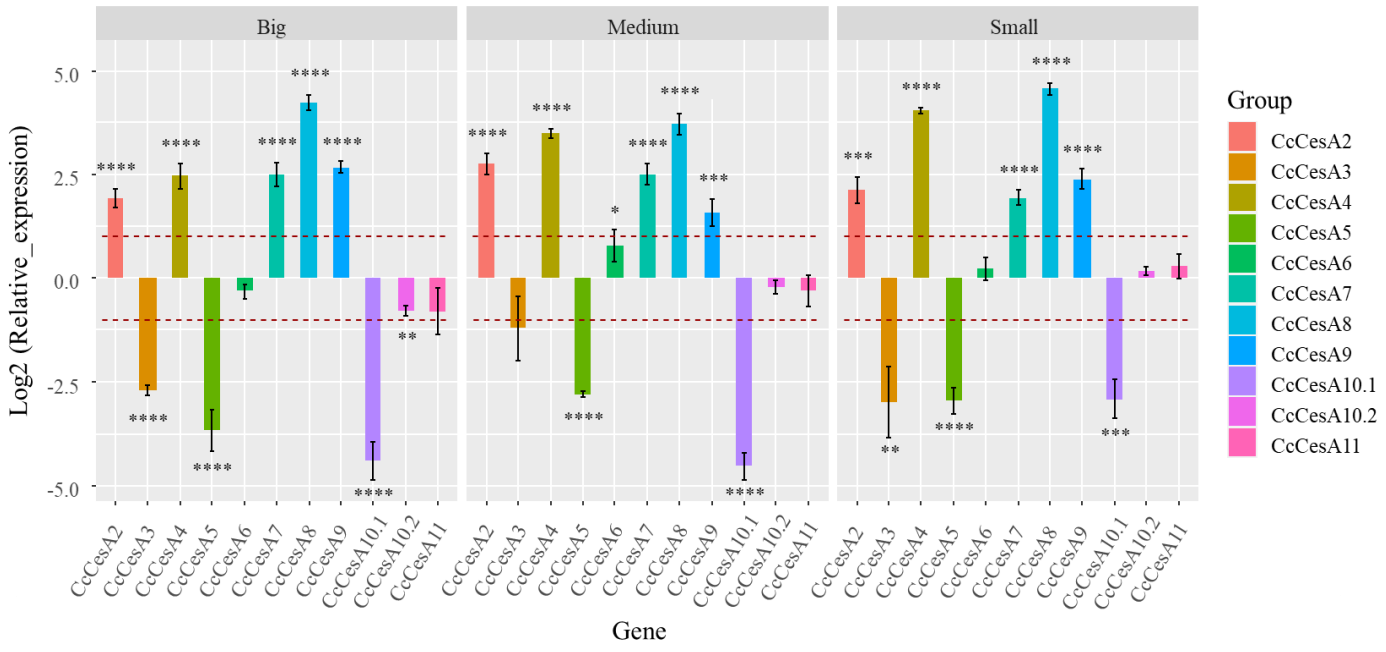

Figure 9. The CcCesA gene family was expressed in small, medium, and large DBH, respectively. Each treatment was controlled by CcCesA1 (relative expression level was close to 1). The two red dotted lines are located at 1 and −1 on the Y-axis. Error bars indicate standard error of four biological replicates. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4. Discussion

4.1. Evolution of the CcCesA Gene Family

A total of 11 CcCesA genes was identified from the *C. citriodora* genome, and the number of CcCesA genes was similar to that of some other plants, such as 12 for *Vitis vinifera* [34], 11 for *Trifolium pratense* [35], 13 for *Brassica rapa* [36], and 10 for *A. thaliana* [37], but there are also significant differences in some plants, such as 26 for *Glycine max* [38]. Previous studies demonstrate that differences in the number of genes in families may be related to polyploidy [39].

