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Genome-Wide Identification and Characterization of Calmodulin and Calmodulin-like Genes Family in Tea Plant and Their Roles under Abiotic Stress

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Abstract: As an important Ca^{2+} sensor, calmodulin (CaM) and calmodulin-like protein (CML) play core roles in plant growth, development, and response to environmental stimuli. The *CaM/CML* gene family has been well characterized in various plant species, such as *Arabidopsis thaliana*, rice, and tomato; however, in the tea plant, the *CaM/CML* gene family has not been systematically and comprehensively characterized. In the present study, a total of 5 CsCaM and 60 CsCML proteins were identified from the tea plant genome, which were unevenly distributed on the 14 chromosomes of the tea plant. All the proteins contained two to four EF-hand domains. Meanwhile, an integrated analysis of physicochemical properties, sequence structure, motif identification, phylogeny, gene duplication, promoter *cis*-elements, and RNA-seq expression profiles in the *CsCaM/CML* gene family was performed. Transcriptome analysis revealed that *CsCaM/CMLs* were differentially expressed in different tissues of the tea plant, suggesting their potential roles in plant growth and development. The expression profiles associated with various stress treatments revealed that *CsCaM/CML* genes were involved in a wide range of abiotic factors, including cold and drought stress. Quantitative real-time PCR (qRT-PCR) was also used to validate the differences in expression under abiotic stress. Overall, these findings enhanced our understanding of *CsCaM/CML* genes and provided useful information for further research into their molecular functions in abiotic stress response, and in multiple physiological processes in the tea plant.

Keywords: CsCaM/CML gene family; genome-wide identification; expression analysis; abiotic stress; *Camellia sinensis*

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1. Introduction

Calcium (Ca^{2+}) in plants acts as an important intracellular second messenger and is extensively involved in regulating plant growth and development as well as mediating responses to various biotic and abiotic stresses. The Ca^{2+} signal is not only the core regulator of plant cell physiology but also the plant cellular response to the environment [1–3]. When plants are stimulated, for instance by phytohormones, salt, heat, cold, drought, and pathogen attack, they can rapidly cause calcium transients and calcium oscillations via increasing the Ca^{2+} concentration in the cytoplasm that generate calcium signal transduction and help coordinate adaptive responses [4]. In the process, the perception is that the decoding of transient changes in calcium signal by Ca^{2+}-binding protein sensors is a key regulatory step in the calcium signaling pathway, and four major classes of Ca^{2+} sensors are presented in plants, namely calmodulins (CaMs), CaM-like proteins (CMLs) [5], calcineurin B-like proteins (CBLs) [6], and Ca^{2+}-dependent protein kinases (CDPKs/CPKs) [7]. The EF-hand domain, which is responsible for Ca^{2+} binding, is found in all four types of Ca^{2+} sensors. After binding of Ca^{2+} ions, the Ca^{2+} sensors experience a change in protein

conformation, which triggers plant responses and amplifies the signal by modulating their activity or capacity to interact with downstream proteins [8,9].

CaMs are highly conserved and the most well-studied Ca^{2+}-binding proteins, present in all eukaryotes and comprising 4 EF-hand motifs, while CMLs are only observed in plants. CMLs share at least 16% of their amino acid identity with typical CaMs, which usually contain 1–6 EF-hand domains and no other identifiable functional domains [10,11]. As a result of genome sequencing in several plant species, the *CaM/CML* gene family has been characterized at the genome-wide level. A total of 7 *AtCaM* and 50 *AtCML* have been identified in *Arabidopsis* [10], 5 *OsCaM* and 32 *OsCML* in rice (*Oryza sativa*) [12], 6 *SpCaM* and 45 *SpCML* in wild tomato (*Solanum pennellii*) [13], 4 *MdCaM* and 58 *MdCML* in apple (*Malus x domestica*) [14], 3 *VviCaM* and 62 *VviCML* in grapevine (*Vitis vinifera*) [15], 25 *BnaCaM* and 168 *BnaCML* in *Brassica napus* [16], 8 *MeCaM* and 48 *MeCML* in cassava (*Manihot esculenta*) [17], and 34 *CaM/CML* genes in lotus (*Nelumbo nucifera*) [18]. However, the bioinformatics of the *CaM* and *CML* families in *Camellia sinensis* have not yet been thoroughly investigated. Despite 5 *CsCML* genes being discovered and described in the tea plant, they were not named following a standard procedure and were not fully investigated [19].

CaM and CMLs play significant roles in regulating a variety of physiological processes. They participate in pollen tube growth [20,21], seed and fruit development [22,23], flowering [24], cell metabolism [25], root growth [26], and other processes [27]. As previously reported, *AtCML39* was involved in regulating seed development, germination, and seedling establishment in *Arabidopsis* [23]; AtCML42 interacted with kinesin-interacting Ca^{2+}-binding protein (KIC) to regulate trichome branching [28]. *AtCaM7* was implicated in light-mediated gene expression, and overexpression *AtCaM7* was observed to promote photomorphogenic growth in transgenic plants [29]. Loss and gain of function mutants of *cml24* in *Arabidopsis thaliana* exhibited late and early flowering, respectively [30]. In rice, the OsCaM1-associated OsMKK1-OsMKK6 cascade was found to induce root auxin levels and promote lateral root growth under salt stress [26]. *GhCaM7* promoted cotton fiber elongation by modulating the generation of reactive oxygen species (ROS) [31].

Likewise, a great number of studies have shown that the *CaM* and *CML* genes are crucial for abiotic and biotic stress responses. *AtCML8*, *CML37*, *CML38*, and *CML39* were responsive to pathogenic bacteria, salinity, and hormonal treatment in *Arabidopsis* [32,33]; *AtCML9* was regulated by abscisic acid (ABA) and *Pseudomonas syringae* infection [34]; *AtCML19/42* were involved in the UV stress response of *Arabidopsis thaliana* and were able to prevent UV damage to the plant [35]. In rice, overexpression of *OsCaM1-1* may confer salt stress tolerance through the up-regulation of salt stress response genes [36]; *OsMSR2* (*OsCML24*) significantly enhanced drought and salt tolerance through an ABA-mediated pathway [37]; *OsCam1-1*, *OsCML4*, *5*, *8*, and *11* were induced by osmotic and salt stresses [38]. Apart from *Arabidopsis* and rice, studies on *CaM* and *CML* genes in other plants under abiotic stress have also been reported. *ShCML44* from tomato [39], *MdCML3* from apple [14], *TaCML36* in wheat [40], *VaCML21* of grapevine [41], *ZmCaM* and *ZmCML* genes of maize [42] were all induced under different abiotic and biotic stresses.

The tea plant (*Camellia sinensis*) is an evergreen economic plant that grows well in normal temperature, high humidity, and acid soil (pH 4.5–5.5) environments [43]. However, due to the recent occurrence of extreme weather events, the tea plant typically cannot overwinter safely or grow healthily when damaged by drought, heat, salinity, low temperature, or cold spells. Thus, an increasing number of studies have been concentrated on the molecular mechanisms of tea plant stress responses. CaM and CML proteins are types of Ca^{2+} sensors found in plants that play significant roles in mediating plant tolerance to abiotic stress. Nonetheless, detailed characterization and expression patterns of the *CsCaM* and *CsCML* genes family in the tea plant are largely unknown. Herein, we carried out a genome-wide identification and characterization analysis of *CsCaM/CMLs*, analyzing expression patterns of the tissue-specific profiles and different abiotic stresses. The results will be useful for further exploring the function of CaMs/CMLs in the growth, development, and calcium signaling response of the tea plant in the future.

2. Materials and Methods

2.1. Plant Materials and Treatments

One-year-old tea seedlings (*C. sinensis* cv. Longjing 43) were pre-cultured in a growth chamber at the Tea Research Laboratory of Henan Agricultural University (Zhengzhou, China). The growth conditions were optimized to a photoperiod of 16 h light (25 ± 1 °C, 240 μmol m^{-2} s^{-1}) and 75% relative humidity. After two weeks of adaptive growth, the tea seedlings were exposed to adversity stress treatments, including low temperature (10 °C), drought (20% PEG 6000), high salt (200 mmol/L NaCl), and exogenous ABA (100 μmol/L) to investigate the response of CsCaMs and CsCMLs. The third leaf below the top bud of the tea plant was randomly taken after 0, 4, 12, and 24 h treatments. All the samples were frozen in liquid nitrogen immediately and stored at −80 °C for further use. Each treatment was tested in three biological replicates.

2.2. Genome-Wide Identification of CsCaM and CsCML Gene Family in the Tea Plant

The amino acid sequences of the *Arabidopsis thaliana* CaM and CML family from the TAIR website (https://www.arabidopsis.org/ (accessed on 2 May 2021)) were used as queries to BLAST against the tea plant genome database (TPIA, http://tpdb.shengxin.ren/ (accessed on 15 June 2021)) to identify novel CsCaM and CsCML genes with E-value < 1 $\times 10^{-5}$. Subsequently, we used 'calmodulin', 'EF hand', 'calmodulin-like protein', and 'PF13499' as keywords to search for homology in the tea genome database. The obtained candidate protein sequences were verified using the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 4 July 2021)), SMART (http://smart.embl-heidelberg.de/ (accessed on 8 July 2021)), PfamScan (https://www.ebi.ac.uk/Tools/pfa/pfamscan/ (accessed on 12 July 2021)), and InterPro (http://www.ebi.ac.uk/interpro/ (accessed on 19 July 2021)) to eliminate the genes with incomplete domains, and the remaining sequences were used in further analyses.

