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Genome-Wide Identification, Expression Profile, and Alternative Splicing Analysis of *CAMTA* Family Genes in Cucumber (*Cucumis sativus* L.)

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Abstract: The calmodulin-binding transcription activator (*CAMTA*), as one of the most distinctive families of transcription factors, plays an important role in plant growth and development and in the stress response. However, it is currently unknown whether *CAMTA* exists in cucumbers and what its function is. In this study, we first identified four *CAMTA* genes in the cucumber genome using a genome-wide search method. Subsequently, we analyzed their physical and chemical properties, gene structure, protein domains, and phylogenetic relationships. The results show that the structure of *CsCAMTAs* is similar to that of other plants, and a phylogenetic analysis divides them into three groups. The analysis of cis-acting elements shows that most *CsCAMTAs* contain a variety of hormones and stress-related elements. The RT-PCR analysis shows that *CsCAMTAs* have different expression levels in different tissues and can be induced by IAA, ABA, MeJA, NaCl, and PEG. Finally, we analyzed the expression pattern of *CsCAMTAs'* alternative spliceosomes under salt and drought stress. The results show that the expression levels of the different spliceosomes are affected by the type of stress and the duration of stress. These data indicate that *CsCAMTAs* participate in growth and development and in the stress response in cucumbers, a finding which lays the foundation for future *CsCAMTAs'* functional research.

Keywords: cucumber; *CAMTA*; genome-wide characterization; function analysis

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1. Introduction

As one of the most important secondary messengers in plant signal transmission [1], Ca^{2+} signals play an important role in plant growth, development, and response to external stimuli [2]. External environmental stimuli can cause spatial and temporal changes in cytosolic-free Ca^{2+} concentration ((Ca^{2+}) cyt), thereby stimulating a series of downstream reactions. Subsequently, calmodulin (CaM), calcium-dependent protein kinase (CDPK) and calcineurin B-like protein (CBL) that are present in the cell bind to Ca^{2+} and convert extracellular signals into intracellular signals [3]. In the downstream of the CaM signaling pathway, CaM can combine with a variety of transcription factors (TFs) to cause highly specific responses. The calmodulin-binding transcription activator (*CAMTA*), as one of them, exists widely in organisms and plays a very important role [4].

CAMTAs, also named signal responsive (SR) proteins or ethylene-induced CaM-binding (EICBP) proteins, are the maximal and most distinctive TF family, which can be regulated by CaM [4]. Yang and Poovaiah (2000) first reported *CAMTAs* as a non-specific DNA-binding active protein. *CAMTAs* have several conserved functional domains, including a unique DNA binding domain (CG-1), a transcription-associated immuno globulin-like domain (TIG), an ankyrin repeats (ANK), an isoleucine glutamine domain (IQ), and a Ca^{2+} dependent CaM binding domain (CaMBD) [5,6]. So far, the existence of *CAMTA* has been identified in many eukaryotes, including *Arabidopsis* [5], rice [7], tomatoes [8], tobacco [9], maize [10], soybeans [11], strawberries [12], wheat [13], and flax [14].

The important role of *CAMTAs* in plant growth, development, and resistance to biotic and abiotic stresses is gradually being explored. Yuan et al. [15] found that the expression of auxin response factor 18 (*ARF18*) and DWARF4 (*DWF4*), positively correlated with plant growth mediated by auxin and brassinosteroid (BR), was inhibited in the *AtCAMTA3/AtSR1* mutant. This indicates that *AtCAMTA3/AtSR1* may participate in plant growth and development through auxin- and BR-mediated signaling pathways. In addition, *AtCAMTA3* was found to play an important role in resisting pathogen invasion [16]. Under low temperature conditions, *AtCAMTA3* could also improve the freezing resistance of *Arabidopsis* by binding to the conserved motif 2 (CM2) and positively regulate the expression of *CBF2* (cold-induced gene) [17]. Under drought stress, *GmCAMTA12* regulated the drought tolerance mechanism of *Arabidopsis* and soybeans by producing an ABA response and interacting with multiple stress response genes [18]. Aluminum (Al) treatment could induce *AtCAMTA2* to activate the expression of Al-activated malate transporter 1 (*ALMT1*), thereby regulating the tolerance mechanism of *Arabidopsis* to toxic metal [19]. *CAMTA6* could directly or indirectly regulate the expression of many salt-responsive genes in *Arabidopsis* germinating seeds, thereby regulating the salt stress response [20]. Alternative splicing (AS) seems to play an important role in the functioning of *CAMTA* family members. The study found that *PtCAMTAs* have a variety of AS forms, and the expression levels of different alternative splicing forms under cold stress are also different, which indicates that AS may play a key role in responding to environmental stimuli [21].

Cucumber is one of the most important economic crops in the world, and it plays a vital role in providing people with rich nutrients. As the most important transcription factor, *CAMTAs* have still not been reported in cucumber. In the current study, we identified four *CAMTA* gene family members in the whole genome sequence of the cucumber, and analyzed their chromosomal location, physical and chemical properties, subcellular location, gene structure, protein structure, and phylogenetic tree. Regarding the expression pattern of *CsCAMTAs*, we determined the expression level of *CsCAMTAs* in different tissues, under different stress and different hormone treatments. We also analyzed the AS forms of *CsCAMTAs* under stress. The objective of this study is to lay the foundation for future research on the role of *CAMTAs* in cucumber growth, development, and resistance to stress.

2. Materials and Methods

2.1. Identification of the CAMTA Family Members in Cucumber

First, the cucumber genome sequence and protein sequence information file were downloaded from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/; accessed on 12 February 2021) database. The hidden Markov model (HMM) of the calmodulin-binding transcription activator was downloaded from the plant genome database (https://phytozome.jgi.doe.gov/; accessed on 20 February 2021). Second, using the HMM model as a template, the hmmsearch function of HMMER3.0 was used to compare all protein sequences of the cucumber to obtain the target protein. To determine further whether the identified protein belongs to the *CAMTA* gene family, we used SMART (http://smart.embl-heidelberg.de/; accessed on 20 February 2021) to analyze the protein domain and deleted the ones that did not contain GC-1, ANK repeats, and the IQ domains protein. Finally, we obtained the members of the cucumber *CAMTA* gene family, and used Expasy (https://web.expasy.org/protparam/; accessed on 18 March 2021) to analyze the physical and chemical properties of these genes. The subcellular localization of *CsCAMTAs'* protein is predicted using the online software PlantmPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/; accessed on 18 March 2021) [22].