In a multispecies phylogenetic tree, genes within a subgroup usually have similar functions [40]. Based on the functional studies of CesAs in *A. thaliana* [11], CesA genes in groups B and D of the phylogenetic tree may be involved in the synthesis of SCWs, which means that four genes in *C. citriodora* may have this function. However, one-to-one orthologous genes of the triple subunit (AtCesA4, 7, and 8) have been widely identified not only in eudicots but also in monocots [41]. In addition, three *E. grandis* genes were found in groups B and D, which was consistent with previous results [42]. In *Hordeum vulgare*, three genes, HvCesA4, HvCesA7, and HvCesA8, were expressed during SCW formation [43]. Although the sequence alignment showed that HvCesA7 and HvCesA5 are paralogs, the HvCesA5 transcript level was extremely low in all tissues. For gene families, it is difficult to determine true ortholog between different species based on sequence similarity alone. Many previous studies have determined the orthologs of CesA based on phylogenetic analysis [44]. Therefore, some researchers searched for the CQIC motif in the zinc finger domain to determine whether the gene regulates the synthesis of PCWs [11]. The same result about the CQIC motif can be seen in this research (Figure S2).

Gene duplication can produce functional differences that are critical for environmental adaptation and speciation [45], and can help determine whether a gene family has expanded or remained conserved over the course of evolution [38]. Different researchers define gene duplication differently, especially the percentage of identity of sequence alignment [32,46]. Gene duplication mechanisms, including segmental duplication, tandem duplication, whole-genome duplication, and translocation (reverse transcription and duplication translocation), are important factors in biological evolution [47,48]. We looked for gene duplication events as a means to further understand the pattern of CesA gene family expansion. Gene duplication analysis based on sequence alignment homology revealed two pairs of segmental duplication and one pair of tandem duplication in *C. citriodora*. Another method of demonstrating plant-gene duplication is phylogenetic clustering, based on which, Nawaz et al. [49] noted that segmental duplication is common in eudicots. Repeated gene pairs do not necessarily represent the development of new protein types or the expansion of functional diversity. In order to verify the regulation of SCW gene pair CcCesA4/5, expression analysis and function prediction are required.

Syntenic analysis can provide evidence of functional linkages between members of a gene family among related species [50]. In this study, two homologous gene pairs were identified between *C. citriodora* and *Arabidopsis*, namely CcCesA3/AtCesA1 and CcCesA8/AtCesA8, which was consistent with the phylogenetic tree. Therefore, we hypothesized that the gene function of CcCesA8 is very similar to that of AtCesA8, which is a gene regulating the SCW.

4.2. Differences in Transmembrane Structure

We found that CcCesA1, 3, 5, and 8 lacked two transmembrane domains at the N-terminal. In general, cellulose synthase genes have one or two transmembrane domains at the N-terminal [5]. Whether the absence of an N-terminal transmembrane domain will affect gene function is still worth studying and considering. CesA transmembrane regions may be involved in cellulose biosynthesis. It has been reported that mutations in *A. thaliana* with varying degrees of resistance under the action of cellulose biosynthesis inhibitors are attributed to amino acid substitution in the transmembrane region of primary wall CesAs [51]. From the analysis of various aspects in this article, especially the expression

analysis, it cannot be concluded that there is anything special about the missing transmembrane domain genes. While some scholars consider deletion of the cellulose synthase gene, the N-terminal transmembrane domain is not an accidental phenomenon [22].

4.3. The Gene Structures of CcCesAs Are Conserved

The number of introns is usually related to the sensitivity of gene transcription regulation. There are many introns in this study. The more introns there are, the slower they evolve [52]. Generally, introns decrease over time and become more adaptable to the environment [53]. More introns are thought to have acquired new functions during evolution. Therefore, the CcCesA gene family of *C. citriodora* has a slow evolutionary process and is sensitive to environmental changes. Meanwhile, the number of introns indirectly reflects the number of exons, and the number of exons in this paper is between 12 and 14, which is consistent with the study of CesAs gene in Eudicots [49]. CesA gene structure has also been studied by intron phase, indicating that the 0 phase is dominant [11].

4.4. Cis-Element and Gene-Expression Patterns

The results of cis-element analysis in this article focus on plant hormones. Among the genes regulating the secondary wall, CcCesA5 has a small number of elements, which may affect the synthesis of the SCW. Cellulose biosynthesis is regulated through a complex network involving multiple transcription factors, among which Myeloblastosis (MYB) transcription factor plays an important role in the regulation of secondary wall cellulose biosynthesis. Some sites associated with MYB-binding could be found in all PtCesA gene promoter sequences associated with the secondary wall of poplar [41]. In *A. thaliana*, AtMYB46 activates the expression of these genes by binding to the Myeloblastosis 46 responsive element (M46RE) in the promoter of ATcesA4, 7, and 8, thereby increasing cellulose content [54]. In this article, in addition to CcCesA2, 3, and 11, other genes can also be found related MYB elements, corresponding to the conserved motif CAACCA (Table S4). Whether the transcription factor MYB can bind to the MYB element in the upstream promoter of CcCesA is still unknown, highlighting the urgent need for further verification [55].

