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Genome-Wide Identification and Expression Analysis of the *CesA/Csl* Gene Superfamily in Alfalfa (*Medicago sativa* L.)

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Abstract: The cellulose synthase (*CesA*) and cellulose synthase-like (*Csl*) superfamily encodes critical enzymes involved in processing plant cellulose and hemicellulosic polysaccharides. The alfalfa (*Medicago sativa* L.) genome was sequenced in recent years, but this superfamily remains poorly understood at the genome-wide level. We identified 37 members of the *CesA/Csl* family from the alfalfa genome in this study as well as their chromosomal locations and synteny. We uncovered 28 *CesA/Csl* expressed across all tissues and *CslD* genes specifically expressed in the root. In addition, cis-acting element analysis showed that *CesA/Csl* contained several abiotic stress-related elements. Moreover, transcriptomic analysis of alfalfa seedlings demonstrated the involvement of this superfamily in responses to cold, drought, and salt stresses. Specifically, *CslD* increased expression in cold conditions and decreased under osmotic stress, highlighting its potential role in stress adaptation. The findings offer valuable information for the practical exploration of the functions of *CesA/Csl* during plant development and the development of enhanced tolerance to different stress conditions.

Keywords: alfalfa; *CesA/Csl*; cell wall; forage quality; phylogenetic analysis; gene expression

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1. Introduction

Polysaccharide polymers, including cellulose, hemicellulose, and pectin, form the nanoscale network structure of the cell wall that determines plant shape, size, and specific physicochemical properties [1]. Cellulose is composed of β-1,4-linked chains of glucan units that are synthesized by cellulose synthase complexes (CSC) located at the plasma membrane and organized into microfibrils. Cellulose constitutes the main load-bearing element of the cell wall, making up approximately 20~30% of the dry weight of the primary cell wall and 50% of the secondary wall [2,3], respectively. Native cellulose primarily occurs in para-crystalline structures resulting from inter- and intra-chain hydrogen bonds and van der Waals forces [4,5].

The cellulose synthase gene superfamily can be found across all land plants. The first candidate gene belonging to this superfamily was found in embryophytes and identified by homology analysis to bacterial cellulose synthase genes [6,7]. The machinery necessary for cell wall synthesis evolved multiple times in prokaryotes while, in eukaryotes, this process was acquired from cyanobacteria via numerous lateral gene transfers during endosymbiosis [8]. In higher plants, cellulose synthase genes were first isolated from developing cotton (*Gossypium hirsutum*) fibers [6]. Later, the gene family identified from cotton and rice (*Oryza sativa*) was named *CesA* [2].

The cellulose synthase gene superfamily also includes the cellulose synthase-like gene family and is composed of membrane-embedded glycosyltransferases and 18 enzymatically active *CesA* proteins that form a cellulose synthase complex (CSC) which moves through the plane of the plasma membrane as it synthesizes a cellulose microfibril [3,9,10]. The

*CesA*s have conserved motifs around three aspartic residues that, along with the conserved QXXRW motif, construct the active site on the cytoplasmic surface of the plasma membrane. The new glucan chain is synthesized inside a cavity created by a total of eight transmembrane domains. In addition, the cytoplasmic N-terminal region of the proteins contains two zinc finger domains [11,12].

Angiosperms typically contain 10–20 *CesA* genes [13]. *Arabidopsis thaliana* has 10 *CesA* genes, including 3 *CesA* isoforms essential for the synthesis of primary (*AtCesA1*, *AtCesA3*, and *AtCesA6-like* (*CesA2*, *CesA5*, *CesA6*, and *CesA9*)) and secondary (*AtCesA4*, *AtCesA7*, and *AtCesA8*) cell walls [14,15]. *CesA2*, *CesA5*, and *CesA9* are partially redundant with *CesA6*, as demonstrated by mutant and co-immunoprecipitation analysis [16,17].

The *Csl* family has nine subfamilies, *CslA* to *CslH* and *CslJ* [18,19]. While *CslA*, *CslC*, and *CslD* are common to all land-growing plants, some are restricted to certain groups of plants: *CslB* and *CslG* are exclusive to dicotyledons and gymnosperms, whereas *CslF* and *CslH* are specific to monocots [20,21]. Initially, *CslJ* was solely found in cereals; however, recent studies have demonstrated its extensive occurrence in both dicots and monocots [2,18]. In Arabidopsis, there are six families consisting of 30 *Csl* genes that are classified into them. These families are known as *CslA*, *CslB*, *CslC*, *CslD*, *CslE*, and *CslG*, respectively [22]. Finally, *Csl* proteins encode processive glycosyl transferases (GTs) based on the familiar motifs DXD, D, and QXXRW [23].

Alfalfa is commonly referred to as the 'Queen of Forages' due to its high nutritional value. It contains significant levels of crude protein, secondary metabolites, and mineral composition [24]. High-quality forage production is the fundamental basis of both livestock and dairy industries. There are several component traits to forage quality, including protein concentration, percentage of non-digestible protein, animal intake as indicated by low levels of neutral detergent fiber (NDF), digestibility of the NDF fraction as well as other indicators of feed digestibility, such as fiber content, like acid detergent fiber (ADF), and acid detergent lignin (ADL) [25]. NDF is associated with the structural components of plants, particularly the cell wall. Generally, low NDF values are desirable as they tend to increase as forage matures. ADF is the least digestible plant constituent, mostly composed of cellulose and lignin, whereby forages with lower ADF concentrations are generally better sources of energy. In contrast, ADL affects the ability of animals to digest grass. As plants mature, their lignin content increases and their digestibility decreases. The fiber content and digestibility are significant factors affecting alfalfa quality [26]; reducing fiber content should improve digestibility and animal performance.

In this study, we identified and studied 37 protein-coding genes in alfalfa using phylogenetics, gene structure, chromosomal distribution, and collinearity analyses between species. Moreover, we investigated the unique expressions of *CesA/Csl* genes in diverse tissues and alfalfa's response to abiotic stress through transcriptomic analysis. Finally, to simulate cellulose synthase gene family expression in alfalfa at different cutting intervals, we employed reverse transcription quantitative PCR (qRT–PCR) analysis on alfalfa stems and leaves at different phenological stages. Our findings revealed the features of *CesA/Csl* superfamily members in alfalfa and provide a foundation for further investigations on their functions.

2. Materials and Methods

2.1. Plant Material Preparation

The alfalfa plants of the Zhongmu No.1 cultivar were grown under standard greenhouse conditions. Stem cuttings were rooted in vermiculite for 14 days and then transferred to 15 cm diameter pots containing pasteurized soil. We set conditions as follows: vermiculite (1:1) mixture with a photoperiod of 16 h of light and 8 h of darkness, day/night temperature of 25 ± 2 °C, and water as needed. Plants were divided into two groups: one harvested at the branching stage and the other harvested during the first flowering stage. During leaf harvesting, new and old leaves were differentiated by selecting leaves more than two nodes down from the stem tip as new and leaves two nodes up from the stem base as old. The stems collected were categorized into those in elongation and those after

elongation. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. Identification and Characterization of Cellulose Synthase Genes

The alfalfa (cv. Zhongmu No.1) reference genome sequences and protein database were downloaded from Figshare [27]. The sequences of the *Arabidopsis CesA/Csl* comprising 10 *CesA*s and 30 *Csl*s were obtained from the TAIR database (https://www.arabidopsis.org/ (accessed on 11 February 2023)) and subsequently used as search queries in BLAST with a cutoff E-value of 1×10^{-20} against the alfalfa genome. Subsequently, the feature domain of the *CesA/Csl* family (PF00535 and PF03552) was retrieved from Pfam (http://pfam-legacy.xfam.org/ (accessed on 12 February 2023)) and utilized as a query sequence. The HMMER3 [28] (E value = 1×10^{-10}) was then used to query the *CesA/Csl* genes from the alfalfa genome. The verification of all candidate genes was confirmed utilizing Pfam (http://pfam.xfam.org/ (accessed on 15 February 2023)) and SMART (http://smart.embl-heidelberg.de/ (accessed on 15 February 2023)) to verify that the core domains were present. Then, genes were named following the sequence of their arrangement on the chromosomes. TBtools was used to identify coding sequences (CDSs) [29]. The physicochemical properties of the proteins of *CesA/Csl* genes were calculated using the ExPASy-ProtParam tool (http://web. expasy.org/protparam/ (accessed on 2 March 2023)).