2.3. Physicochemical Properties, Conserved Domain, Gene Structure, and Phylogenetic Relationships of CsCaM and CsCML Genes

The amino acid sequence length (aa), predicted molecular weight (D), and isoelectric point (pI) of CsCaM/CML family members were investigated using the ExPASy ProtParam tool (http://web.expasy.org/protparam (accessed on 17 August 2021)). The conserved motifs of CsCaM/CML family members were analyzed using the MEME tool (http://meme-suite.org/tools/meme (accessed on 21 August 2021)). The exon and intron structure of each CsCaM and CsCML gene was illustrated using Gene Structure Display Server (GSDS2.0, http://gsds.gao-lab.org/ (accessed on 1 September 2021)). The sequences of CaM/CML family members from the tea plant (CsCaM/CML), *Arabidopsis* (AtCsCaM/CML), rice (OsCsCaM/CML), and cabbage (BrCaM/CML) were performed to establish a phylogenetic tree by MEGA 7.0 with the neighbor-joining (NJ) method and 1000 bootstrap replicates.

2.4. Promoter Analysis

In order to identify the *cis*-acting elements in the promoter sequences of the *CsCaM* and *CsCML* genes, we isolated the upstream 2000-bp region of the translation start site from the TPIA database and subjected it to the PlantCARE tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 15 September 2021)).

2.5. Chromosomal Location and Collinearity Analysis

The identified tea plant *CsCaM/CML* family genes were examined for chromosomal location and gene collinearity based on the GFF file from the TPIA (tpdb.shengxin.ren/index.html (accessed on 29 September 2021)) and mapped using TBtools software [44]. The Multiple Collinearity Scan (MCScanX) toolkit was used to compare tea plant genome sequences and analyze tandem repeats and fragment repeats [45].

2.6. Expression Analysis of CsCaM and CsCML Gene Family

To analyze the tissue-specific expression patterns of *CsCaM/CMLs* in the tea plant, which includes root, flower, stem, apical bud, young leaf, fruit, mature leaf, and old leaf, the published transcriptome data were obtained and analyzed from the TPIA database. Moreover, gene expression data under cold and drought stresses were analyzed to better understand the probable function of *CsCaM/CMLs* in response to abiotic stress. The transcriptomic data were calculated and analyzed using TBtools software [44].

2.7. RNA Extraction and qRT-PCR Assays

Total RNA of the tea plant was extracted using RNAprep Pure Plant Kit (Tian Gen Biochemical Technology Co., Ltd., Beijing, China). The cDNA was generated using the PrimeScript™ RT reagent Kit (TaKaRa, Japan) according to the manufacturer's protocol. Quantitative reverse-transcription PCR (qRT-PCR) analysis was conducted using the Cham Q Universal SYBR qPCR Master Mix (Vazyme, China) on the Applied Biosystems 7500 FAST platform (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences used for qRT-PCR are listed in Table 1. For data normalization, the *CsPTB* (GenBank accession number: GAAC01052498.1) gene served as the internal control, and the relative expression was calculated according to the $2^{-\Delta\Delta Ct}$ method [46]. The qRT-PCR program was as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, and 60 °C for 34 s.

Table 1. Primers for qRT-PCR.

Gene Name	Primer Sequences (5′–3′)
CsCML1	F: TCTCAGGCACATCCTCACCA; R: ATACTGGTGAGAATGTGCCTGAG
CsCML3	F: AAGATTGGAGAAAGGGACAGTAAG; R: AGTCATCCACAGTTACTTCTCCATC
CsCML12	F: GTGTTAGTTGGTCTTGGGTATGAAA; R: ACACAACCTCTCATCATTGACCTAA
CsCML33	F: TCCAAGCACCTCAAGCCC; R: TACTGGTGAGAATGTGCCTGAGA
CsCML39	F: TGGACTCCGATGGAAGCCTAAC; R: GCCTCGCTCATATCGGGTAAAA
CsCML42	F: AGTTGATACTGATGGAAATGGGAC; R: CTTCATCTGTTATTCTCTCTCCCAA
CsCML51	F: AAGAACAACGACGGCTTCATAA; R: CCATCACCATTAGAATCCACCTT
CsPTB	F: ACCAAGCACACTCCACACTATCG; R: TGCCCCCTTATCATCATCCACAA

3. Results

3.1. Genome-Wide Identification of CaM and CML Genes in the Tea Plant

In total, 5 *CsCaMs* and 60 *CsCMLs* were identified from the tea plant genome, and named according to homology with *AtCaM/CML* genes (Table 2). The CsCaM/CML proteins ranged in length from 86 (CsCML48) to 340 (CsCML19) amino acids, and their molecular weight (MW) ranged between 9.94 and 35.29 kDa. The theoretical isoelectric points (pI) values of the CsCaM and CsCMLs ranged from 3.95 to 6.32, with CsCML60 having the lowest (pI 3.95) and CsCML7/55 having the highest (pI 7.74), indicating that these two proteins were more basic than the others. In addition to CsCML19/52/53/54, which were hydrophobic proteins with a mean hydrophilic value greater than 0, the other proteins were hydrophilic proteins ranging from −0.023 (CsCML8) to 0.829 (CsCML3). The results indicated that the CsCaM/CML protein family of tea are mostly acidic and hydrophilic.

Table 2. Characteristics and names of the CsCaM and CsCML proteins identified in the *Camellia sinensis* genome.

Gene name	Gene ID [1]	CDS [2]	AA [3]	DMW [4]	pI [5]	GRAVY [6]	EF-Hands [7]
CSS0003231.2	CsCaM1	552	183	20,913.41	4.68	−0.801	4
CSS0032965.1	CsCaM2	450	149	16,833.64	4.10	−0.619	4
CSS0045497.1	CsCaM3	450	149	16,847.67	4.11	−0.619	4
CSS0013557.1	CsCaM4	450	149	16,847.67	4.11	−0.619	4
CSS0005129.1	CsCaM5	450	149	16,833.64	4.10	−0.619	4
CSS0026594.1	CsCML1	444	147	16,478.71	4.72	−0.359	3
CSS0018159.1	CsCML2	501	166	18,237.10	4.87	−0.728	4
CSS0046781.1	CsCML3	510	168	19,174.36	4.82	−0.829	4
CSS0033756.1	CsCML4	435	144	16,061.25	4.54	−0.396	4
CSS0029813.1	CsCML5	576	191	21,799.47	4.21	−0.211	2
CSS0006572.1	CsCML6	447	158	17,470.39	4.72	−0.637	4
CSS0041481.1	CsCML7	516	171	18,995.88	7.74	−0.447	3
CSS0005295.1	CsCML8	582	193	21,586.52	4.33	−0.023	2
CSS0005567.1	CsCML9	558	185	21,313.48	5.23	−0.436	4
CSS0044479.1	CsCML10	540	179	20,170.17	5.15	−0.381	4
CSS0011314.1	CsCML11	456	151	16,771.97	4.40	−0.317	4
CSS0038900.1	CsCML12	456	151	16,771.97	4.40	−0.317	4
CSS0016893.1	CsCML13	504	167	18,742.05	5.33	−0.427	3
CSS0032997.1	CsCML14	462	153	17,515.67	4.07	−0.330	3
CSS0000487.1	CsCML15	462	153	17,558.76	4.13	−0.304	3
CSS0022279.1	CsCML16	402	133	15,226.13	4.44	−0.377	2
CSS0000476.1	CsCML17	471	156	17,786.14	4.31	−0.290	3
CSS0006005.1	CsCML18	471	156	17,788.17	4.25	−0.270	3
CSS0046243.1	CsCML19	1020	339	35,286.53	4.14	0.161	3
CSS0017640.1	CsCML20	558	185	21,234.38	5.36	−0.506	4
CSS0001406.1	CsCML21	558	185	21,211.34	5.23	−0.505	4
CSS0033780.1	CsCML22	453	150	17,023.32	4.38	−0.509	4
CSS0024416.1	CsCML23	690	229	26,354.00	4.63	−0.445	4
CSS0047932.1	CsCML24	501	166	18,021.71	4.34	−0.454	3
CSS0012943.1	CsCML25	501	166	18,109.77	4.32	−0.501	3
CSS0046747.1	CsCML26	582	193	21,163.77	4.50	−0.382	4
CSS0019921.1	CsCML27	705	234	25,234.74	4.58	−0.029	4
CSS0041234.1	CsCML28	459	152	17,256.35	4.08	−0.422	4
CSS0037530.1	CsCML29	459	152	17,212.28	4.09	−0.438	4
CSS0036108.1	CsCML30	459	152	17,328.42	4.08	−0.457	4
CSS0038701.1	CsCML31	693	230	26,266.87	4.59	−0.408	3
CSS0037305.1	CsCML32	693	230	26,261.85	4.60	−0.403	3
CSS0025958.1	CsCML33	444	147	16,538.72	4.69	−0.397	3
CSS0018156.1	CsCML34	477	158	17,028.53	4.35	−0.543	4
CSS0043064.1	CsCML35	483	160	17,093.61	4.24	−0.501	3
CSS0046384.1	CsCML36	684	227	26,169.92	5.47	−0.441	4
CSS0038594.1	CsCML37	639	212	24,257.84	4.85	−0.373	4
CSS0023779.1	CsCML38	483	160	18,310.36	4.25	−0.457	3
CSS0017237.1	CsCML39	483	160	17,550.88	4.38	−0.139	4
CSS0002201.1	CsCML40	687	238	26,577.87	6.30	−0.440	2
CSS0018824.1	CsCML41	459	152	17,183.34	4.32	−0.493	4