qRT-PCR is an effective method to study gene expression in different organs, tissues, developmental stages, and experimental treatments. CesA genes controlling secondary wall synthesis have been shown to be concentrated in stem expression, especially in xylem, in many species such as bread wheat [11], *Miscanthus* × *giganteus* [56], and flax [57]. Therefore, we conducted studies on stem tissue. Previous studies have concluded that the biomass of wood is mainly secondary xylem, which is developed from vascular cambium, but there are few studies on cambium. We found differences in DBH within the same tree age, and we wanted to look for factors affecting this difference in the genes that regulate cellulose synthesis, but the growth process of lumber by a variety of different gene regulation mechanisms, in addition to the regulation of gene interactions themselves, such as cell-wall assembly, requires harmonized deposition of cellulose and matrix polysaccharides [58]. There are also environmental influences on wood growth, such as abiotic stress [59], which explains the phenomenon of different DBH of the same tree age. From the analysis of individual genes, except for the insignificant expression of different DBH of CcCesA10.1, other CcCesA genes may cause different DBH sizes of *C. citriodora*. From the analysis of multiple genes, we found that among the genes responsible for secondary cell wall synthesis (CcCesA2, 4, 5, and 8), CcCesA8 had the most significant relative expression, while CcCesA5 had the lowest expression, suggesting that CcCesA5 did not regulate secondary wall synthesis. In the three types of trees with different DBH, CcCesA8 was the most expressed, followed by CcCesA4 and CcCesA2. It was noteworthy that CcCesA7 and 9 were also involved in the synthesis of SCWs. In conclusion, CcCesA2, 4, and 8 are worthy of further study, for example, to explore the expression of genes in different developmental stages [16], or expression in the stem of genes at different heights above the ground [56], and the response to abiotic stress.

Gene expression patterns may be influenced by promoter regions that contain binding sites for transcription factors that may influence differences in gene function. In *Gossypium* species, the abscisic acid responsive element (ABRE) was detected in the promoters of highly expressed GhCesA genes, implying ABA may upregulate the expression of these genes [60]. In this study, the ABRE was the most numerous, but it was not present in CcCesA8. However, there was a unique element in CcCesA8 responsible for circadian control that may enhance CcCesA8 expression.

In the study of wood properties, cellulose is an important research subject, and CesA has been proved to affect the synthesis of cellulose [3]. This study showed that CcCesA8 is an important gene affecting wood properties in *C. citriodora*. CcCesA8 can significantly regulate secondary cell wall synthesis, indicating that CcCesA8 plays an important role in regulating growth and wood quality. Therefore, CcCesA8 can provide molecular markers for related traits, which has potential application value in marker-assisted breeding. In future studies, this gene can be used to assist in the screening of *C. citriodora* with excellent wood properties.

5. Conclusions

Although CesA genes have been identified and functionally characterized in other species, the evolution of these genes in *C. citriodora* varieties has not been studied. In this study, we performed a rigorous genome-wide analysis of the CesA genes in *C. citriodora*. By constructing a multi-species evolutionary tree, the genes that may be involved in secondary cell wall synthesis were grouped and further analyzed. Through chromosome localization and replication event analysis, it was concluded that the CcCesA family may be extended due to segmental duplication and tandem duplication. Our qRT-PCR and promoter analysis revealed gene expression profiles and implied the specificity of CesA8. The results of this study may help to elucidate the evolutionary relationships and functional differences between CesA genes in *C. citriodora*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14030618/s1, Figure S1: Motif sequences identified in CcCesA proteins of *C. citriodora*, Figure S2: Alignments of the deduced amino acid sequences of CcCesAs in *C. citriodora*, Table S1: Pairwise identities and divergence between paralogous pairs of CcCesA genes from *C. citriodora* and details about duplication gene, Table S2: CesA genetic pairs of *C. citriodora* with *E. grandis* and *A. thaliana*, Table S3: The number of cis-elements for each function of each gene, Table S4: Raw data for cis-elements of 12 CcCesA genes in *C.citriodora*.

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