2.3. Phylogenetic, Chromosomal Location and Synteny Analysis

The MUSCLE software (v5.1) was used to perform multiple sequence alignments with default settings. We constructed a phylogenetic tree from this alignment using the neighbor-joining method with a bootstrap value of 1000, as implemented in MEGA11 [30]. The tree was visualized and annotated in the R package Ggtree [31]. TBtools was utilized to analyze and visualize the chromosomal distribution of identified *CesA/Csl* family genes as well as the syntenic relationships among *M. Sativa*, *M. Truncatula*, and *Arabidopsis*.

2.4. Gene Structure and Motif Composition Analysis

The alfalfa reference genome (cv. Zhongmu No.1) was used to extract the genome sequences of *CesA/Csl* genes and predict their exon–intron structures. The conserved motifs of *CesA/Csl*s were predicted using the MEME Suite 5.5.1 (http://meme-suite.org/tools/meme (accessed on 8 March 2023)) with the motif length in the range from 6 to 300 residues and maximum number of motifs to 5 and allowed any number of repetitions [32].

2.5. Promoter Cis-Acting Element Analysis

The putative promoter region was extracted as 2000-bp sequences upstream of the start codon of each *CesA/Csl* gene and was submitted to the PlantCARE [33] database to predict cis-acting elements.

2.6. Transcriptome Data Analysis

Transcriptome data for six alfalfa tissues (flowers, leaves, elongating stems, pre-elongating stems, nodules, and roots) and for treatments of low temperature (4 °C, 2, 24, and 48 h), drought (400 mM mannitol, 1, 3, and 12 h) or salinity (250 mM NaCl, 1, 3, and 12 h) exposed at the seedling stage were obtained from the SRA database on NCBI (SRP055547, SRP144299, SRP145557) [34,35]. HISAT2 [36] was used to map the alfalfa reference genome (cv. ZhongmuNo.1) and obtain the SAM files. The count value of the transcriptome data that matched the genome data was calculated using featureCounts software (v2.0.1) [37]. Gene expression levels were estimated using the FPKM value obtained through the R package DESeq2 (v1.40) [38].

2.7. qRT-PCR

RNA was extracted from the leaf and stem at different growing stages using the Eastep® super total RNA extraction kit (Promega, Shanghai, China). The corresponding cDNA was

then obtained using the HiScript® III All-in-one RT Super Mix (Vazyme, Nanjing, China). The NCBI Primer-BLAST tool was utilized to design primers for the *CesA/Csl* genes. The qRT–PCR experiment was performed using the CFX384 Touch Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). cDNA was diluted at a concentration of 100 ng/μL. The qRT–PCR reaction system consisted of 10 μL of Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), 0.2 mM of each primer, and 2 μL of template in a volume of 20 μL. The primers used to amplify each gene are shown in Table S1. The gene expression of *MsActin* was used for data normalization. Relative gene expression levels were calculated using the $2^{-\Delta\Delta t}$ method [39]. Three replicates were designed for each experiment. Post hoc Duncan's multiple range test for multiple comparisons was used for analysis of variance (ANOVA).

3. Results

3.1. Basic Information on the CesA/Csl Genes in Alfalfa

In all, 37 *CesA* and *Csl* genes were identified in alfalfa. Table 1 shows that the protein sequences varied in length, ranging from 371 aa (*MsCslB1*) to 1572 aa (*MsCesA1*). Likewise, the protein molecular weights range between 41.98 kDa (*MsCslB1*) and 175.18 kDa (*MsCesA1*).

Table 1. The sequence of 37 *CesA*/*Csl* genes identified in alfalfa.

No.	Gene Rename	Gene Accession	Chromosome	Location		Amino Acid	MW (kDa)	pI
				Start	End			
1	*MsCslD1*	MsG0180001606.01	Chr1	24,040,717	24,045,076	1146	128.34	5.82
2	*MsCslD2*	MsG0180001607.01	Chr1	24,051,752	24,056,169	1137	127.31	8.22
3	*MsCslA1*	MsG0180003417.01	Chr1	62,051,942	62,059,793	488	56.27	8.79
4	*MsCslA2*	MsG0180003418.01	Chr1	62,061,668	62,066,995	440	50.81	9.08
5	*MsCslA3*	MsG0180003419.01	Chr1	62,073,889	62,083,620	538	62.18	8.78
6	*MsCslA4*	MsG0180003420.01	Chr1	62,091,072	62,096,754	492	56.87	9.39
7	*MsCslG1*	MsG0180004053.01	Chr1	72,523,965	72,527,724	711	81.75	6.82
8	*MsCslA5*	MsG0180005031.01	Chr1	85,959,191	85,963,009	530	60.97	8.89
9	*MsCesA1*	MsG0280006922.01	Chr2	7,998,792	8,011,529	1572	175.18	6.02
10	*MsCesA2*	MsG0280008107.01	Chr2	25,539,931	25,545,645	1044	118.98	7.20
11	*MsCslD3*	MsG0280008146.01	Chr2	26,016,637	26,025,349	1178	133.37	8.13
12	*MsCslB1*	MsG0280010585.01	Chr2	71,770,428	71,788,821	371	41.98	8.18
13	*MsCslB2*	MsG0280010586.01	Chr2	71,792,138	71,802,870	498	55.55	8.02
14	*MsCslB3*	MsG0280010587.01	Chr2	71,809,645	71,814,024	698	79.32	7.94
15	*MsCesA3*	MsG0380012697.01	Chr3	22,927,630	22,933,990	774	87.01	6.09
16	*MsCslE1*	MsG0380014552.01	Chr3	56,426,859	56,438,397	833	96.37	6.73
17	*MsCslE2*	MsG0380014553.01	Chr3	56,444,913	56,453,340	419	47.72	8.65
18	*MsCslE3*	MsG0380014558.01	Chr3	56,484,562	56,490,490	595	68.10	8.54
19	*MsCslE4*	MsG0380014561.01	Chr3	56,507,839	56,510,348	476	53.93	6.05
20	*MsCslC1*	MsG0380017240.01	Chr3	93,932,951	93,936,593	702	80.35	8.03
21	*MsCesA4*	MsG0380017435.01	Chr3	96,225,074	96,231,632	1089	122.97	6.56
22	*MsCslB4*	MsG0480020393.01	Chr4	40,435,695	40,441,148	380	43.20	8.72
23	*MsCslA6*	MsG0480020597.01	Chr4	44,402,146	44,408,477	542	62.53	9.07
24	*MsCslD4*	MsG0480021653.01	Chr4	61,289,304	61,292,141	719	81.45	8.86
25	*MsCslD5*	MsG0480021859.01	Chr4	65,279,346	65,280,787	454	51.10	8.34
26	*MsCesA5*	MsG0480023097.01	Chr4	80,793,999	80,812,063	1362	151.46	5.64
27	*MsCesA6*	MsG0480023279.01	Chr4	83,142,696	83,149,308	1098	124.09	6.37
28	*MsCesA7*	MsG0480023418.01	Chr4	84,957,277	84,963,896	1098	124.08	6.37
29	*MsCslD6*	MsG0580025441.01	Chr5	18,677,897	18,682,489	1122	126.02	5.8
30	*MsCslC2*	MsG0680030621.01	Chr6	6,190,384	6,194,874	641	74.24	8.95
31	*MsCslA7*	MsG0780041074.01	Chr7	86,131,839	86,141,384	1154	130.89	8.59
32	*MsCesA8*	MsG0780041211.01	Chr7	88,265,473	88,272,679	1049	118.17	8.00
33	*MsCslD7*	MsG0780041340.01	Chr7	89,777,194	89,781,609	1171	130.94	8.22
34	*MsCslD8*	MsG0780041693.01	Chr7	94,013,062	94,017,095	1050	118.13	7.91
35	*MsCslC3*	MsG0880042124.01	Chr8	4,096,083	4,102,789	698	80.08	8.92
36	*MsCesA9*	MsG0880043508.01	Chr8	25,944,537	25,952,543	1075	120.77	7.69
37	*MsCesA10*	MsG0880044994.01	Chr8	52,543,467	52,549,606	1002	114.28	8.92

The locations of *CesA/Csl* genes were obtained and mapped to the different chromosomes (Figure 1). The results showed the 37 genes are distributed across the eight chromosomes, mostly on chromosomes 1, 2, 3, and 4. We also found a small cluster on chromosome 7 and at least eight genes on chromosome 1, mostly clustered on the lower arm of the chromosome. Interestingly, we found only a single gene on chromosomes 5 and 6, in the upper arm of each chromosome.