Table 2. *Cont.*

Gene name	Gene ID [1]	CDS [2]	AA [3]	DMW [4]	pI [5]	GRAVY [6]	EF-Hands [7]
CSS0033073.1	CsCML42	447	148	16,881.83	4.06	−0.399	4
CSS0029226.1	CsCML43	585	194	21,265.68	4.46	−0.438	4
CSS0033360.1	CsCML44	492	183	20,290.86	4.86	−0.363	4
CSS0020659.1	CsCML45	495	164	18,192.37	4.55	−0.390	4
CSS0024302.1	CsCML46	690	229	25,736.15	6.32	−0.417	2
CSS0036849.1	CsCML47	447	148	17,026.25	4.88	−0.825	3
CSS0042140.1	CsCML48	258	85	9941.18	4.46	−0.569	2
CSS0021840.1	CsCML49	447	148	16,864.84	4.06	−0.304	4
CSS0004986.1	CsCML50	489	162	17,926.04	4.48	−0.359	4
CSS0002820.1	CsCML51	648	216	23,653.66	4.47	−0.254	3
CSS0038574.1	CsCML52	633	210	23,387.23	4.07	0.060	2
CSS0013916.1	CsCML53	423	140	15,001.98	4.16	0.057	3
CSS0039564.1	CsCML54	423	140	15,016.01	4.16	0.059	3
CSS0025741.1	CsCML55	516	171	18,995.88	7.74	−0.447	3
CSS0046428.1	CsCML56	639	212	24,245.83	4.85	−0.357	4
CSS0034436.1	CsCML57	483	160	17,262.94	4.40	−0.457	3
CSS0034378.1	CsCML58	471	156	17,787.21	4.27	−0.259	3
CSS0004793.1	CsCML59	471	156	17,817.21	4.29	−0.286	3
CSS0031482.1	CsCML60	450	149	16,933.79	3.95	−0.473	4

[1] GeneID number in the Genome Database for *Camellia sinensis* (TPIA, tpdb.shengxin.ren/index.html (accessed on 2 May 2021)); [2] Length of the coding region in base pairs; [3] Number of amino acids; [4] DMW, molecular weight, Da; [5] pI, theoretical isoelectric point; [6] Average hydrophilicity of the protein; [7] Number of EF-hands based on the prediction by InterProScan.

3.2. Conserved Motif and Gene Structure Analysis

Ca^{2+} is bound by the EF-hand motif, which is the main domain of calcium-binding proteins. As shown in Figure 1A,B, the CsCaM/CML protein family all possessed the conserved EF-hand domains, which had been identified and analyzed in several model plants such as *Arabidopsis* and rice. CaM proteins were relatively conserved, and all five CsCaM proteins in the tea plant contained four typical EF-hand domains, while there was some variation among CsCMLs, containing two to four typical EF-hand domains. According to these results, all identified CsCaM and CsCML proteins contain typical EF-hand domains, suggesting that they may have a function in binding Ca^{2+}.

Different combinations of introns and exons are the imprints of gene evolution. The distribution of exon–intron arrangement was examined to reveal the structural diversity of *CsCaM/CMLs*. As illustrated in Figure 1C, *CsCaML1* contained two introns, while *CsCaM2/3/4/5* contained only one intron. Among the studied 60 *CsCMLs*, 27 *CsCMLs* coding genes did not contain any introns, whereas the rest of the *CsCMLs* contained one to four introns, with *CsCML40* having the longest intron fragment and *CsCML19/23/31* containing four introns. These introns play an important role in the regulation of gene expression and transcription in plants.

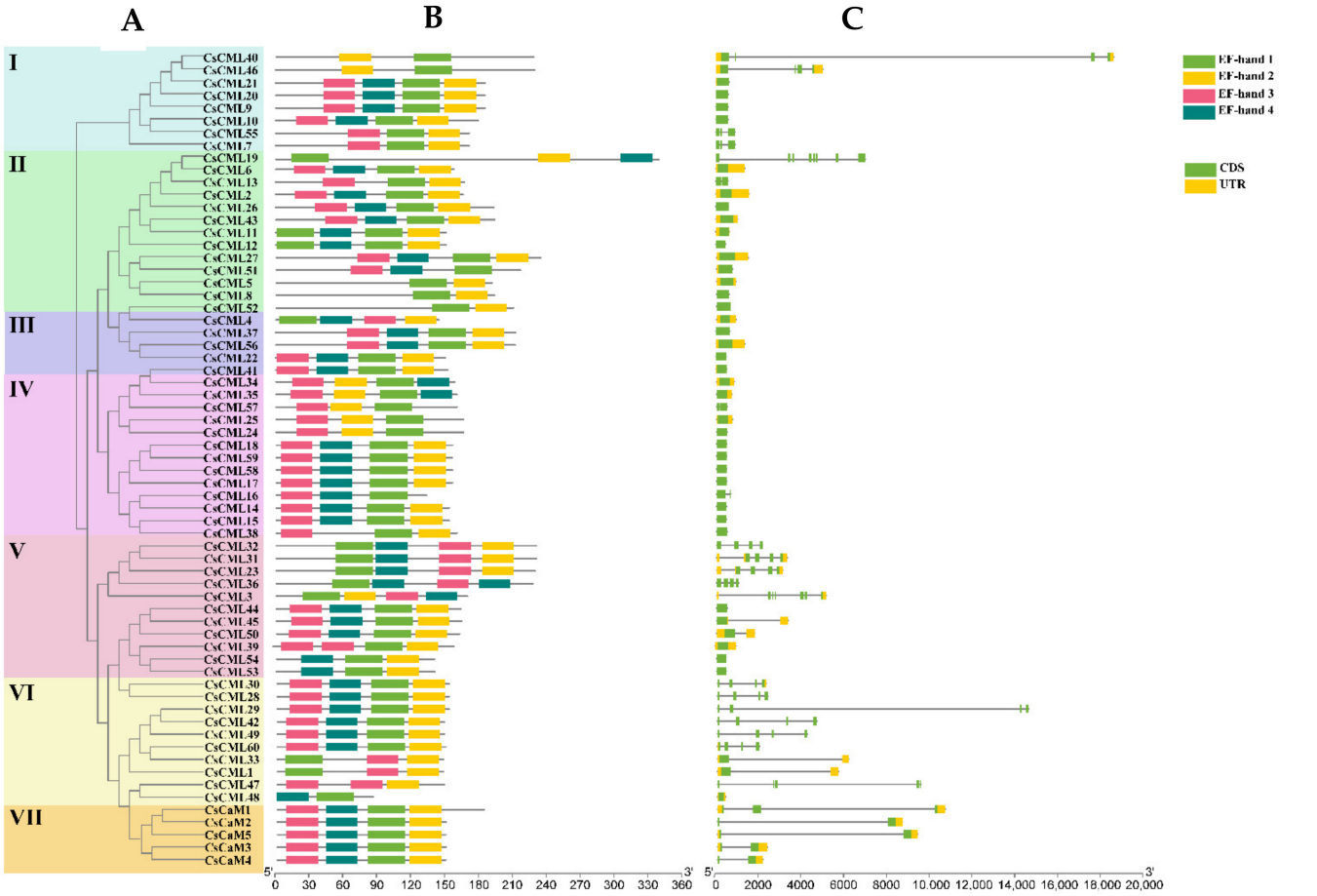

Figure 1. Phylogenetic relationships, motif compositions, and gene structure of *Camellia sinensis* CaMs and CMLs. (**A**) Phylogenetic tree and classification of CsCaM and CsCML proteins. The CsCaM and CsCML can be divided into seven clades which are denoted as subgroups 1–7 from top to bottom. (**B**) Schematic representation of the conserved EF-hand motifs among the CsCaM and CsCML proteins as obtained by MEME analysis. Each color represents a specific motif. (**C**) Exon/intron organization of tea *CsCaM* and *CsCML* genes. The exons, introns, and UTRs are represented by yellow boxes, fold lines, and green boxes, respectively.

3.3. Cis-Acting Element Analysis of CsCaM/CML Promoter

To understand the potential transcriptional regulatory mechanisms of the *CsCaM/CMLs*, the 2000 bp sequences of the *CsCaM/CML* promoter regions were analyzed by PlantCARE, and data visualization of the screened cis-elements was performed using TBtools. A variety of *cis*-elements were identified in the *CsCaM/CML* family, including environmental stress elements, hormone signals, plant growth, and development-related factors (Figure 2). Among them, environmental stress elements contained defense and stress response (TC-rich repeats), low-temperature responsive (LTR), drought-inducibility (MBS), anaerobic induction (ARE), and light responsive elements (GT1-motif/ACE/G-box). Hormone-responsive cis-acting elements were also present, such as MeJA (CGTCA-motif/TGACG-motif), IAA (TGA-element/AuxRR-core), SA (TCA-element), ABA (ABRE), and GA (GARE-motif/P-box). Two *cis*-acting elements involved in plant growth and development were associated with palisade mesophyll cell differentiation (HD-Zip 1) and meristem expression (CAT-box). These findings suggested that *CsCaM/CML* family genes may be more widely involved in the plant response to environmental stresses as well as their growth and development.