2.2. Gene Structure and Protein Conserved Domain Analysis

The exon-intron structure information of the *CsCAMTA* genes was extracted from the genome gff3 annotation file through Tbtools software. This information is visualized through the Gene Structure Display Server (GSDS v2.0; http://gsds.gao-lab.org/; accessed

on 2 April 2021) [23]. We used SMART (http://smart.embl-heidelberg.de/; accessed on 15 April 2021) to analyze the protein domain and subsequently used Illustrator for BioSequence (IBS) software to draw a schematic diagram of the protein domain.

2.3. Phylogenetic Tree and Cis-Acting Elements Analysis

The protein sequences of *Arabidopsis*, tomatoes, rice, and soybeans were obtained from the *Arabidopsis* Information Resource (TAIR), the plant genome resources (Phytozome), and NCBI. The multiple sequence alignment of proteins was performed by ClustalW. We used the Neighbor-joining (NJ) method of MEGA-X, set the bootstrap replicates value to 1000, and constructed a phylogenetic tree of 39 *CAMTA* protein sequences [24]. The EvolView tool (http://www.evolgenius.info; accessed on 10 May 2021) was used to draw the phylogenetic tree [25]. The up-stream 2000 bp DNA sequences of *CsCAMTA* genes were obtained from the cucumber genome sequence, and cis-acting elements in the promoter region were analyzed in the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; accessed on 19 May 2021) [26].

2.4. Transcript Analysis of CsCAMTA Genes in Different Plant Tissues

The expression data of the cucumber *CAMTA* gene in different periods and different organs were downloaded from the Short Read Archive (SRA) database of NCBI (accession number: SRP071224), and RNA-Seq data of the cucumber was analyzed in reference to the method of Wei et al. [27]. The expression data were converted with Log (RPKM + 1) to calculate gene expression levels. The heat map of the expression profile of *CsCAMTAs* was produced by using HemI [28].

2.5. Transcript Analysis of CsCAMTA Genes under Different Abiotic Stresses and Hormones

2.5.1. Plant Materials and Treatments

Cucumber seeds ('Chinese long' inbred line 9930) were provided by Shenzhen Genomics Institute, Chinese Academy of Agricultural Sciences. We soaked the seeds in water at 55 °C for 15 min, then placed them on damp filter paper where they germinated overnight in the incubator at 25 °C. After the cotyledons were fully expanded, the seedlings were moved to the hydroponic box for cultivation. Yamazaki cucumber nutrient solution was used and replaced every three days. The environment of the growth room was controlled to have a photoperiod of 14/10 h (light/dark), an air temperature of 28/18 °C (day/night), and a light intensity of 200 μmol.m^{-2}s^{-1}. Stress treatments were carried out at the two-leaf seedling stage including PEG6000 (15%), NaCl (150 mM). We collected leaf samples for RT-PCR experiments after treating for 0, 3, 6, 12, 24, and 48 h. [18]. During the hormone treatment, we transferred the two-leaf stage seedlings to a nutrition solution containing IAA (10 μM), ABA (100 μmol/L), and methyl (Me)-JA (100 μmol/L), and then collected leaves at 0, 3, 6, 12, 24, 48 h for RT-PCR experiments [21]. Collected samples were immediately frozen in liquid nitrogen and stored at −80 °C for analysis.

2.5.2. RNA Extraction and Quantitative RT-PCR

Total RNA was isolated using the MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China). The FastQuant First Strand cDNA Synthesis Kit (Tiangen, Beijing, China) was used to synthesize cDNA according to the manufacturer's protocol. RT-PCR was performed using the SuperReal PreMix Plus kit (TIANGEN, Beijing, China) and Roche LightCycler instrument. There were three biological replicates per treatment. The primers used for RT-PCR were designed using prime5 software, as shown in Table S1. The cucumber *ACTIN* gene was used to normalize relative expression levels. The $2^{-\Delta\Delta Ct}$ method was used to analyze the data.

3. Results

3.1. Identification and Characterization of Calmodulin-Binding Transcription Activator (CAMTA) Genes in Cucumber

Based on the completed genome sequences, using biological information technology to analyze and identify the cucumber genome, we found that there are four *CAMTA* gene family members in the cucumber. According to the degree of similarity with the aligned sequences, they were named *CsCAMTA1–4* in order (Table 1). The *CsCAMTA* genes distribute on Chr-4, -6, -7, and the amino acid length varies from 916 (*CsCAMTA4*) to 1102 (*CsCAMTA1*). Then, we analyzed their physicochemical properties and found that the pI of *CsCAMTAs* all concentrate between 5.59 (*CsCAMTA1*) and –7.59 (*CsCAMTA2*). *CsCAMTA1* and *CsCAMTA3* are slightly acidic, while *CsCAMTA2* and *CsCAMTA4* are weakly alkaline. Due to their grand average of hydropathicity being less than 0 and the instability index being greater than 75, they belong to hydrophilic labile proteins. In addition, subcellular localization prediction results showed that all *CsCAMTAs* exist in the nucleus.

Table 1. Characteristics of *CAMTA* transcription factors in cucumber.

Gene	Gene ID	Chr. No.	Chr. Location	Length (aa)	Mol. Wt. (kDa)	pI	Instability Index	Grand Average of Hydropathicity	Subcellular Localization
CsCAMTA1	CsaV3_7G006500	7	4030549–4044110	1102aa	122,677.8	5.59	75.30	−0.452	Nucleus.
CsCAMTA2	CsaV3_4G025820	4	15185970–15195755	943aa	105,027.08	7.59	81.28	−0.422	Nucleus.
CsCAMTA3	CsaV3_6G008250	6	6628761–6640726	962aa	107,532.40	5.83	77.58	−0.491	Nucleus.
CsCAMTA4	CsaV3_6G022470	6	15401167–15413107	916aa	104,047.12	7.41	86.12	−0.384	Nucleus.

Note: pI, isoelectric point. Mol. Wt., molecular weight.

3.2. Genomic Structure and Protein Domain Analysis of CsCAMTA Members

GSDS, a gene structure analysis software, was used to analyze the structure of *CsCAMTA* members, and we found that the number of introns in *CsCAMTAs* is between 11 and 12 (Figure 1). Among them, the intron number of *CsCAMTA1* and *CsCAMTA3* is 12; the intron number of *CsCAMTA2* and *CsCAMTA4* is 11. The gene structures of different members are relatively similar.