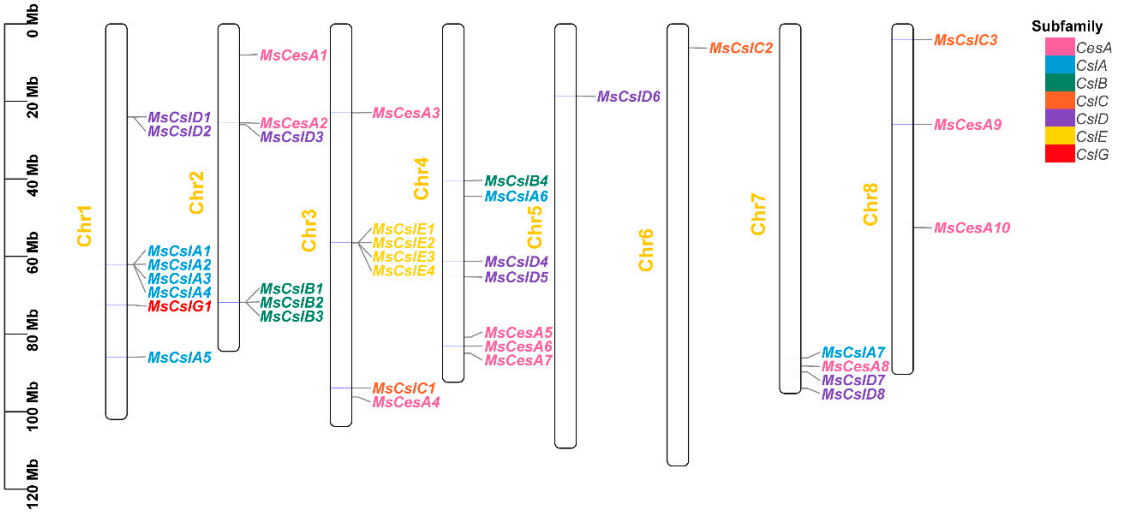

Figure 1. Chromosomal location of *CesA/Csl* members in the alfalfa genome. Genes from the same subfamily are shown in the same color, corresponding to the phylogenetic tree of Figure 2.

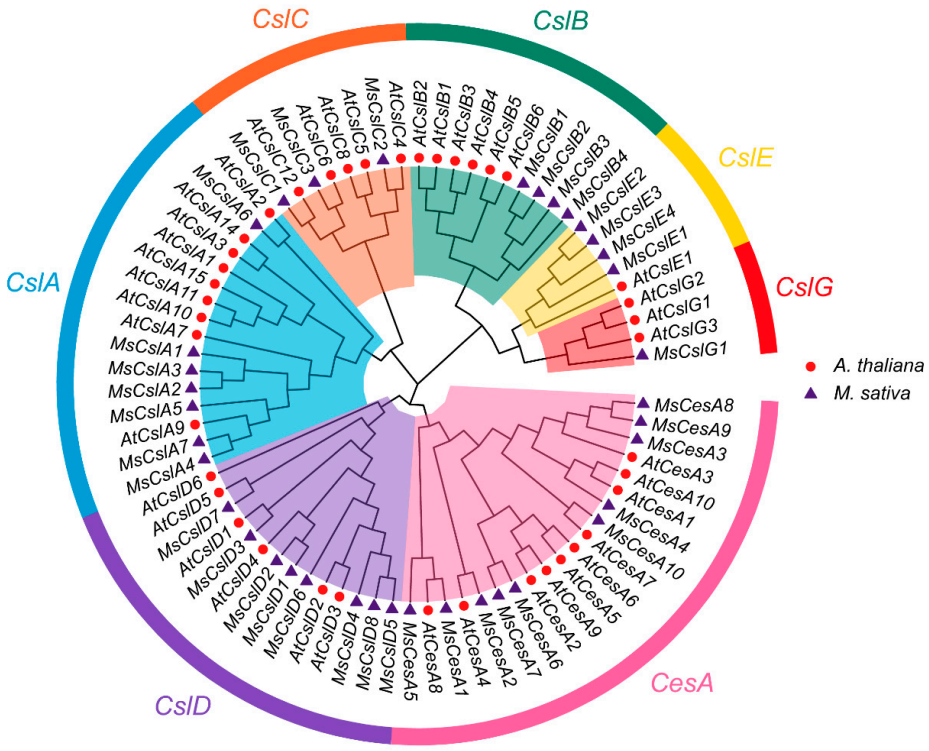

Figure 2. Phylogenetic relationships and subfamily *CesA/Csl* protein designations in alfalfa and *Arabidopsis*.

3.2. Phylogenetic Analysis

In order to investigate the evolutionary relationships among *CesA/Csl* proteins in alfalfa, we constructed a phylogenetic tree of the 37 genes with 40 *CesA/Csl* protein se-

quences obtained from the *Arabidopsis* database using MEGA11. The cellulose synthase homologues of both species were divided into a single *CesA* subfamily and six *Csl* families (i.e., *CslA*-*CslE* and *CslG*, according to their classification in Arabidopsis), as depicted in Figure 2.

The 37 *CesA/Csl* proteins were unevenly distributed among the seven clades, indicating that they experienced dynamic changes since they last shared a common ancestor. We found 10 alfalfa *CesA*, which are essential for cell wall synthesis in *Arabidopsis*. This indicates *CesA* members might also be responsible for cell wall synthesis in alfalfa.

3.3. Motifs and Gene Structure

We predicted and confirmed conserved motifs of the *CesA/Csl* proteins using the MEME software (v5.5.1). Motif 1, including QXXRW, is the most conserved motif and found in most *CesA/Csl* proteins. Motif 9, also including QXXRW, is expected in *CslA*s. We found that there are similar motifs present in both *CesA* and *CslD* proteins, including motifs 1 to 8 and 10, which have the highest number among all subfamilies (9). The majority of proteins in the *CslD*s share a comparable motif composition with the *CesA*s.

The conserved domains of the cellulose synthase are contained within the 27 proteins belonging to the *CesA*, *CslB*, *CslD*, *CslE*, and *CslG* subfamilies (PLN02638, PLN02400, PLN02915, PLN02915, PLN02436, PLN2189, PLN2248, PLN02190, and PLN02893). The *CslA* and *CslC* clades, which were compared, included 10 proteins with GT2 domains (CesA_CaSu_A2 and Glyco_tranf_GTA_type) (Figure S1). The *CslA* and *CslC* clades, which had a similar motif composition, had a distinct composition from other subfamilies (Figure 3A). Most proteins with similar motif compositions were classified in the same subfamilies and could be responsible for similar functions.