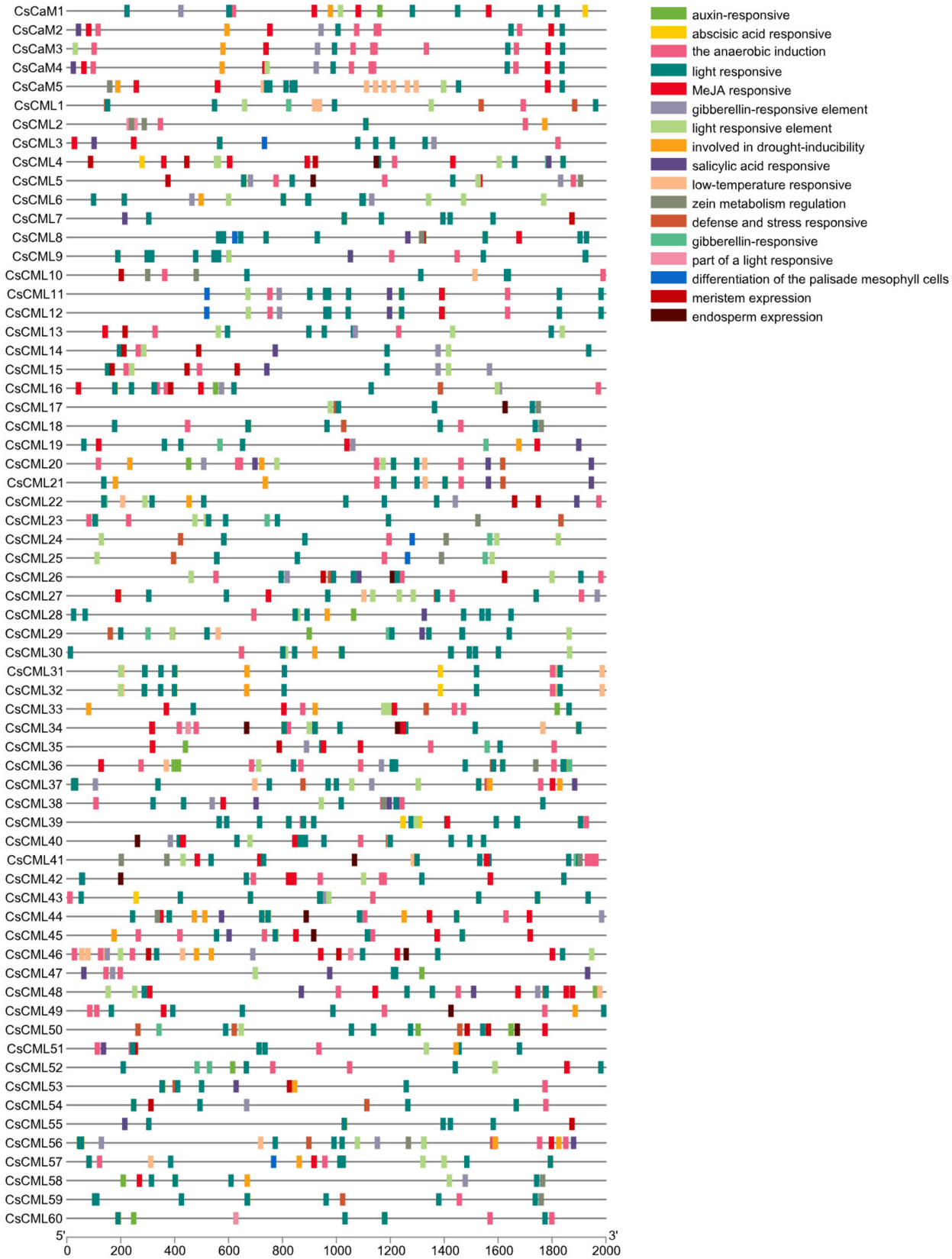

Figure 2. *Cis*-element analysis of tea *CsCaM* and *CsCML* gene promoters. The 2000 bp sequence upstream of the *CsCaM/CML* start codon was analyzed using online software Plant CARE. Binding sites in the promoter region are represented by boxes of different colors, and the graph shows the number of binding sites.

3.4. Phylogenetic Analysis of CsCaM and CsCML Families

To clarify the potential functions of CaM and CML family proteins in the tea plant and *Arabidopsis*, we established an unrooted tree using MEGA 6.0. CsCaM/CML proteins were classified into 7 subgroups based on their similarities and relationships with Arabidopsis members, which contained 8, 13, 5, 13, 11, 10, and 5 members, respectively (Figure 3). Furthermore, the evolutionary relationships of CaM/CMLs in different plant species were investigated by constructing another phylogenetic tree using the CaM/CMLs sequences from *C. sinensis*, *A. thaliana*, *O. sativa*, and *B. oleracea* (Figure 4). CsCaMs clustered with AtCaMs, OsCaMs, and BrCaMs, but CsCML47/48 and AtCML12 clustered with AtCaM1 and OsCaM1 respectively; similarly, CsCML was related to other species of CML. These results demonstrate that the CaM/CML proteins of the tea plant, *Arabidopsis*, rice, and cabbage are highly homologous.

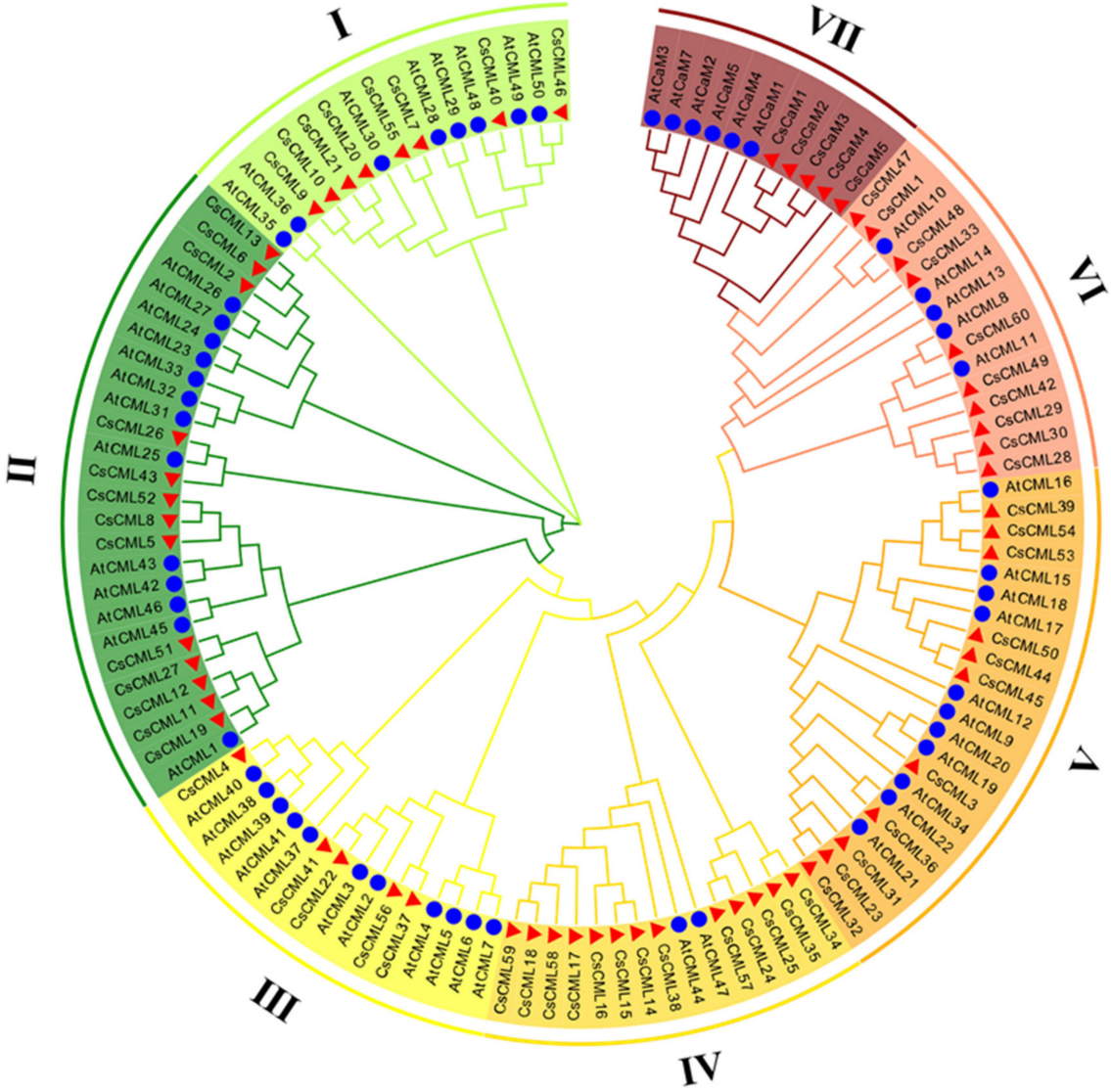

Figure 3. Phylogenetic analysis of *Camellia sinensis* and *Arabidopsis* CaM and CML proteins. A total of 65 CaM and CML proteins from tea (5 CaMs and 60 CMLs) and 57 from *Arabidopsis* (7 CaMs and 50 CMLs) were aligned using DNAMAN. The phylogenetic tree was constructed using the MEGA 7.0 program by the neighbor-joining method with bootstrap values 1000 using protein sequences. The red triangles represent *Camellia sinensis*. The blue circles represent *Arabidopsis*.