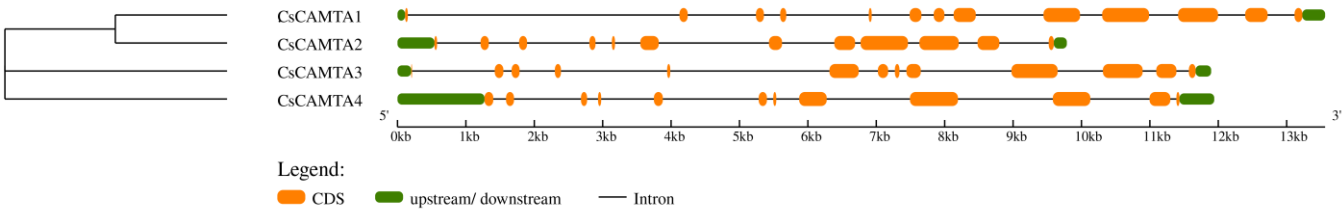

Figure 1. Gene structure of the *CsCAMTA* genes. The untranslated regions (UTRs) are indicated by thick green boxes; the exons are indicated by thick orange boxes; the introns are indicated by black lines.

For a better understanding of these genes, we analyzed the structure of the proteins encoded by these genes. The results show that the four *CsCAMTA* members all contain a CG-1 DNA binding domain (Pfam03859), ankyrin repeats (Pfam12796), IQ motifs (Pfam00612), and CAMBD (Figure 2). According to the existence of TIG (Pfam01833), *CsCAMTAs* can be divided into two groups. *CsCAMTA1*, *CsCAMTA3*, and *CsCAMTA4* belong to one group, while *CsCAMTA2* belongs to another group, suggesting that there are differences in the types of functional domains of *CsCAMTAs*, implying that there may be differences in their functions. The number of ANK domains and IQ motifs in *CsCAMTAs* varies from 1 to 3.

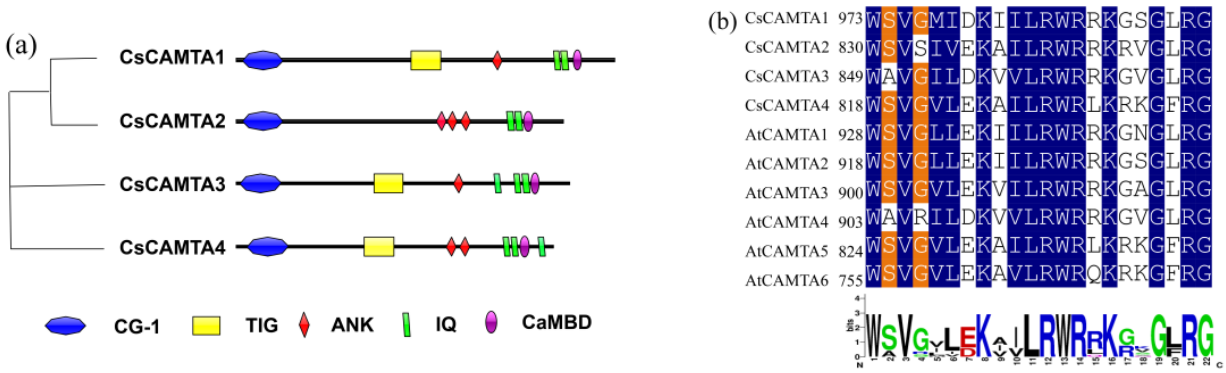

Figure 2. Protein conserved domain of the *CsCAMTAs* (**a**): Schematic diagram of all domains (**b**): Alignment of conserved CaMBD of *CsCAMTAs* with 6 *AtCAMTAs*. The full name of the abbreviation: CG-1, sequence-specific DNA-binding domain; TIG, transcription-associated immunoglobulin-like domain; ANK, ankyrin repeat domain; IQ, isoleucine glutamine motif; CaMBD, calmodulin-binding domain.

3.3. Phylogenetic Analysis of CAMTA Family Genes

To understand the evolutionary history or genetic relationship of *CsCAMTA* members better, we used the NJ algorithm to construct a phylogenetic tree among *Arabidopsis*, tomatoes, rice, soybeans, and cucumbers. As shown in Figure 3, 39 *CAMTAs* (6 *AtCAMTAs*, 7 *SlSRs*, 7 *OsCAMTAs*, 15 *GmCAMTAs*, 4 *CsCAMTAs*) are divided into three groups. Among them, *CsCAMTA1* and *CsCAMTA2* belong to group A and have the highest homology with *GmCAMTA5* and *GmCAMTA7*. *CsCAMTA4* belongs to group B and has a closer relationship with *GmCAMTA8*. *CsCAMTA3* is in group C and has upper homology with *AtCAMTA4*. It can be seen from the entire evolutionary tree that *CsCAMTAs* have the highest homology with *GmCAMTAs*, relatively low homology with *SlSRs* and *AtCAMTAs*, and the lowest homology with *OsCAMTAs*.

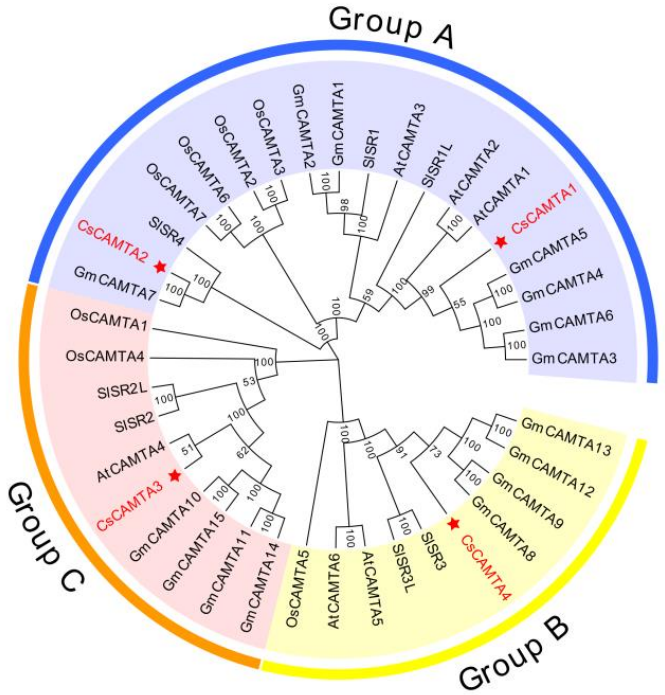

Figure 3. Phylogenetic relationship of the *CAMTA* homologs in different species. Cucumbers are marked as a red five-pointed star.