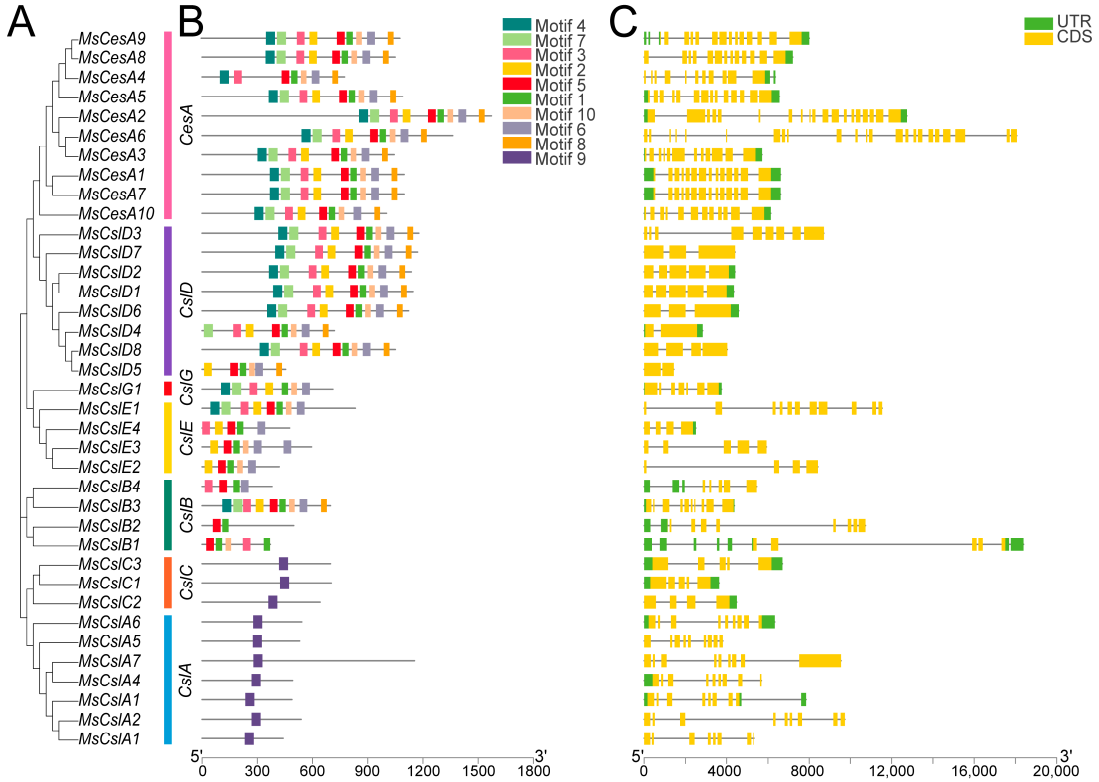

Figure 3. Structural characteristics of *CesA/Csl*s in alfalfa. (**A**) Phylogenetic analysis of *CesA/Csl* proteins. (**B**) Predicted motifs of *CesA/Csl* proteins. (**C**) Exon–intron structures of *CesA/Csl* genes. UTRs will be displayed as green rectangles, exons will be shown as yellow rectangles, and solid lines will denote introns. Additionally, the length of the respective proteins and genes is indicated by the scale bars.

3.4. Promoter Analysis

The promoter region of each alfalfa *CesA/Csl* gene was extracted by taking 2000-bp sequences upstream of their start codon. These sequences were submitted to the PlantCARE database to predict cis-acting elements (Figure 4). Assembling the common cis-elements, we found that almost all *CesA/Csl* gene promoters contain light-responsive elements. This suggests they may be involved in the regulation of the light response regulatory network. We also discovered several stress response elements, such as MREs, MBSs, LTRs, and TC-rich repeats. These elements are related to various stress factors, namely light, drought, cold, defense, and stress responsiveness. In addition, hormone-regulating elements, such as the CGTAC motifs, ABREs, and TCA elements, were also identified and may be associated with MeJA, ABA, and SA response. These results suggest that these factors may affect the transcriptional levels of *CesA/Csl*.

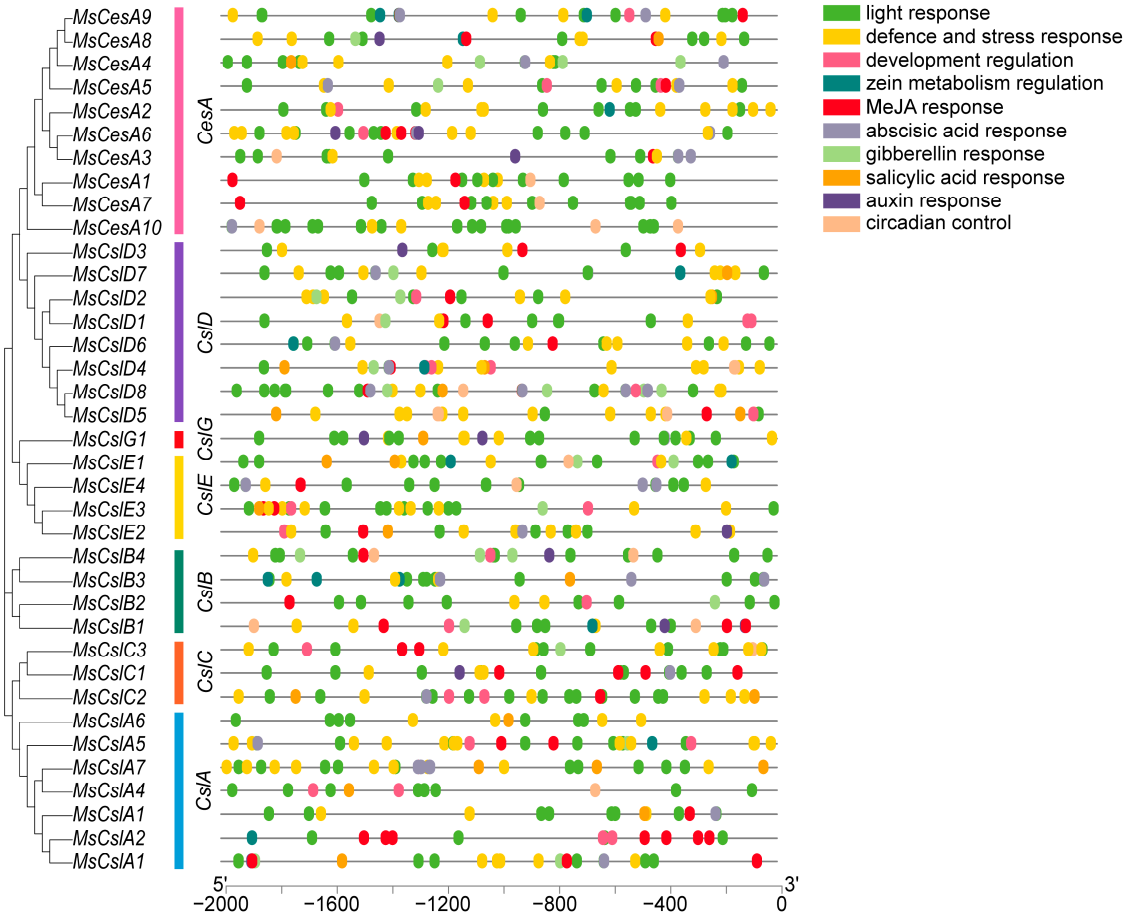

Figure 4. Analysis of cis-acting elements in *CesA/CSL*. Boxes filled with different colors represent different functions.

3.5. Collinearity Analysis

In order to gain a deeper understanding of the possible evolutionary events related to the *CesA/Csl*s across various plants, three comparative syntenic maps were constructed for *M. sativa*, *A. thaliana*, and *M. truncatula* (Figure 5). Fifteen collinear pairs of *CesA/Csl* genes were identified between *M. sativa* and *A. thaliana*, and 30 were identified between *M. sativa* and *M. truncatula*. The amount of homologous gene pairs between the genes *CesA/Csl* of *M. sativa* and *M. truncatula* was twice that of the homologous gene pairs between *M. sativa* *CesA/Csl*s and *AtCesA/Csl*s.

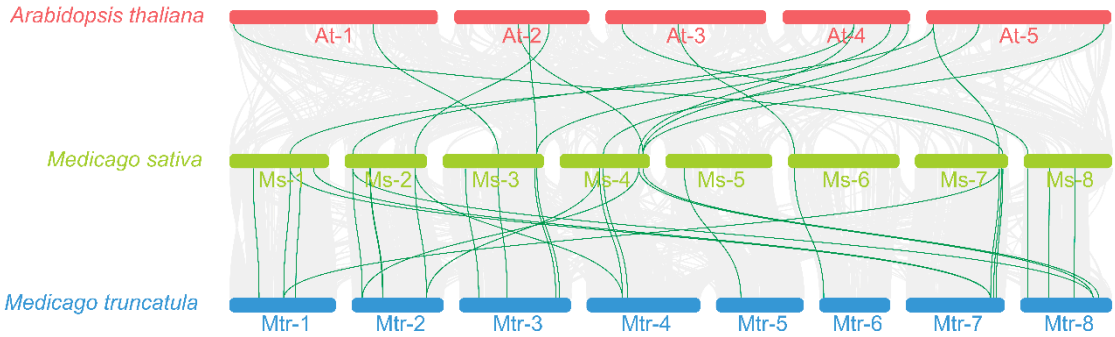

Figure 5. Collinearity analysis between *A. thaliana*, *M. sativa* and *M. truncatula*. Grey lines in the background indicate collinear blocks within *A. thaliana*. The green lines of *A. thaliana*, *M. sativa*, and *M. truncatula* highlight syntenic *CesA/Csl* gene pairs. Chromosomes of these representative plant species are shown in different colors. The species names with the prefixes 'At', 'Ms,' and 'Mtr' represent *A. thaliana*, *M. sativa*, and *M. truncatula* genomes, respectively.