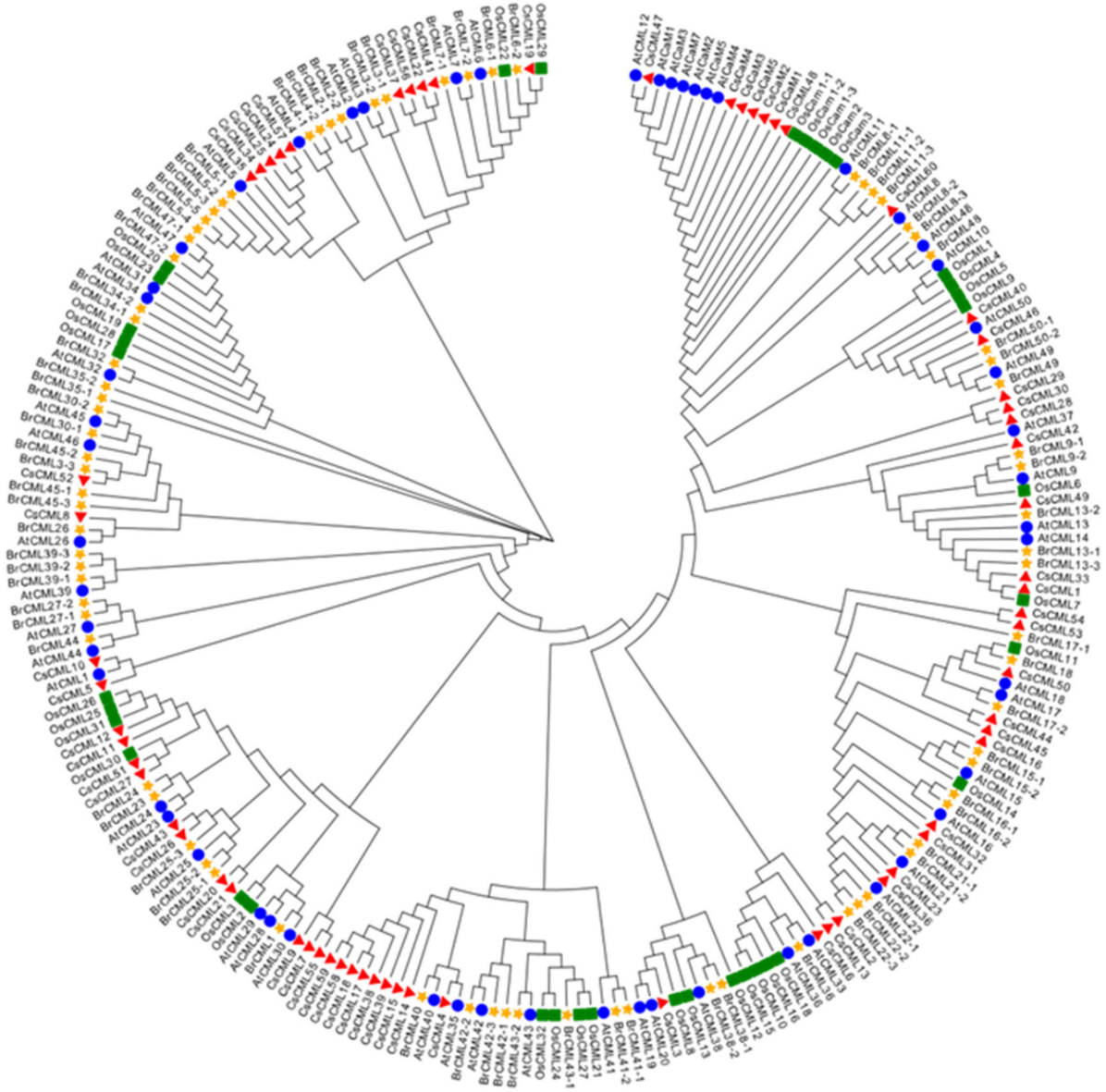

Figure 4. Phylogenetic relationships of CaM/CML genes from Camellia sinensis and other species. The phylogenetic tree was constructed based on sequence alignment of CaM/CML homologs from *Camellia sinensis*, *Arabidopsis*, rice, and cabbage using the neighbor-joining method with bootstrapping analysis in MEGA 7.0 (bootstrap:1000). The red triangles represent *Camellia sinensis*; the blue circles represent *Arabidopsis*; the green squares represent rice; the yellow stars represent cabbage.

3.5. Chromosomal Localization and Collinearity Analysis

Chromosomal (Chr) localization of 65 *CsCaM/CML* genes based on tea plant genome information was analyzed using TBtools software (Figure 5). MCscanX software was used to evaluate collinearity diagrams among *CsCaM/CML* gene members. The results revealed that 58 genes were distributed unevenly on the 14 chromosomes with no genes distributed on Chr 8, while the other 7 genes, including *CsCML43/55/56/57/58/59/60*, could not be matched on a particular chromosome, but were present on the contig. Chr 4 harbored the most *CsCMLs*, while Chr 15 contained the fewest, with only one member. *CsCaMs* were mainly located in the middle of Chr 13, while *CsCaM1* was located in the 3′ region of Chr 11. Genes were distributed unevenly on chromosomes, suggesting that there has been genetic variation in tea plants during evolution.

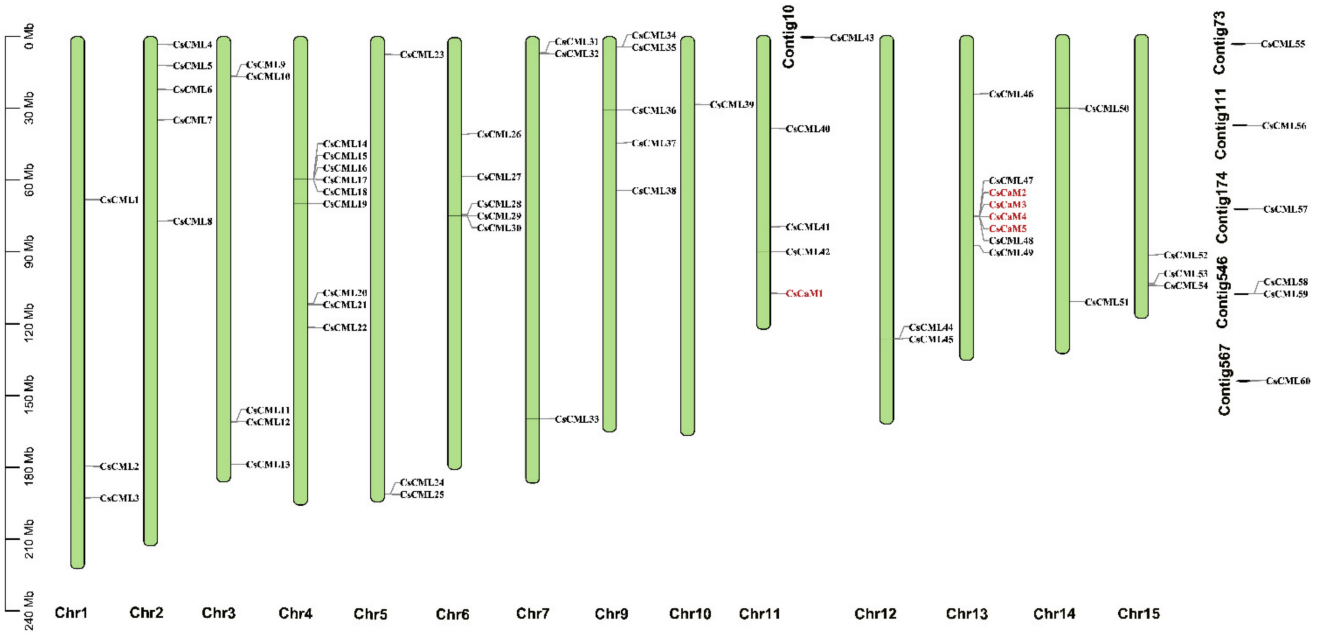

Figure 5. The distribution of *CsCaM* and *CsCML* genes in tea chromosomes. A total of 65 *CsCaM* and *CsCML* genes were mapped onto the grapevine genome. Names were assigned based on their location in tea chromosomes. The ones in red are *CsCaM* genes.

Gene duplications affect plant evolution, inheritance, and variation, and have a close relationship with gene expression and transcriptional regulation. Collinearity analysis was performed for exploring duplications within the *CsCaMs* and *CsCMLs* family (Figure 6). There were 14 gene duplication pairs among the 65 *CsCaM/CML* genes, with duplication of sequences occurring between each chromosome, but no duplication on Chr12. Interestingly, among these duplicated fragments, *CsCML4* and *CsCML25* both had a duplication relationship with *CsCML8*, and both *CsCML5* and *CsCML10* were duplicated with *CsCML21*, while *CsCML4/5/8* were all located on Chr 2, *CsCML10* on Chr3, and *CsCML21* on Chr4. It is speculated that the duplication of gene sequences may be transmitted between chromosomes through individual genes, and the gene duplication phenomenon has important implications for the evolution of CsCaM/CML proteins.

3.6. Expression Profiles of CsCaM and CsCML Genes in Different Tissues of Tea Plant

To determine the spatiotemporal expression patterns of the *CsCaM/CMLs* genes, the expression patterns of 65 *CsCaM/CML* transcripts in different tissues were obtained from TPIA datasets and further analyzed (Figure 7). As visualized by heatmap plotting, in addition to *CsCML16*, *17*, *18*, *49*, *57*, *58*, and *59* showing minimal expression/no expression in different tissues, most *CsCaM* and *CML* genes were found to be constitutively expressed in tea plant tissues, albeit at different levels. *CsCML1*, *14*, *33*, and *52* showed maximum relative expression in buds, while *CsCaM5* presented the highest expression level in young leaves, *CsCaM1* and *CsCML35* displayed a high level of expression in old leaves, and *CsCaM2* and *CsCML36* expressed more in stems. Moreover, other *CsCML* genes were relatively expressed (values > 2) mainly in flowers, mature leaves, and roots of tea plants, containing 9, 6, and 11 gene members, respectively. The findings demonstrated that *CsCaM/CMLs* may play pivotal roles in the morphological establishment of tea plant flowers and the growth and development of leaves and roots. Generally, the expression profiles of most genes in the same cluster were similar but not identical, suggesting that their functions were redundant and partially differentiated.