3.4. Cis-Acting Regulatory Elements in the Promoters of the CsCAMTAs

To explore the possible response mechanism of *CsCAMTAs* to various external stimuli, we used the Plant CARE database to analyze the 2000 bp, cis-acting element concentrated distribution region of four *CsCAMTA* genes' promoter regions. The results show that the predicted cis-acting elements can be divided into three categories: light signal response, hormone signal response, and abiotic stress response (Table 2). In this region, *CsCAMTA* members contain 6–12 cis-acting elements (Figure 4). Among them, anaerobic responsive element (ARE) exists in all four *CsCAMTAs*. It suggests that *CsCAMTAs* may function when an anaerobic reaction occurs. The abscisic acid (ABA)-response element (ABRE), auxin response element (TGA-element), and MeJA-responsive element (CGTCA-motif) are all present in *CsCAMTA1*, indicating that it may be sensitive to hormones. *CsCAMTA3* has a drought response element (MBS), low-temperature-response element (LTR), and ARE, indicating that it plays a crucial role in the response to abiotic stress.

Table 2. Summary of cis-acting elements of *CsCAMTA* genes.

Element	Sequence	Description
G-box	CACGTC	cis-acting regulatory element involved in light responsiveness
W-box	TTGACC	cis-acting regulatory element involved in light responsiveness
Circadian	CAAAGATATC	cis-acting regulatory element involved in circadian control
TGA-box	TGACGTAA	auxin-responsive element
ABRE	(C/T)ACGTG(G/T)	cis-acting element involved in the abscisic acid responsiveness
TGACG-motif	TGACG	cis-acting regulatory element involved in the MeJA-responsiveness
CGTCA-motif	CGTCA	cis-acting regulatory element involved in the MeJA-responsiveness
AuxRR-core	GGTCCAT	cis-acting regulatory element involved in auxin responsiveness
MBS	CAACTG	MYB binding site involved in drought-inducibility
LTR	CCGAAA	cis-acting element involved in low-temperature responsiveness
ARE	AAACCA	cis-acting regulatory element essential for the anaerobic induction

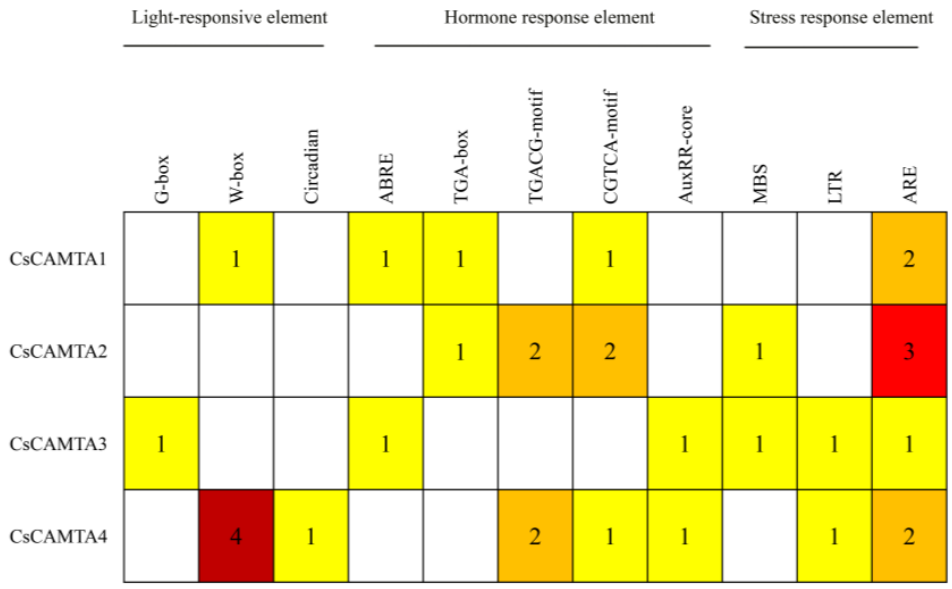

Figure 4. Numbers of elements in the upstream 2 kb regions of *CsCAMTA* genes. The different colors and numbers of the grid indicate the numbers of different Cis-acting regulatory elements in these *CsCAMTA* genes.

3.5. Expression Profiles Analysis of CsCAMTA Genes

3.5.1. Tissue-Specific Expression Patterns of the *CsCAMTA* Genes

To determine the tissue-specific expression pattern of *CsCAMTAs*, we analyzed the expression levels of *CsCAMTAs* in different tissues at different growth stages of the cucum-

ber. The results show that the four *CsCAMTA* genes express in various tissues at different levels (Figure 5; FPKM > 0). Among them, *CsCAMTA1*, *CsCAMTA3*, and *CsCAMTA4* are constitutive expressions (FPKM > 1 in all samples). *CsCAMTA1* has the highest expression in the roots of 4-week-old seedlings; *CsCAMTA2* and *CsCAMTA3* have the highest expression levels in female flowers; *CsCAMTA4* has the highest expression in 12-week-old cucumber roots. *CsCAMTA1* and *CsCAMTA3* have low expression levels in young leaves, while *CsCAMTA3* and *CsCAMTA4* have the lowest expression levels in 1-week-old fruits. This indicates that *CsCAMTAs* may mainly act in cucumber roots, stems, leaves, and female flowers, but have weak effects on the growth and development of fruits.