3.6. Analysis of the Expression of CesA/Csl Genes in Various Tissues

We investigated the function of *CesA/Csl*s by analyzing their expression patterns in six tissues from an RNA-seq database. As shown in Figure 6A, 3 *CslA* genes were not expressed in any of the examined tissues. These genes might be upregulated in other tissues or under specific biotic or abiotic conditions. Other 34 expressed genes showed varying transcript abundance across tissues, suggesting their functions may be different. We also identified two genes (*MsCslD2*, *MsCslD1*) expressed in a single tissue, respectively, in stem and root (Figure 6A); a single gene expressed in two different tissues (*MsCslB4*); and 28 genes expressed in all tissues. These findings indicate that these genes play a crucial role in the growth and development of lucerne. We performed qRT–PCR to further validate their expression patterns at different harvest stages (Figure 6B–E). The *MsCesA5* gene was expressed in all tissues but was mostly in the old stem (Figure 6B), in particular in cut alfalfa as compared to flowering one (Figure 6C). We found contrasting results in the case of *MsCslD7* (Figure 6D). These results were consistent with transcriptomic data.

3.7. Expression Analysis of CesA/Csls under Abiotic Stress

We found *CesA/Csl* promoter sequences contain several abiotic stress-related elements and thus aimed to understand the possible involvement of the identified *CesA/Csl* genes in stress response. To achieve this, we analyzed the expression patterns of the identified genes in response to cold, drought, and salt using an RNA-seq database at the alfalfa seeding stage. As shown in Figure 7, we found two *CslD* genes (*MsCslD1*, *MsCslD2*) that were not expressed in any of the treatment and control groups. Under cold stress (Figure 7A), the expression levels of *CesA/Csl*s were observed to form two distinct clusters: one group composed of nine genes with continuously increasing expression and eight genes with increased and then decreased expression; and another group with nine genes with increased and then decreased expression and six genes with a slowly increased expression before reaching equilibrium. In the mannitol treatment (Figure 7B), the genes were divided into two groups: one group containing two genes with sharply decreased expression and fifteen genes that changed slightly before decreasing expression; and another group with eight genes showing fluctuating and then sharply increased expression and seven genes with increased and then decreased expression. Finally, under salt stress (Figure 7C), two distinct clusters were observed in the expression levels of *CesA/Csl* genes: one group composed of eighteen genes decreasing in expression at different time points and five genes increasing and then decreasing expression slightly; and another group including six genes increasing slowly and then increasing sharply at 24 h and two genes decreasing and then increasing slowly in expression.

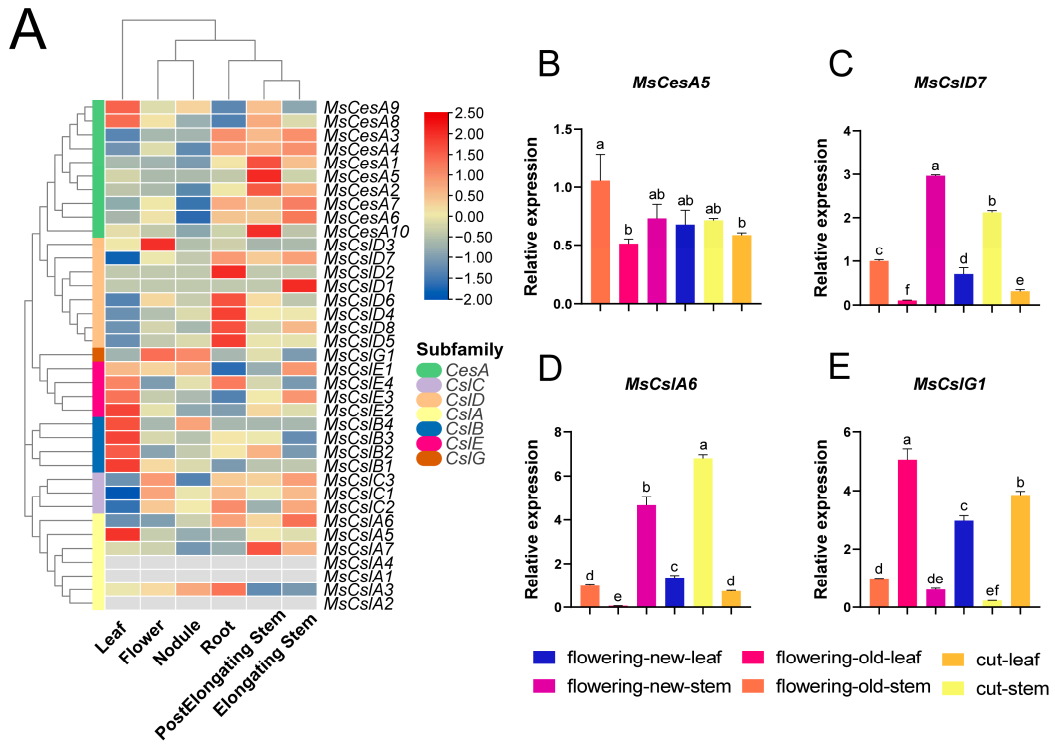

Figure 6. Expression patterns of *CesA/Csl*s in different alfalfa tissues. (**A**): Heatmap of the *CesA/Csl* genes in various tissues (leaf, elongating stem, post-elongating stem, root, flower, and nodule). The Z-score algorithm was used to normalize expression levels by row. The relative expression level is indicated by the color scale on the right side of the heatmap. The gradient from blue to red represents an increase in the level of expression. (**B**–**E**): Expression patterns of *CesA/Csl*s in different tissues. Relative expression of target genes in different cuts. Each tissue's expression level was normalized by expression level of the flowering old stem. The values represent the means ± SEM of three biological replications. Statistically significant differences were indicated by different lowercase letters ($p < 0.05$).

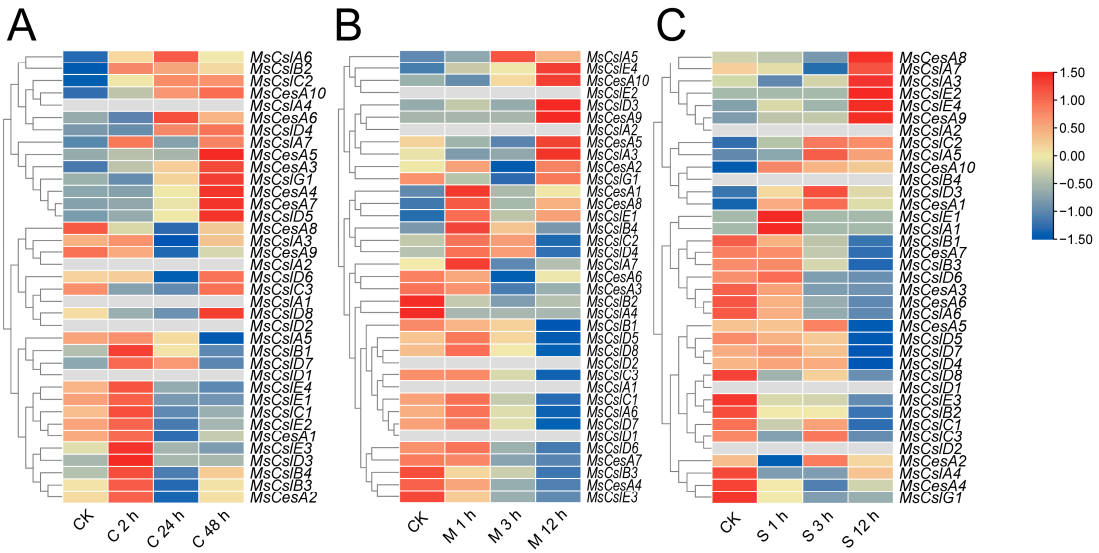

Figure 7. Expression analysis of *CesA/Csl* response to different abiotic stresses. (**A**): cold stress, (**B**): drought stress, (**C**): salt stress. The Z-score algorithm was used to normalize expression levels by row. The relative expression level is indicated by the color scale on the right side of the heatmap. Increasing expression levels are represented by a color gradient from blue to red.

We also found two *CesA* genes (*MsCesA5*, *MsCesA10*), one *CslG* gene (*MsCslG1*), one *CslC* gene (*MsCslC2*) and one *CslA* gene (*MsCslA7*) with elevated expression under both cold and drought stress; and two *CesA* genes (*MsCesA9*, *MsCesA8*) and one *CslE* gene (*MsCslE4*) with elevated expression in both salt and drought stress.