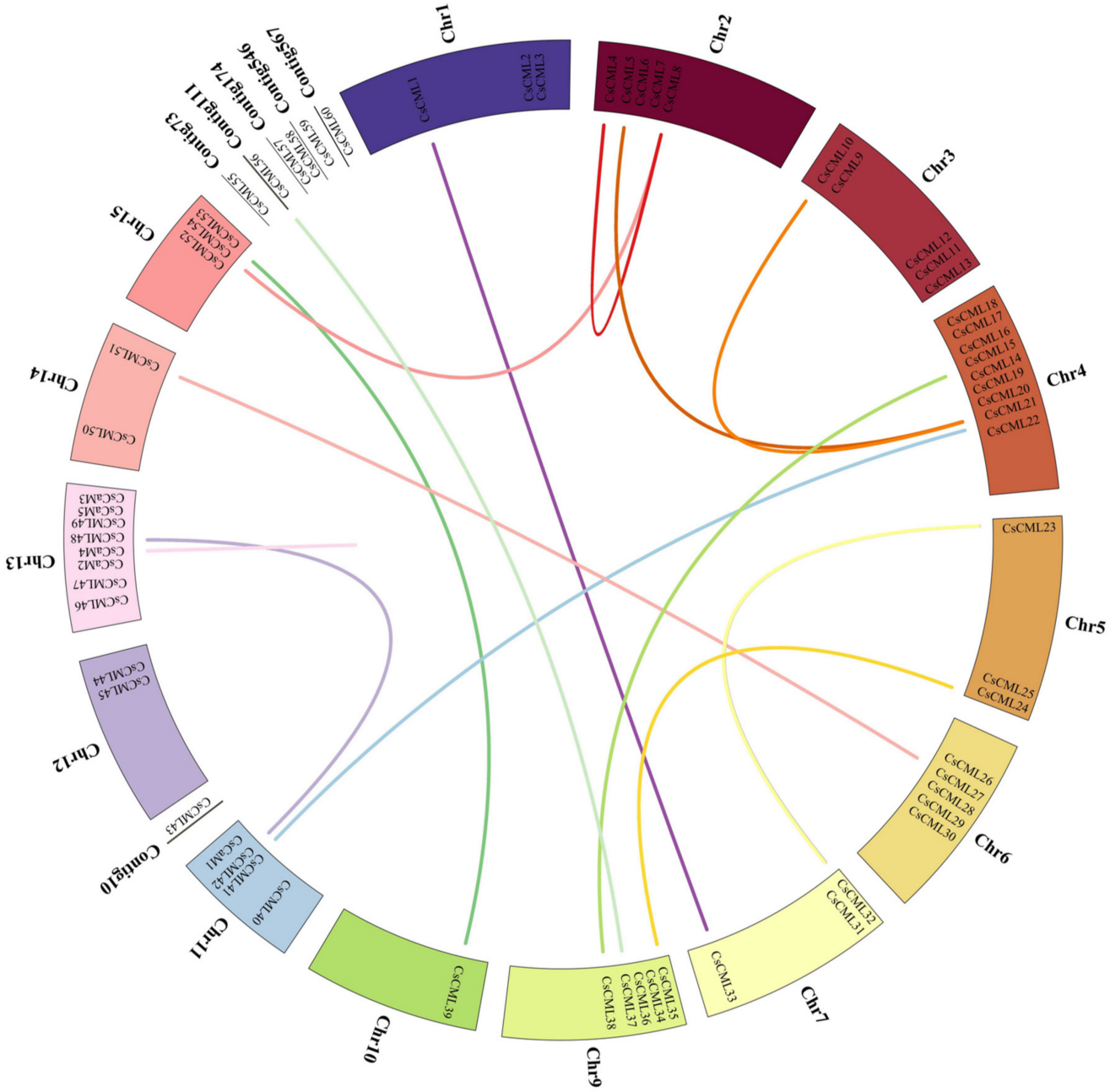

Figure 6. Collinearity analysis of tea *CsCaM* and *CsCML* genes. Among the 65 *CsCaMs/CsCMLs* genes, 14 segmentally duplicated tea plant *CsCaM/CML* genes were mapped onto 14 chromosomes.

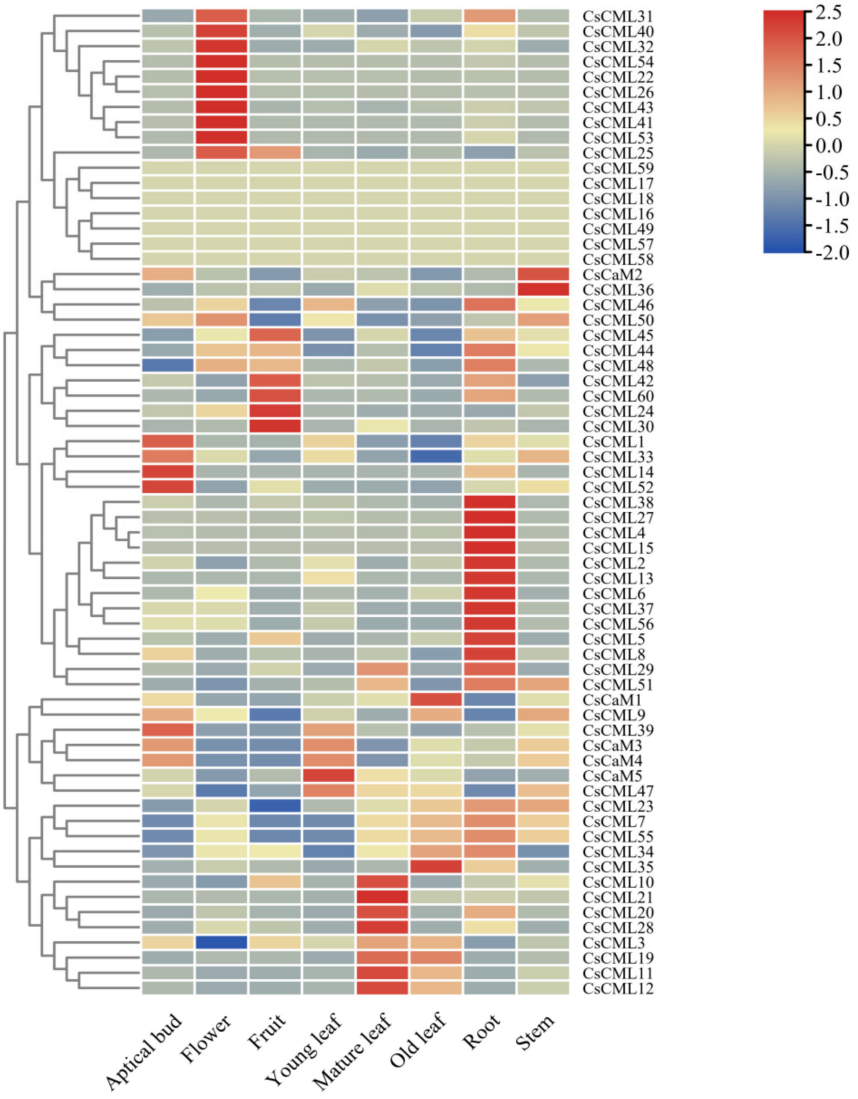

Figure 7. Expression profiles of *CsCaM* and *CsCML* genes in different tissues of the tea plant. Differential expression patterns of *CsCaM* and *CsCML* genes in root, flower, stem, terminal bud, young leaf, fruit, mature leaf, and old leaf of the tea plant.

3.7. Expression Patterns of CsCaM and CsCML Genes under Abiotic Stress in the Tea Plant

To obtain further insights into the potential functions of *CsCaM* and *CsCML* genes in the tea plant, we analyzed the expression levels of the *CsCaM/CMLs* in response to abiotic stress. As shown in Figure 8A, after cold acclimation (CA) treatment, results revealed that the expression patterns of *CsCML2*, *6*, *11*, *12*, *22*, *27*, *32*, *34*, and *CsCaM2* were up-regulation (value > 1.5) at 6 h, while *CsCML8*, *9*, *19*, *23*, *29*, *45*, *48*, *52*, and *CsCaM5* genes were down-regulated after treatment. Both *CsCaM1* and *CsCML7*, *15*, *21*, *35*, and *55* were highly expressed after 7 d of low-temperature stress. *CsCML1*, *4*, *25*, *33*, *36*, *37*, *40*, *41*, *43*, *44*, *50*, *56*, and *57* showed the highest expression level after the de-acclimation (DA) treatment. However, the rest of the genes showed no expression in response to cold treatment.

Under drought stress, their expression patterns were classified into four types: (1) genes such as *CsCML28*, *35*, *41*, *45*, *47*, and *CsCaM2* or *CsCML1*, *7*, *15*, *27*, and *55* were notably activated after 24 h or 48 h of the drought stress, and displayed a significant increase in expression levels. Subsequently, these genes were down-regulated after 48 h or 72 h of the PEG treatment, respectively; (2) type two, including *CsCML14*, *22*, *40*, *52*, and *CsCaM1* were continuously up-regulated under drought stress; (3) type three was a set of genes such as *CsCML3*, *5*, *6*, *10*, *11*, *12*, and *25*, showing a significant decreased in expression levels;

(4) type four consisted of the remaining 10 *CsCaM/CMLs*, whose expression levels were not detected under drought stress (Figure 8B). The results confirmed that the *CsCaM* and *CsCML* genes expressed differently under various stress conditions and participated in the regulation of abiotic stress responses. It is noteworthy that several *CsCMLs* respond to multiple stresses, and *CsCML7/11/15/21/55* were upregulated by cold and drought.