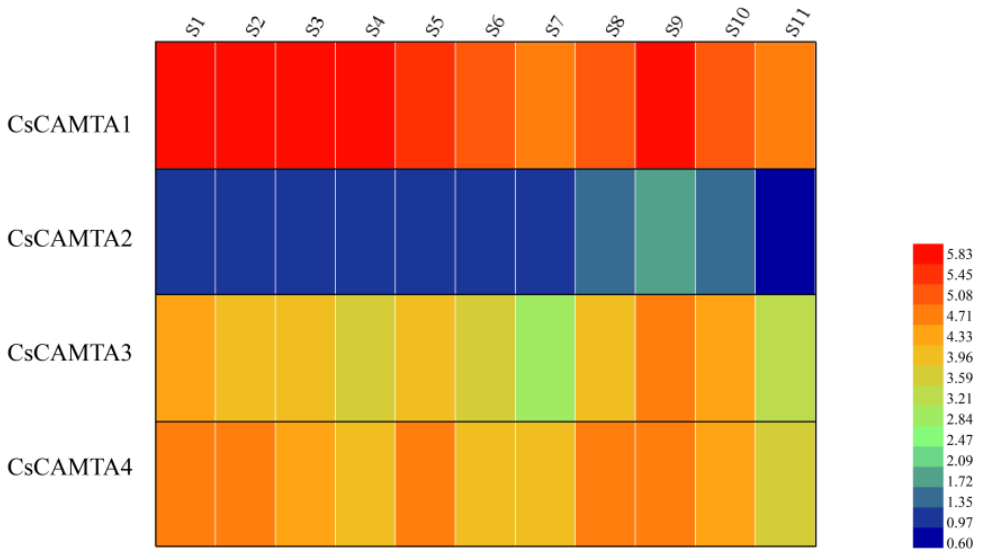

Figure 5. Tissue-specific expression analysis of *CsCAMTAs*. RNA-seq data were obtained from NCBI (accession number: SRP071224). S1, roots of 4-week-old seedlings; S2, stem of 4-week-old seedlings; S3, cotyledon of 4-week-old seedlings; S4, true leaf of 4-week-old seedlings; S5, root; S6, stem; S7, young leaf; S8, old leaf; S9, female flower; S10, male flower; S11, flesh of 1-week-old fruit. Red and blue indicate high and low levels of expression level, respectively. The color bar represents the expression values.

3.5.2. Expression Patterns of *CsCAMTA* Genes under Hormone and Abiotic Stress

To understand the expression pattern of *CsCAMTA* genes under different hormones and abiotic stresses, we treated cucumber seedlings with three hormones (IAA, ABA, MeJA) and two stress factors (NaCl, PEG). The results are shown in Figures 6 and 7. From Figure 6, we can see that the expression of *CsCAMTA1* is significantly up-regulated after 3 h of IAA and MeJA stimulation, while it takes 6 h to increase the expression of *CsCAMTA1* during ABA treatment. The performance of *CsCAMTA2* is significantly different from other *CsCAMTAs*, and its expression is significantly down-regulated after 3 h of treatment with the three hormones. The expression patterns of *CsCAMTA3* and *CsCAMTA4* are similar. At 6 h after IAA treatment, the expression levels of both are up-regulated, and at 48 h after MeJA treatment, the expression levels reach their maximum. However, the difference is that *CsCAMTA4* is down-regulated after 3 h ABA treatment.

Under abiotic stress treatment, different *CsCAMTA* genes were up-regulated or down-regulated to varying degrees after a certain period of stress (Figure 7). Under the salt stress, *CsCAMTA1*, *CsCAMTA3*, and *CsCAMTA4* show a trend that there is no significant change in the short-term (0, 3, 6, 12, and 24 h) stress, but they are all up-regulated in the long-term (48 h) stress. After 24 h of salt stress, the expression of *CsCAMTA1* is significantly up-regulated to 3.0-fold relative to the control, while *CsCAMTA3* and *CsCAMTA4* are up-regulated by more than 4.0-fold and 6.0-fold, respectively. However, short-term salt stress inhibited the expression of *CsCAMTA2*, and there is no significant change after

long-term stress. Under drought stress, *CsCAMTA1* and *CsCAMTA3* are down-regulated after 3 h stress, but a slow upward trend appears after long-term stress. There is no significant change in *CsCAMTA2*. *CsCAMTA4* is significantly up-regulated after 48 h under both stresses.

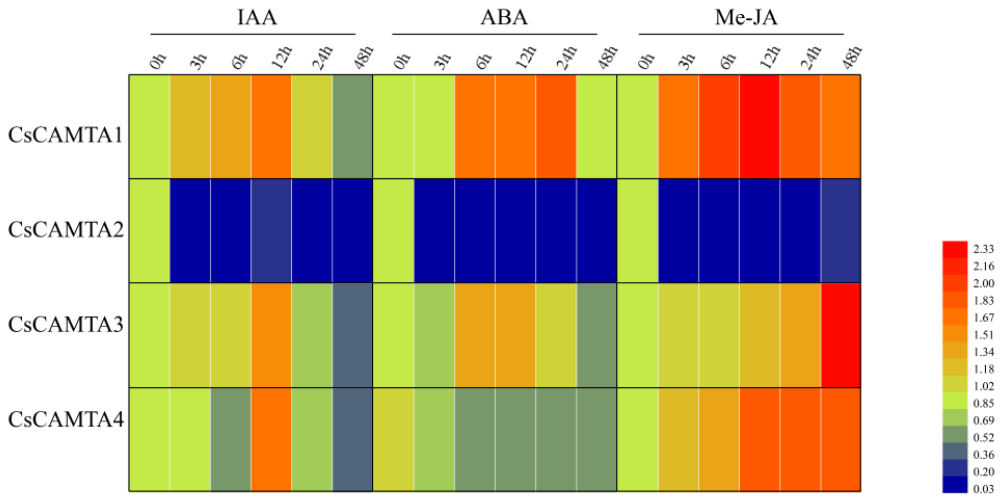

Figure 6. The expression pattern of *CsCAMTA* genes in leaves treated with indole-3-acetic acid (IAA), abscisic acid (ABA), and methyl jasmonate (MeJA). The sampling time points were 0, 3, 6, 12, 24, and 48 h. Red and blue indicate high and low levels of expression level, respectively. The color bar represents the expression values.