4. Discussion

The plant *CesA/Csl* belongs to the glycosyltransferase type 2 (GT2) family, which is widely distributed across plants and participates in cell wall biogenesis by synthesizing cellulose and hemicellulose [22]. The gene families responsible for cellulose synthesis, namely cellulose synthase (*CesA*) and cellulose synthase-like (*Csl*), have received extensive research and have been well-documented in various plants, such as rice (*Oryza sativa*) [40], Arabidopsis (*Arabidopsis thaliana*) [17], bread wheat [41], cotton (*Gossypium hirsutum*) [42], and tomato (*Solanum lycopersicum*) [15]. Although the complete genome sequence for alfalfa is available, there has not been a comprehensive analysis of the whole genome or an extensive study of the *CesA/Csl* gene family in this species. In this study, we have, for the first time, discovered 37 genes from the alfalfa genome (Table 1). Our study involved basic bioinformatical, phylogenetic, evolutionary, and expression analyses, which verified the crucial contribution of the *CesA/Csl* genes to alfalfa's growth, development, and tolerance to stress.

Characterizing the phylogeny of homologs from different plant species has immense potential for predicting gene function. This study's multiple sequence alignments and neighbor-joining phylogenetic tree construction revealed seven *CesA/Csl* subfamilies (Figure 2), which is consistent with the number of subfamilies of soybean (*Glycine max*) studied previously [43]. Of these, ten *CesA/Csl* genes were clustered as *CesA* gene family and eight were classified in the *CslD* gene family, making them the two largest subfamilies of the gene family. The *CslDs* are is phylogenetically similar to the *CesAs*, suggesting that the two subfamilies have a shared ancestry, which is in line with previous reports [44]. The *CslA* and *CslC* subfamilies are closely related to single-copy gene homologs found in six chlorophyte green algae species that have an evolutionary origin distinct from the other *Csl* clades [45]. This confirms that the evolution of these genes likely occurred several times independently and led to functional diversification [46].

The analysis of gene structure and replication allows the identification of conserved introns and exons essential for evolution and helps understand the evolution and differentiation of gene family members, their conservation levels, or episodes of functional diversification [47–49]. We showed that most *CesA/Csl* genes have a similar structure across sub-families, suggesting evolutionary conservation (Figure 3). The examination of motifs and conserved domains also revealed strong conservation among the subfamilies in alfalfa. The *CesA* and *CslB*, *CslD*, *CslE*, and *CslG* subfamilies, among the most highly conserved, contained the cellulose synthase-conserved domains. In addition, the *CslA* and *CslC* subfamilies containing the GT2 domains were distinct from those of the other subfamilies (Figure S1). The genes of the *CesA*, *CslB*, *CslD*, *CslE*, and *CslG* subfamilies share similar structures. However, *CslA* and *CslC* subfamilies contain a single motif, leading to major structural differences when compared to the other five subfamilies, confirming the findings of previous studies [50,51].

Transcriptional regulation plays the largest role in the activation and repression of expression and is largely controlled by gene promoters and cis-acting elements [52]. We found that a large number of cis-acting elements were discovered in the predicted promoter regions of the *CesA/Csl* genes, including the light-responsive, several types of stress-responsive, and hormone-regulating elements.

The biosynthesis of cellulose and hemicellulose in the plant cell walls during growth is significantly influenced by *CesA/Csl* genes. *MsCslD2*, a member of the *CslD* clade, was present only in the root and showed tissue-specific expression, while other *CslD* subfamily genes were highly expressed in the root. This observation implies that *CslD* is important for root development. In contrast, *MsCslD1* is not found in the root but is highly expressed

in the elongating stem. Previous studies indicated that *CslD* proteins may be involved in cellulose synthesis in root hairs and stem growth [53–56], which is in agreement with our results. In addition, *CesA/Csl* genes promote plant response to abiotic stress [51,57]. Our RNA-Seq data indicated most of the cellulose synthase family genes appear to respond differently to distinct abiotic stresses (Figure 7). In particular, the *CslD* genes (*MsCslD7*, *MsCslD6*, *MsCslD4*, *MsCslD8*, and *MsCslD5*) are down-regulated in osmotic stress and up-regulated in cold stress. Previous studies addressed this topic by investigating abiotic stress responses. Specifically, *AtCslD*5 plays a key role in osmotic stress tolerance, a function that may involve regulating ROS under stress [57] and *OsCslD*4, homologous to the *AtCslD*5 gene, is involved in salt stress response in rice by mediating abscisic acid (ABA) levels to enhance osmotic stress tolerance [58]. The modulation of cellulose synthesis is likely to have been an important factor in polysaccharide metabolism and plant adaptation to salt stress [59]. Cellulose synthase gene mutations or RNAi interference can reduce cellulose content and increase salt tolerance [59,60]. These processes both lead to an increase in soluble sugars, while drought and salt tolerance can be increased by increasing soluble sugar accumulation in plants [61].

5. Conclusions

In this study, 37 *CesA/Csl* genes were identified from the alfalfa genome and classified into *CesA* and 5 *Csl* subfamilies (*CslA*, *CslB*, *CslD*, *CslE*, and *CslG*). Phylogenetic analysis revealed that the *MsCesA/Csl* gene families exhibit significant homology to that found in in *Arabidopsis*. Expression pattern analysis showed that the *CesA/Csl*s have distinct expression patterns in different tissues and when experiencing various abiotic stresses. Most *CesA/Csl* genes were expressed in all tissues while CslDs were primarily expressed in the roots and stems, indicating their possible involvement in cellulose synthesis during growth. In addition, cis-acting element analysis showed that *CesA/Csl* contain several abiotic stress-related elements. Transcriptomic analysis of alfalfa seedlings under abiotic stress showed 32, 32, and 33 *CesA/Csl* genes that respond to cold, drought, and salt stress, respectively. The expression levels of the *CslD* genes were elevated under cold stress and decreased in drought and salt conditions. The subfamily *CslD* may participate in stress adaptation. These results will aid in identifying the role of *MsCesA/Csl* in modifying cell wall composition in alfalfa to improve forage quality.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture13091658/s1, Figure S1: Conserved domains of *CesA/Csl* genes; Table S1: Primers used to amplify *CesA/Csl* and reference genes using qRT-PCR.