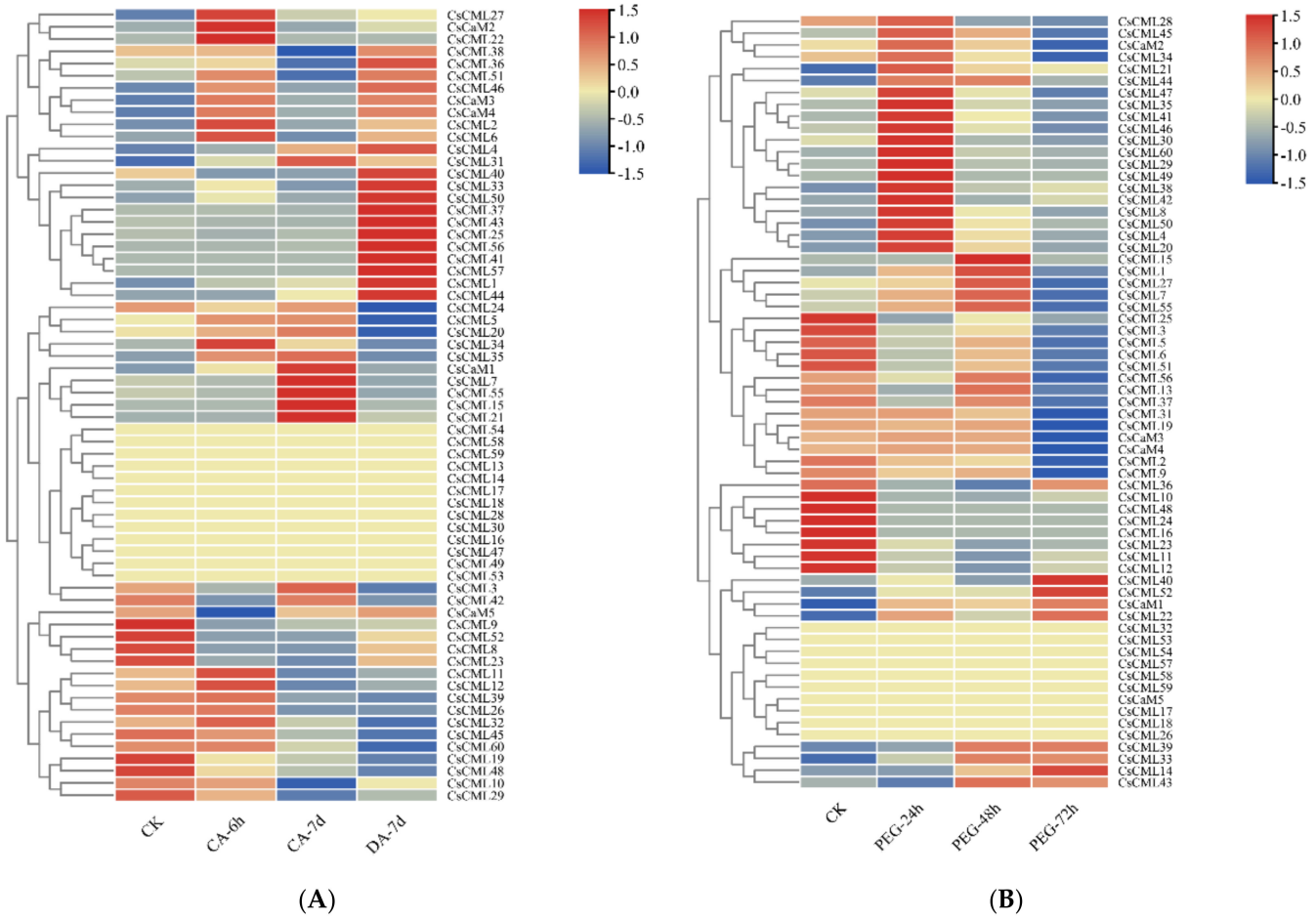

Figure 8. Expression profiles of *CsCaM* and *CsCML* genes under cold and drought stress. (**A**) The cold stress expression pattern of the tea plant was not adapted to 25~20 °C (CK), fully adapted to 10 °C for 6 h (CA−6 h) and 10~4 °C for 7 days (CA−7d), and recovered for 7 days (DA−7d) at 25~20 °C. (**B**) Expression patterns of tea plants treated with 25% polyethylene glycol (PEG) for 0 h, 24 h, 48 h, and 72 h under drought stress.

3.8. Expression Analysis of the Selected Genes in Response to Abiotic Stress by qRT-PCR

In order to confirm the results from TPIA, seven CsCML genes were chosen and submitted to qRT-PCR expression analysis in response to multiple abiotic stress treatments, including low temperature, high salt, drought, and ABA (Figure 9). Under low-temperature stress, the expression levels of six *CsCML* (*CsCML1/3/33/39/42/51*) genes were significantly upregulated on the whole, while *CsCML12* was repressed at first, and then increased at 24 h. Among the six genes, *CsCML39* was the most sensitive to low temperature with the highest expression and may have an important role in the low-temperature response. In addition, the expression levels of *CsCML12* and *CsCML42* significantly increased under salt and drought stress, while the other genes did not change significantly. Meanwhile, *CsCML42* showed a maximum change in expression under ABA treatment, which indicated that *CsCML42* was sensitive to ABA stimulation. Overall, *CsCML39* was the most sensitive to low temperature with the highest expression and may have an important role in the low-temperature response, while *CsCML12* showed a trend of decreasing followed by increasing

under low temperature, high salt, and drought stresses. These results demonstrated that *CsCaM/CML* family members may have distinct biological functions in tea plants. In general, *CsCML* expression profiles revealed that all the collected genes respond to one or more stresses with varying expression patterns.

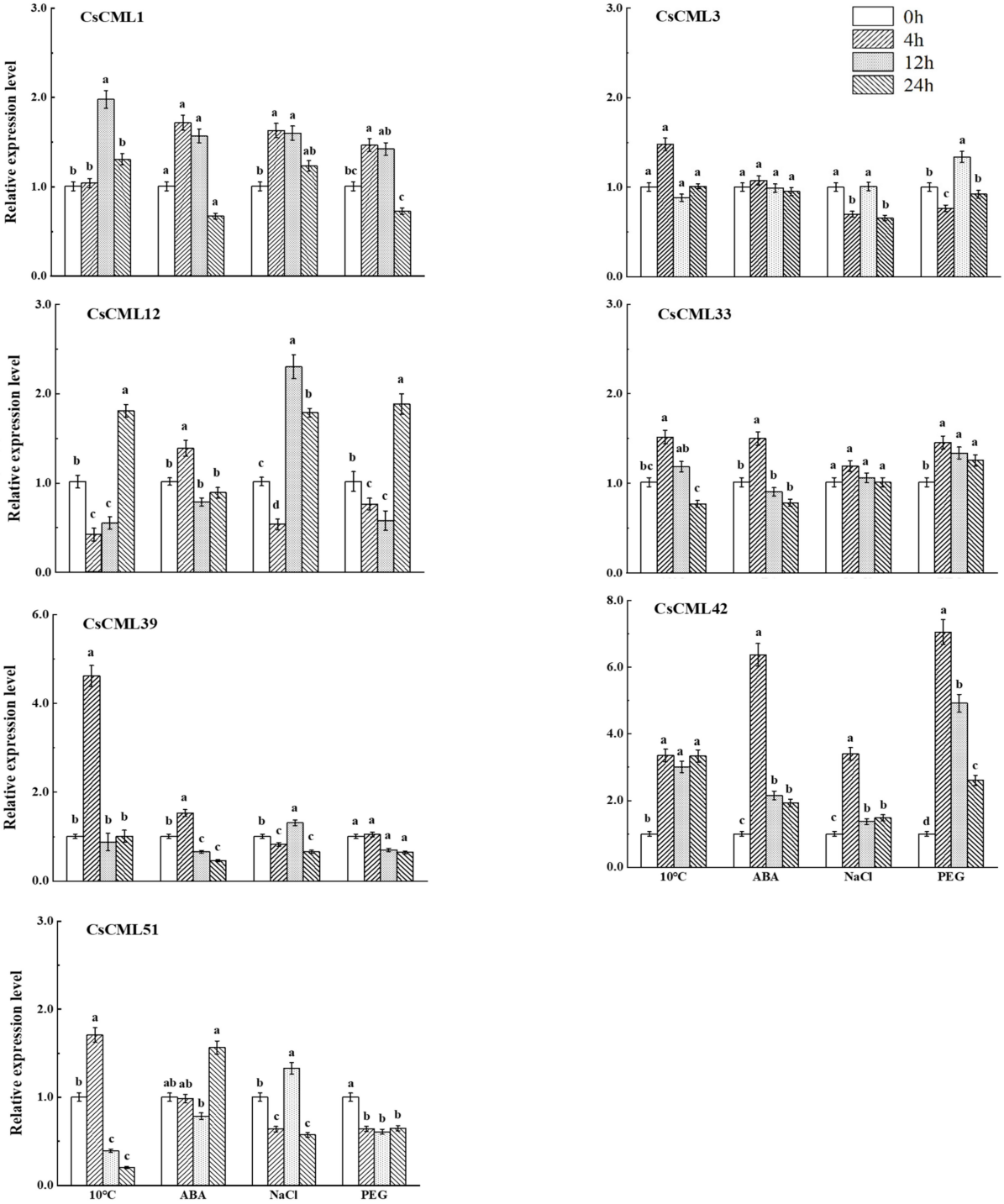

Figure 9. Response of seven *CsCML* genes to abiotic stress in tea leaves. The untreated plants were treated with low temperature (10 °C), drought (20% PEG 6000), high salt (200 mmol/L NaCl) and ABA (100 μmol/L). Samples were taken at 0, 4, 12, and 24 h after stress treatment. Three biological replicates were set at each time. Gene expression was detected using qRT-PCR. Different lowercase letters indicate significant differences between different time periods under the same treatment ($p < 0.05$).