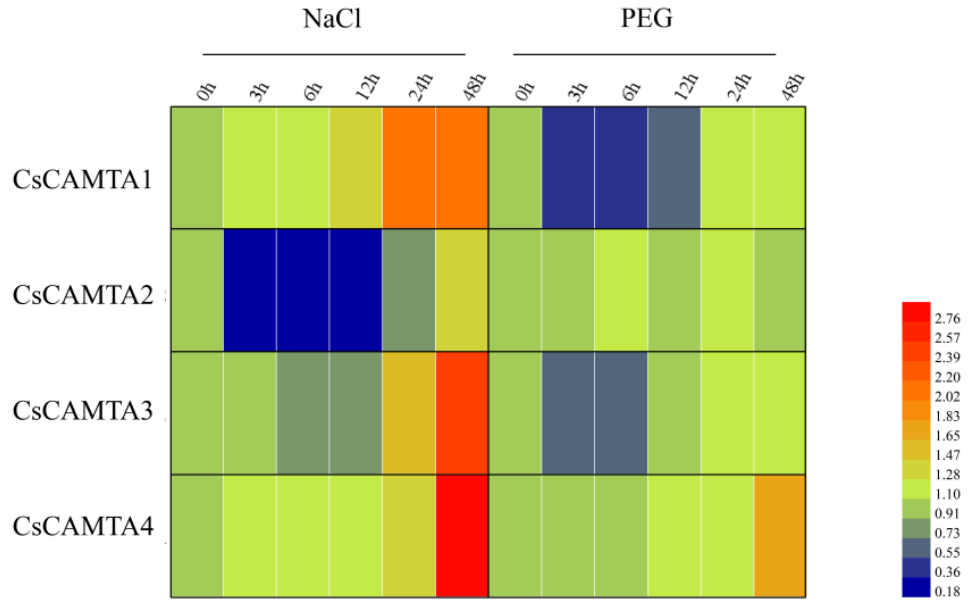

Figure 7. The expression pattern of *CsCAMTA* genes in leaves treated with NaCl and PEG. The sampling time points were 0, 3, 6, 12, 24, and 48 h. Red and blue indicate high and low levels of expression level, respectively. The color bar represents the expression values.

3.6. Alternative Splicing Analysis for CsCAMTA Genes

To understand the expression patterns of different transcripts produced by alternative splicing in response to stress responses of *CsCAMTA* genes, we designed specific primers for different transcripts of *CsCAMTA* genes based on the annotation information of NCBI.

Under stress treatment, the expression pattern of each splicing isoform of *CsCAMTA* genes in leaves is similar to that of normal transcripts, but some splicing isoforms show

specific expression patterns at different periods. As shown in Figure 8, under salt stress, most genes are down-regulated under short-term treatment and are significantly induced after long-term treatment (48 h), such as *CsCAMTA1.3*, *CsCAMTA2.3*, *CsCAMTA3.2*, etc. In contrast, *CsCAMTA1.2* is induced after 6 h of NaCl treatment but is down-regulated after 48 h. This may indicate that the sensitivity of different gene spliceosomes to stress is various, and the period of the function is different. Compared with salt stress, *CsCAMTA2.1* and *CsCAMTA4.1* are induced quickly after 3 h of PEG treatment (Figure 9), suggesting that they are more sensitive to drought stress. *CsCAMTA3.1* plays a major role in salt stress, but under drought stress, *CsCAMTA3.2* has a more significant effect. These results indicate that alternative splicing plays a crucial role in *CsCAMTAs'* response to stress.

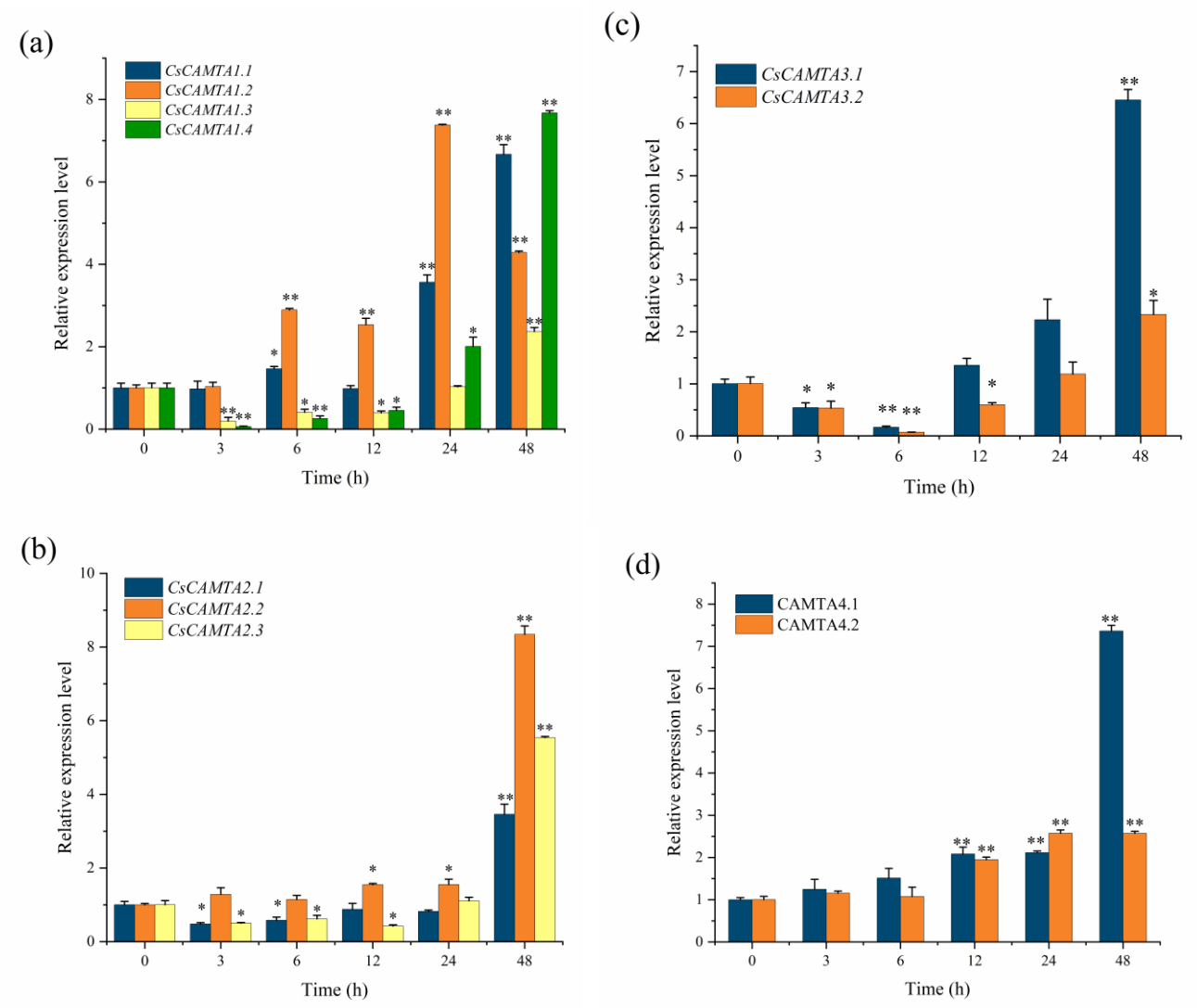

Figure 8. Expression analysis of alternative splicing form of *CsCAMTA* genes in leaves under salt stress. (**a**) *CsCAMTA1*; (**b**) *CsCAMTA2*; (**c**) *CsCAMTA3*; (**d**) *CsCAMTA4*. Error bars represent the standard deviation of the three biological replicates. The asterisk (*) indicates that the expression level of the stress group is significantly different from that of the control group (* $p < 0.05$, ** $p < 0.01$, one-way ANOVA, Tukey test).