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References

1. Zhang, B.; Gao, Y.; Zhang, L.; Zhou, Y. The Plant Cell Wall: Biosynthesis, Construction, and Functions. *J. Integr. Plant Biol.* **2021**, *63*, 251–272. [CrossRef] [PubMed]
2. Little, A.; Schwerdt, J.G.; Shirley, N.J.; Khor, S.F.; Neumann, K.; O'Donovan, L.A.; Lahnstein, J.; Collins, H.M.; Henderson, M.; Fincher, G.B.; et al. Revised Phylogeny of the *Cellulose Synthase* Gene Superfamily: Insights into Cell Wall Evolution. *Plant Physiol.* **2018**, *177*, 1124–1141. [CrossRef] [PubMed]
3. Turner, S.; Kumar, M. Cellulose Synthase Complex Organization and Cellulose Microfibril Structure. *Philos. Trans. A Math. Phys. Eng. Sci.* **2018**, *376*, 20170048. [CrossRef] [PubMed]
4. Polko, J.K.; Kieber, J.J. The Regulation of Cellulose Biosynthesis in Plants. *Plant Cell* **2019**, *31*, 282–296. [CrossRef] [PubMed]
5. Rongpipi, S.; Ye, D.; Gomez, E.D.; Gomez, E.W. Progress and Opportunities in the Characterization of Cellulose—An Important Regulator of Cell Wall Growth and Mechanics. *Front. Plant Sci.* **2018**, *9*, 1894. [CrossRef] [PubMed]
6. Pear, J.R.; Kawagoe, Y.; Schreckengost, W.E.; Delmer, D.P.; Stalker, D.M. Higher Plants Contain Homologs of the Bacterial CelA Genes Encoding the Catalytic Subunit of Cellulose Synthase. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 12637–12642. [CrossRef] [PubMed]
7. Arioli, T.; Peng, L.; Betzner, A.S.; Burn, J.; Wittke, W.; Herth, W.; Camilleri, C.; Höfte, H.; Plazinski, J.; Birch, R.; et al. Molecular Analysis of Cellulose Biosynthesis in *Arabidopsis*. *Science* **1998**, *279*, 717–720. [CrossRef]
8. Popper, Z.A.; Michel, G.; Hervé, C.; Domozych, D.S.; Willats, W.G.T.; Tuohy, M.G.; Kloareg, B.; Stengel, D.B. Evolution and Diversity of Plant Cell Walls: From Algae to Flowering Plants. *Annu. Rev. Plant Biol.* **2011**, *62*, 567–590. [CrossRef]
9. Nixon, B.T.; Mansouri, K.; Singh, A.; Du, J.; Davis, J.K.; Lee, J.-G.; Slabaugh, E.; Vandavasi, V.G.; O'Neill, H.; Roberts, E.M.; et al. Comparative Structural and Computational Analysis Supports Eighteen Cellulose Synthases in the Plant Cellulose Synthesis Complex. *Sci. Rep.* **2016**, *6*, 28696. [CrossRef]
10. Duncombe, S.G.; Chethan, S.G.; Anderson, C.T. Super-Resolution Imaging Illuminates New Dynamic Behaviors of Cellulose Synthase. *Plant Cell* **2022**, *34*, 273–286. [CrossRef]
11. Kurek, I.; Kawagoe, Y.; Jacob-Wilk, D.; Doblin, M.; Delmer, D. Dimerization of Cotton Fiber Cellulose Synthase Catalytic Subunits Occurs via Oxidation of the Zinc-Binding Domains. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11109–11114. [CrossRef] [PubMed]
12. Kaur, S.; Dhugga, K.S.; Gill, K.; Singh, J. Novel Structural and Functional Motifs in *Cellulose Synthase* (CesA) Genes of Bread Wheat (*Triticum Aestivum*, L.). *PLoS ONE* **2016**, *11*, e0147046. [CrossRef] [PubMed]
13. Pancaldi, F.; van Loo, E.N.; Schranz, M.E.; Trindade, L.M. Genomic Architecture and Evolution of the *Cellulose Synthase* Gene Superfamily as Revealed by Phylogenomic Analysis. *Front. Plant Sci.* **2022**, *13*, 870818. [CrossRef] [PubMed]
14. Taylor, N.G.; Howells, R.M.; Huttly, A.K.; Vickers, K.; Turner, S.R. Interactions among Three Distinct CesA Proteins Essential for Cellulose Synthesis. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1450–1455. [CrossRef]
15. Song, X.; Xu, L.; Yu, J.; Tian, P.; Hu, X.; Wang, Q.; Pan, Y. Genome-Wide Characterization of the Cellulose Synthase Gene Superfamily in *Solanum Lycopersicum*. *Gene* **2019**, *688*, 71–83. [CrossRef] [PubMed]
16. Desprez, T.; Juraniec, M.; Crowell, E.F.; Jouy, H.; Pochylova, Z.; Parcy, F.; Höfte, H.; Gonneau, M.; Vernhettes, S. Organization of Cellulose Synthase Complexes Involved in Primary Cell Wall Synthesis in *Arabidopsis Thaliana*. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 15572–15577. [CrossRef]
17. Persson, S.; Paredez, A.; Carroll, A.; Palsdottir, H.; Doblin, M.; Poindexter, P.; Khitrov, N.; Auer, M.; Somerville, C.R. Genetic Evidence for Three Unique Components in Primary Cell-Wall Cellulose Synthase Complexes in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 15566–15571. [CrossRef]
18. Yin, Y.; Johns, M.A.; Cao, H.; Rupani, M. A Survey of Plant and Algal Genomes and Transcriptomes Reveals New Insights into the Evolution and Function of the Cellulose Synthase Superfamily. *BMC Genom.* **2014**, *15*, 260. [CrossRef]
19. Marcotuli, I.; Colasuonno, P.; Blanco, A.; Gadaleta, A. Expression Analysis of Cellulose Synthase-like Genes in Durum Wheat. *Sci. Rep.* **2018**, *8*, 15675. [CrossRef]
20. Farrokhi, N.; Burton, R.A.; Brownfield, L.; Hrmova, M.; Wilson, S.M.; Bacic, A.; Fincher, G.B. Plant Cell Wall Biosynthesis: Genetic, Biochemical and Functional Genomics Approaches to the Identification of Key Genes. *Plant Biotechnol. J.* **2006**, *4*, 145–167. [CrossRef]
21. Keegstra, K.; Walton, J. β-Glucans—Brewer's Bane, Dietician's Delight. *Science* **2006**, *311*, 1872–1873. [CrossRef] [PubMed]
22. Richmond, T.A.; Somerville, C.R. The Cellulose Synthase Superfamily. *Plant Physiol.* **2000**, *124*, 495–498. [CrossRef] [PubMed]
23. Wang, L.; Guo, K.; Li, Y.; Tu, Y.; Hu, H.; Wang, B.; Cui, X.; Peng, L. Expression Profiling and Integrative Analysis of the CESA/CSL Superfamily in Rice. *BMC Plant Biol.* **2010**, *10*, 282. [CrossRef] [PubMed]
24. Suwignyo, B.; Aristia Rini, E.; Helmiyati, S. The Profile of Tropical Alfalfa in Indonesia: A Review. *Saudi J. Biol. Sci.* **2023**, *30*, 103504. [CrossRef]
25. Oba, M.; Allen, M.S. Evaluation of the Importance of the Digestibility of Neutral Detergent Fiber from Forage: Effects on Dry Matter Intake and Milk Yield of Dairy Cows. *J. Dairy Sci.* **1999**, *82*, 589–596. [CrossRef]
26. Lin, S.; Medina, C.A.; Norberg, O.S.; Combs, D.; Wang, G.; Shewmaker, G.; Fransen, S.; Llewellyn, D.; Yu, L.-X. Genome-Wide Association Studies Identifying Multiple Loci Associated with Alfalfa Forage Quality. *Front. Plant Sci.* **2021**, *12*, 648192. [CrossRef] [PubMed]