4. Discussion

CaM and CMLs are types of calcium-sensor proteins that modulate a variety of developmental processes and stress responses by mediating Ca^{2+} signatures. Among the various calcium sensor proteins reported, CaM and CMLs are the most conserved and the major calcium receptor proteins in plants. Although the analyses of CaM/CML gene family in several plant species have been conducted by genome-wide survey, including *Arabidopsis* [10], rice [12], tomato [13,47], lotus [18], cabbage [48], apple [14], and wheat [40], there has been no systematic study of *CaM/CML* genes in *C. sinensis*. In Ma's report, only 5 *CsCMLs* were identified in the tea plant, although the details of these genes are unclear [19]. In the current study, a genome-wide search method was used that detected a total of 5 *CsCaM* and 60 *CsCML* genes in the tea plant, and various bioinformatics analyses were accomplished.

Conserved motif analysis revealed that all the CsCaMs contained four EF-hand domains, similar to those characterized in *Arabidopsis*. CsCML proteins contained two to four EF-hand structural domains, consistent with previous studies, such as the apple MdCMLs [14] and the cabbage BrCMLs [48]. There may be functional differences among CsCaMs/CMLs owing to the different numbers of the EF-hand motif. Gene structure analysis showed that only 27 *CsCMLs* carried 1–4 introns, most *CsCMLs* were intronless, while *CsCaM* contain 1 or 2 introns, indicating critical evolutionary changes in the *C. sinensis* genome. The presence of few or no introns suggests that they may be quickly transcribed to support an early defense response in the plant under stress [49].

Cis-acting elements are functional elements in the gene promoter region and play important roles in regulating gene expression. According to the prediction of *cis*-acting elements, most *CsCaM* and *CsCMLs* contain various types of *cis*-elements related to plant growth and development, hormone response, light response, and stresses response. *CsCaM* and *CsCMLs* showed different expression patterns under different abiotic stress treatments, which indicated that they may affect tea plant growth in response to abiotic stress. AtCaM5 from *Arabidopsis* binds to IQM4 to participate in dormancy and germination of *Arabidopsis* seeds [50]. Both AtCML15 and AtCML18 are able to interact with the Na^+/H^+ reverse transporter protein AtNHX1, thereby reducing Na^+/H^+ interchange activity and playing a role in adversity stress [51]. The tomato SlCML37 was found to interact with the proteasome maturation factor SlUMP1 to increase the low-temperature stress tolerance [52].

CsCaM and CsCMLs were divided into 7 subgroups based on the phylogenic tree between *Arabidopsis* and the tea plant, each subgroup with a different number of proteins and structure, indicating that the CsCaM/CML family proteins of the tea plant are numerous and have different origins and diverse functions. It is worth noting that five CsCaMs and five AtCaMs were clustered together into one group (VII), indicating their close phylogenetic relationship and high sequence identity (Figure 3). Further, we constructed another evolutionary tree of tea plant CsCaM/CML with *Arabidopsis thaliana*, rice, and cabbage, which indicated that CaM and CML proteins are ubiquitous and conserved among plant species.

Chromosomal localization showed that 65 *CsCaM/CML* genes were unevenly distributed on the 14 chromosomes of *Camellia sinensis* (Figure 5). *CsCaMs* and *CsCMLs* were not distributed on every chromosome, which was also observed in rice [12], apple [14], and maize [42]. Gene duplication is a major cause of gene family expansion [53]. In this study, we performed a synteny analysis based on the CSS genome and mapped *CsCaM/CMLs* onto the identified collinear regions. Different types of duplications were identified in several gene pairs, indicating that gene duplication is a key factor in the expansion of the *CsCaM/CML* gene family. There were 14 pairs of gene duplications in the *CsCaM/CML* genes. Among these duplicated segments, *CsCML4/5/10/25* was duplicated with *CsCML8/21*, *CsCML4/5/8* was located on Chr 2, *CsCML10* on Chr 3, and *CsCML21* on Chr 4. It is hypothesized that duplication of gene sequences may be transmitted between chromosomes through individual genes and is important for the genetic stability of organisms. Collinearity analysis provided additional information on the evolution of *CsCaM/CML*, and tandem

duplication between adjacent chromosomes may have been generated by the evolution of genes to adapt to their environment.

CaM and *CML* genes were reported to be diversely expressed in various plant tissues. In this study, most of the *CsCaM/CML* genes were found to be present in at least one tissue (Figure 7), implying the wide involvement of *CsCaM/CML* genes in tea plant growth and development. Five of the *CsCMLs* were highly expressed in apical buds, suggesting their potential function in growth differentiation and phototropism. Eight of the *CsCMLs* and two *CsCaMs* were highly expressed in leaves, elucidating their role in leaf development. Ten of the *CsCMLs* were abundantly expressed in flowers, revealing their role in reproduction. One of the *CsCML* and one *CsCaM* were highly expressed in stems, suggesting a potential role in stem development. Five of the *CsCMLs* were expressed in fruit, suggesting a prospective role for them in fruit formation. Twelve *CsCMLs* were expressed at high levels in roots, suggesting their possible function during root development. However, *CsCML16*, *17*, *18*, *49*, *57*, *58*, and *59* were not expressed in any tissue, suggesting these genes may be related to other biological process, or due to homoeologous gene silencing. The expression patterns of the *CsCaM/CML* gene family in different tissues revealed function divergence.

The important functions of CaMs/CMLs in plant stress tolerance have been widely reported, such as drought [54], salt [37], cold stress [52], insect [55], and pathogens attack [56]. In *Arabidopsis*, it has been shown that *AtCaM1*, *3* and *4*, as well as *AtCML8/9/20/24/37/38/39*, were involved in resistance to abiotic stresses [24,32–34,57–61] Additionally, *OsCML4* confers drought tolerance in rice by scavenging ROS in a way that is independent of the ABA manner [62]. *GmCaM4* in soybean [63], *MtCML40* in alfalfa [64], and *ShCML44* in tomato [39] were found to be associated with plant tolerance to abiotic stresses.

As a leaf-harvested crop, the tea plant is inevitably confronted with low temperature stresses throughout the whole life cycle. The low temperatures in late autumn and early spring often cause injury to tea plants and then affect its yield and quality, which seriously restricts the development of the tea industry. In this study, most of the *CsCaM/CML* genes were differentially expressed under low temperature stress, demonstrating that these genes are involved in the response of the tea plant to low temperature stress. For example, the expression levels of *CsCML33* and *CsCaM5* were expressed highly in young leaves of tea plant under the low temperature condition, suggesting that these genes were involved in cold tolerance. Previous investigations have confirmed that CMLs are involved in plant responses to cold stress. The expression levels of *AtCML24* and *OsMSR2* could be induced under cold treatment, which might participate in cold-induced Ca^{2+} signal transduction [24,37]. There are also studies showing that $CaCl_2$ treatment alleviates the chilling injury of plant [65,66], which indicated that calcium signaling proteins play an important role in low temperature stress in plants.

Some of the *CsCaM/CML* genes were repressed under drought stress, while the other genes were increased. The expression pattern of these genes after drought stress in tea plants was significantly specific, suggesting that the *CsCaM/CML* gene family has an important role in the response of tea plants to drought stress. Our qRT-PCR analysis of seven *CsCML* genes showed that *CsCML39* had a strong up-regulated expression under low temperature stress, while *CsCML12* showed an increasing trend under low temperature, high salt, and drought stresses. *CsCML42* showed a significant increase in expression in response to all these abiotic stresses and was assumed to play an important role in the pathway. The results indicated that *CsCaMs* and *CsCMLs* in *Camellia sinensis* had functional diversity.

5. Conclusions

This is the first comprehensive and systematic genome-wide analysis of the *CsCaM/CML* gene family in *C. sinensis*. A total of 5 *CsCaM* and 60 *CsCML* genes were identified in the present study. Analysis of the conserved motifs, gene structure, *cis*-acting elements, chromosome location, and phylogenetic relationships indicated that the *CsCaM/CML* gene family was highly conserved during plant evolution. A large number of *cis*-acting elements involved in low temperature response, drought induction, anaerobic response, hormone re-

sponse, and light response can be observed in the promoters of *CsCaM/CMLs*, implying the potential roles in plant growth and tolerance to abiotic stresses. Differential expression of *CsCaM/CMLs* in different tissues indicated their involvement in growth and development. Meanwhile, *CsCaM/CMLs* were differentially expressed under abiotic stresses. qRT-PCR analysis further indicated that *CsCaM/CMLs* genes were involved in the response of the tea plant to abiotic stresses and plant hormones. Notably, this study provides a comprehensive understanding of the CsCaM/CMLs and a solid foundation for further elucidating the molecular mechanisms of CsCaM/CMLs in calcium signaling and stress responses in the tea plant.

Author Contributions: Conceptualization, Q.Z. and R.Z.; methodology, R.K.; software, L.W.; validation, C.L. and F.Z.; formal analysis, R.K.; investigation, L.W.; resources, Q.Z. and F.Z.; data curation, R.K. and C.L.; writing—original draft preparation, R.K.; writing—review and editing, Q.Z. and R.Z.; visualization, R.K.; supervision, Q.Z. and R.Z.; project administration, Q.Z. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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