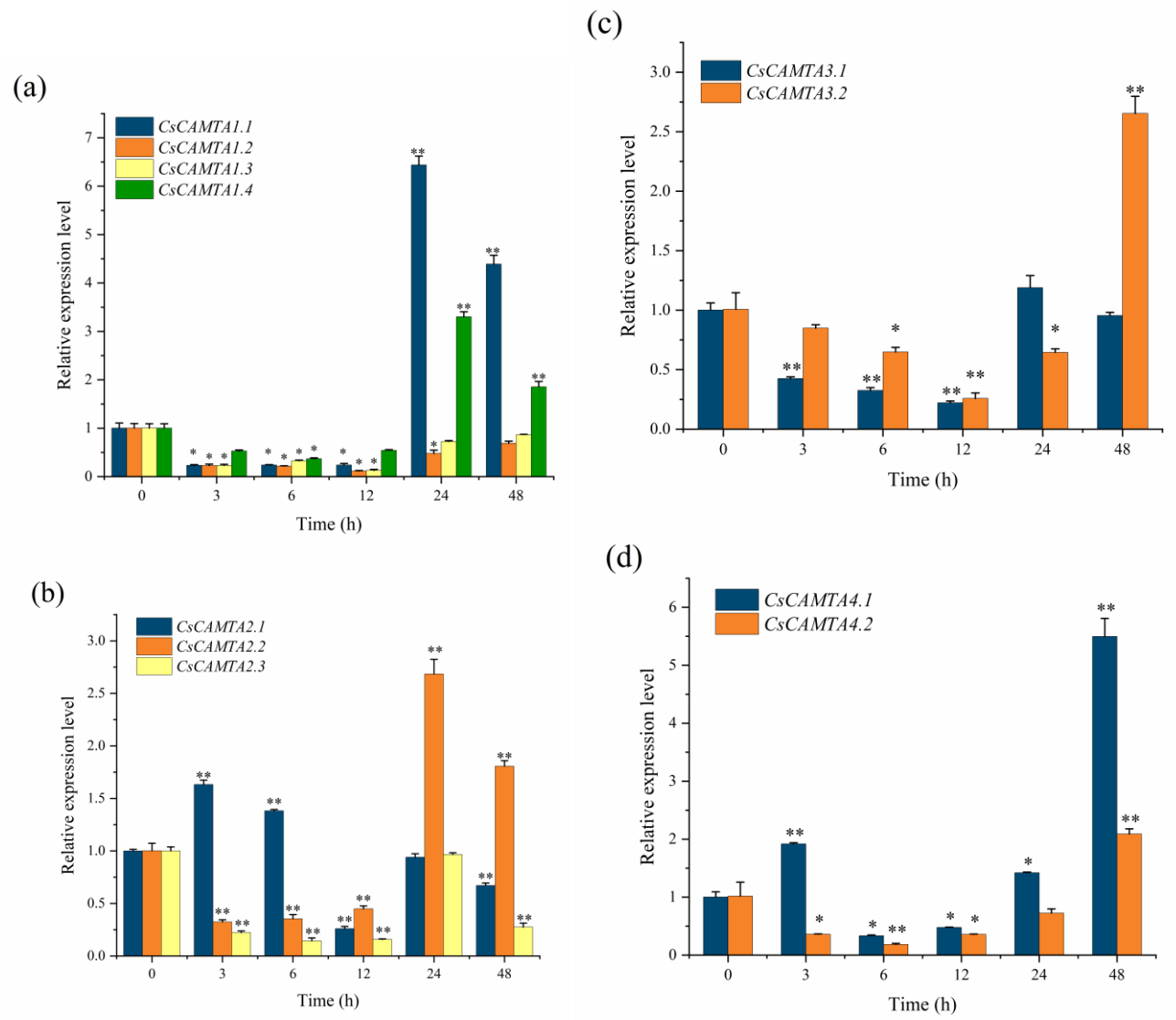

Figure 9. Expression analysis of alternative splicing form of *CsCAMTA* genes in leaves under drought stress. (**a**) *CsCAMTA1*; (**b**) *CsCAMTA2*; (**c**) *CsCAMTA3*; (**d**) *CsCAMTA4*. Error bars represent the standard deviation of the three biological replicates. The asterisk (*) indicates that the expression level of the stress group is significantly different from that of the control group (* $p < 0.05$, ** $p < 0.01$, one-way ANOVA, Tukey test).

4. Discussion

Calcium signaling is a pathway that transmits extracellular signals to an intracellular biological response through changing the intracellular Ca^{2+} concentration [29]. Previous studies have shown that a variety of stimulating factors can cause changes in intracellular Ca^{2+} concentration, thereby mediating important biological response processes [30], including high temperature, low temperature, salt, pathogenic bacteria, reactive oxygen species, and hormones. CaM is the most important multifunctional receptor protein for Ca^{2+} in cells, and it has a high affinity and specificity with Ca^{2+} [3]. Many TFs, including *CAMTA/SR*, *NAC*, *WRKY*, *MYB*, MADS-box, and *bZIP*, have been identified to interact with CAM to regulate plant growth, development, and the biotic and abiotic stress response [1]. Members of the *CAMTA* gene family have been identified in many eukaryotes, and play an important role in plant hormones and the abiotic stress response. In this report, we screened the members of the *CAMTA* family in the cucumber genome and found four in total (Table 1). The size of the *CAMTA* gene family in cucumbers is similar to that in bananas and *Arabidopsis* with five and six members, respectively [5,31]. However, it is far

less than that in soybeans with 15 members [11], wheat with 15 members, and *Brassica napus* with 18 members [13,16]. This indicates that the evolutionary distribution of the *CAMTA* gene family in the entire plant kingdom is uneven. *Brassica napus* and soybeans are tetraploid, so they have undergone multiple rounds of genome-wide replication during the evolution process, resulting in a relatively large number of *CAMTA* members. However, bananas are triploid, and cucumbers and *Arabidopsis* are diploid; thus, they have relatively few members of the *CAMTA* gene family.