27. Shen, C.; Du, H.; Chen, Z.; Lu, H.; Zhu, F.; Chen, H.; Meng, X.; Liu, Q.; Liu, P.; Zheng, L.; et al. The Chromosome-Level Genome Sequence of the Autotetraploid Alfalfa and Resequencing of Core Germplasms Provide Genomic Resources for Alfalfa Research. *Mol. Plant* **2020**, *13*, 1250–1261. [CrossRef]
28. Eddy, S.R. Accelerated Profile HMM Searches. *PLoS Comput. Biol.* **2011**, *7*, e1002195. [CrossRef]
29. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* **2020**, *13*, 1194–1202. [CrossRef]
30. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [CrossRef]
31. Yu, G.; Smith, D.K.; Zhu, H.; Guan, Y.; Lam, T.T.-Y. Ggtree: An r Package for Visualization and Annotation of Phylogenetic Trees with Their Covariates and Other Associated Data. *Methods Ecol. Evol.* **2017**, *8*, 28–36. [CrossRef]
32. Bailey, T.L.; Johnson, J.; Grant, C.E.; Noble, W.S. The MEME Suite. *Nucleic Acids Res.* **2015**, *43*, W39–W49. [CrossRef] [PubMed]
33. Lescot, M.; Déhais, P.; Thijs, G.; Marchal, K.; Moreau, Y.; Van de Peer, Y.; Rouzé, P.; Rombauts, S. PlantCARE, a Database of Plant Cis-Acting Regulatory Elements and a Portal to Tools for in Silico Analysis of Promoter Sequences. *Nucleic Acids Res.* **2002**, *30*, 325–327. [CrossRef]
34. O'Rourke, J.A.; Fu, F.; Bucciarelli, B.; Yang, S.S.; Samac, D.A.; Lamb, J.F.S.; Monteros, M.J.; Graham, M.A.; Gronwald, J.W.; Krom, N.; et al. The *Medicago Sativa* Gene Index 1.2: A Web-Accessible Gene Expression Atlas for Investigating Expression Differences between *Medicago Sativa* Subspecies. *BMC Genom.* **2015**, *16*, 502. [CrossRef] [PubMed]
35. Dong, X.; Deng, H.; Ma, W.; Zhou, Q.; Liu, Z. Genome-Wide Identification of the MADS-Box Transcription Factor Family in Autotetraploid Cultivated Alfalfa (*Medicago Sativa* L.) and Expression Analysis under Abiotic Stress. *BMC Genom.* **2021**, *22*, 603. [CrossRef] [PubMed]
36. Kim, D.; Paggi, J.M.; Park, C.; Bennett, C.; Salzberg, S.L. Graph-Based Genome Alignment and Genotyping with HISAT2 and HISAT-Genotype. *Nat. Biotechnol.* **2019**, *37*, 907–915. [CrossRef]
37. Liao, Y.; Smyth, G.K.; Shi, W. FeatureCounts: An Efficient General Purpose Program for Assigning Sequence Reads to Genomic Features. *Bioinformatics* **2014**, *30*, 923–930. [CrossRef]
38. Love, M.I.; Huber, W.; Anders, S. Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [CrossRef]
39. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001**, *25*, 402–408. [CrossRef]
40. Ouyang, S.; Zhu, W.; Hamilton, J.; Lin, H.; Campbell, M.; Childs, K.; Thibaud-Nissen, F.; Malek, R.L.; Lee, Y.; Zheng, L.; et al. The TIGR Rice Genome Annotation Resource: Improvements and New Features. *Nucleic Acids Res.* **2007**, *35*, D883–D887. [CrossRef]
41. Kaur, S.; Dhugga, K.S.; Beech, R.; Singh, J. Genome-Wide Analysis of the *Cellulose Synthase-like* (Csl) Gene Family in Bread Wheat (*Triticum Aestivum* L.). *BMC Plant Biol.* **2017**, *17*, 193. [CrossRef] [PubMed]
42. Li, Y.; Yang, T.; Dai, D.; Hu, Y.; Guo, X.; Guo, H. Evolution, Gene Expression Profiling and 3D Modeling of CSLD Proteins in Cotton. *BMC Plant Biol.* **2017**, *17*, 119. [CrossRef] [PubMed]
43. Nawaz, M.A.; Rehman, H.M.; Baloch, F.S.; Ijaz, B.; Ali, M.A.; Khan, I.A.; Lee, J.D.; Chung, G.; Yang, S.H. Genome and Transcriptome-Wide Analyses of Cellulose Synthase Gene Superfamily in Soybean. *J. Plant Physiol.* **2017**, *215*, 163–175. [CrossRef]
44. Ermawar, R.A.; Collins, H.M.; Byrt, C.S.; Henderson, M.; O'Donovan, L.A.; Shirley, N.J.; Schwerdt, J.G.; Lahnstein, J.; Fincher, G.B.; Burton, R.A. Genetics and Physiology of Cell Wall Polysaccharides in the Model C4 Grass, *Setaria Viridis* spp. *BMC Plant Biol.* **2015**, *15*, 236. [CrossRef]
45. Yin, Y.; Huang, J.; Xu, Y. The Cellulose Synthase Superfamily in Fully Sequenced Plants and Algae. *BMC Plant Biol.* **2009**, *9*, 99. [CrossRef] [PubMed]
46. Bulone, V.; Schwerdt, J.G.; Fincher, G.B. Co-Evolution of Enzymes Involved in Plant Cell Wall Metabolism in the Grasses. *Front. Plant Sci.* **2019**, *10*, 1009. [CrossRef]
47. Betts, M.J.; Guigó, R.; Agarwal, P.; Russell, R.B. Exon Structure Conservation despite Low Sequence Similarity: A Relic of Dramatic Events in Evolution? *EMBO J.* **2001**, *20*, 5354–5360. [CrossRef]
48. Carmel, L.; Rogozin, I.B.; Wolf, Y.I.; Koonin, E.V. Evolutionarily Conserved Genes Preferentially Accumulate Introns. *Genome Res.* **2007**, *17*, 1045–1050. [CrossRef]
49. Li, G.; Liu, X.; Liang, Y.; Zhang, Y.; Cheng, X.; Cai, Y. Genome-Wide Characterization of the Cellulose Synthase Gene Superfamily in *Pyrus Bretschneideri* and Reveal Its Potential Role in Stone Cell Formation. *Funct. Integr. Genom.* **2020**, *20*, 723–738. [CrossRef]
50. Daras, G.; Templalexis, D.; Avgeri, F.; Tsitsekian, D.; Karamanou, K.; Rigas, S. Updating Insights into the Catalytic Domain Properties of Plant *Cellulose Synthase* (CesA) and *Cellulose Synthase-like* (Csl) Proteins. *Molecules* **2021**, *26*, 4335. [CrossRef]
51. Wang, J.; Li, J.; Lin, W.; Deng, B.; Lin, L.; Lv, X.; Hu, Q.; Liu, K.; Fatima, M.; He, B.; et al. Genome-Wide Identification and Adaptive Evolution of CesA/Csl Superfamily among Species with Different Life Forms in Orchidaceae. *Front. Plant Sci.* **2022**, *13*, 994679. [CrossRef] [PubMed]
52. Hernandez-Garcia, C.M.; Finer, J.J. Identification and Validation of Promoters and *Cis*-Acting Regulatory Elements. *Plant Sci.* **2014**, *217–218*, 109–119. [CrossRef] [PubMed]
53. Favery, B.; Ryan, E.; Foreman, J.; Linstead, P.; Boudonck, K.; Steer, M.; Shaw, P.; Dolan, L. KOJAK Encodes a Cellulose Synthase-like Protein Required for Root Hair Cell Morphogenesis in *Arabidopsis*. *Genes Dev.* **2001**, *15*, 79–89. [CrossRef]

54. Wang, X.; Cnops, G.; Vanderhaeghen, R.; De Block, S.; Van Montagu, M.; Van Lijsebettens, M. AtCSLD3, a Cellulose Synthase-like Gene Important for Root Hair Growth in *Arabidopsis*. *Plant Physiol.* **2001**, *126*, 575–586. [CrossRef] [PubMed]
55. Bernal, A.J.; Jensen, J.K.; Harholt, J.; Sørensen, S.; Moller, I.; Blaukopf, C.; Johansen, B.; de Lotto, R.; Pauly, M.; Scheller, H.V.; et al. Disruption of ATCSLD5 Results in Reduced Growth, Reduced Xylan and Homogalacturonan Synthase Activity and Altered Xylan Occurrence in *Arabidopsis*. *Plant J.* **2007**, *52*, 791–802. [CrossRef] [PubMed]
56. Kim, C.M.; Park, S.H.; Je, B.I.; Park, S.H.; Park, S.J.; Piao, H.L.; Eun, M.Y.; Dolan, L.; Han, C. *OsCSLD1*, a Cellulose Synthase-like D1 Gene, Is Required for Root Hair Morphogenesis in Rice. *Plant Physiol.* **2007**, *143*, 1220–1230. [CrossRef]
57. Zhu, J.; Lee, B.-H.; Dellinger, M.; Cui, X.; Zhang, C.; Wu, S.; Nothnagel, E.A.; Zhu, J.-K. A Cellulose Synthase-like Protein Is Required for Osmotic Stress Tolerance in *Arabidopsis*. *Plant J.* **2010**, *63*, 128–140. [CrossRef]
58. Zhao, H.; Li, Z.; Wang, Y.; Wang, J.; Xiao, M.; Liu, H.; Quan, R.; Zhang, H.; Huang, R.; Zhu, L.; et al. Cellulose Synthase-like Protein OsCSLD4 Plays an Important Role in the Response of Rice to Salt Stress by Mediating Abscisic Acid Biosynthesis to Regulate Osmotic Stress Tolerance. *Plant Biotechnol. J.* **2022**, *20*, 468–484. [CrossRef]
59. Li, S.; Zhang, L.; Wang, Y.; Xu, F.; Liu, M.; Lin, P.; Ren, S.; Ma, R.; Guo, Y.-D. Knockdown of a Cellulose Synthase Gene *BoiCesA* Affects the Leaf Anatomy, Cellulose Content and Salt Tolerance in Broccoli. *Sci. Rep.* **2017**, *7*, 41397. [CrossRef]
60. Ye, Y.; Wang, S.; Wu, K.; Ren, Y.; Jiang, H.; Chen, J.; Tao, L.; Fu, X.; Liu, B.; Wu, Y. A Semi-Dominant Mutation in OsCESA9 Improves Salt Tolerance and Favors Field Straw Decay Traits by Altering Cell Wall Properties in Rice. *Rice* **2021**, *14*, 19. [CrossRef]
61. Cao, H.; Guo, S.; Xu, Y.; Jiang, K.; Jones, A.M.; Chong, K. Reduced Expression of a Gene Encoding a Golgi Localized Monosaccharide Transporter (*OsGMST1*) Confers Hypersensitivity to Salt in Rice (*Oryza Sativa*). *J. Exp. Bot.* **2011**, *62*, 4595–4604. [CrossRef] [PubMed]

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