We analyzed the structure of four *CsCAMTA* genes and found that the number of introns in all *CsCAMTAs* ranges from 11 to 12. This number is almost three times the average number of introns in cucumber genes (4.39), which may lay the structural foundation for the occurrence of AS [32]. The number of introns of *CsCAMTA1* and *CsCAMTA3* is 12; the number of introns of *CsCAMTA2* and *CsCAMTA4* is 11 (Figure 1). This is similar to the results of previous identifications on other species, indicating that members of the *CAMTA* gene family are conservative in gene structure [14]. Just as the *CAMTA* identified in other species, the four *CsCAMTAs* contain all the conserved domains of the typical CAMTA protein, including the CG-1 domain, TIG domain, ankyrin (ANK) repeat domain, IQ domain, and CaM binding domain (CaMBD) (Figure 2). According to the existence of TIG, *CsCAMTAs* are divided into two groups, which are consistent with previous studies [13]. On the one hand, *CsCAMTA* without the TIG domain may affect the DNA non-specific interaction of transcription factors and protein dimerization. On the other hand, the absence of the TIG domain may be the structural basis for the expansion and evolution of its family members [33,34]. According to the results of previous studies, IQ motifs can be combined with CaM in a Ca^{2+}-dependent or Ca^{2+}-independent manner to transmit signal substances, while CAMBD can transmit signals through the combination with Ca^{2+}/CaM complexes [4,35]. Regarding how *CsCAMTAs* interact with CaM, our research found that all *CsCAMTAs* contain IQ motifs and CAMBD, which indicates that *CsCAMTAs* can not only bind to CaM in a calcium-dependent manner but also can bind to CaM in a calcium-independent manner. This may make the signal transmission more stable and accurate.

To understand the relationship between cucumber *CAMTA* members and other species, a phylogenetic tree of *CAMTA* members of cucumbers, *Arabidopsis*, tomatoes, rice, and soybeans was constructed, which divides the 39 *CAMTAs* into three groups. The four *CsCAMTAs* members fell into all three groups (Figure 3), which suggests that the structure and function of *CsCAMTAs* are highly conserved during plant evolution [36]. In group A, the number of *CAMTA* members of cucumbers and other species is more than the number of members distributed in groups B and C, indicating that *CAMTA* is undergoing rapid adaptive evolution in group A [37]. In addition, in the same group, the closest members have similar gene structures and may have similar functions. Previous studies have confirmed that *AtCAMTA1*, *AtCAMTA2*, and *AtCAMTA3* play an important role in plant defense against pathogenic bacteria and in response to low temperatures and salt stress [38]. Thus, we speculate that *CsCAMTA1* and *CsCAMTA2* may have similar functions to them. Later experiments also proved that *CsCAMTA1* is indeed up-regulated under long-term salt stress treatment (Figure 7).

The cis-elements in the promoter region, as the binding sites of transcription factors, play a vital role in the regulation of gene expression, especially for the regulation of gene expression in response to biotic and abiotic stress [39]. We have identified many cis-acting elements in *CsCAMTA* genes, including the W-box, Circadian, TGA-box, ABRE, TGACG-motif, CGTCA-motif, AuxRR-core, MBS, LTR, and ARE (Figure 5). An interesting phenomenon is that *CsCAMTA3* does not have MeJA response elements (TGACG-motif, CGTCA-motif), but it has a clear response to MeJA treatment (Figure 6). *CsCAMTA2* contains a drought response element (MBS), but it does not respond significantly under drought stress (Figure 7). This phenomenon was also found in poplar and soybean *CAMTA* family members [11,21], indicating that the relationship between corresponding elements

and the occurrence of responses is not one-to-one. The specific relationship may need to be further explored in detail.

The function of *CAMTA* in the process of plant growth and development has been extensively studied. In cotton, the positive correlation between the expression of *GhCAMTA2A.2* and *GhCAMTA7A* and fiber strength proved their important role in the development of cotton fiber [40]. Yang et al. [8] found that *SlSRs* act as candidate signal junctions for connection development and ethylene- and calcium-mediated signals during tomato fruit development and maturation. In *Arabidopsis*, *CAMTA1* and *CAMTA5* regulate the expression of the organ development gene *AVP1*, thereby controlling auxin fluxes and then regulating the occurrence of organs [41,42]. In our study, *CsCAMTAs* have different expression intensities and spatially differentiated expressions in different tissues. Compared with *CsCAMTA1*, *CsCAMTA3*, and *CsCAMTA4*, *CsCAMTA2* is expressed very low in almost all tissues. This phenomenon also occurs in rape. *BnCAMTA3A2* and *BnCAMTA3C2* have low expression levels in almost all tissues [16], which may be due to redundant functions of these genes or transcriptional silencing/post-transcriptional silencing effects [43]. The expression levels of *CsCAMTA3* and *CsCAMTA4* in female flowers and roots are significantly higher than their expression levels in fruits. This is similar to the high expression levels of wheat *TaCAMTA1-D* and *TaCAMTA3-D* in reproductive ears and seedling buds, respectively [13]. Such expression patterns exist in almost all *CAMTA* families that have been identified, indicating that *CsCAMTAs* are involved in the growth and development of cucumbers at various growth stages, but the period and intensity of action between members are different, and the specific functions need to be further verified.

The response of *CAMTA* gene family members to hormonal and abiotic stresses has been reported in many species. In *Arabidopsis*, *CAMTA1* takes part in auxin signaling and responds to salt stress [44]. A previous study also found that the expression of maize *ZmCAMTAs* is regulated by stress-related hormone signaling molecules (IAA, SA, ABA, and JA), which suggests that *ZmCAMTAs* may respond to stress through hormone signaling pathways [10]. In our experiments, *CsCAMTA1* and *CsCAMTA3* are significantly increased after 48 h of salt stress treatment. Meanwhile, ABA and MeJA significantly increase the expression of *CsCAMTA1* and *CsCAMTA3*, which indicates that *CsCAMTA1* and *CsCAMTA3* might increase the salt tolerance of cucumber seedlings through ABA and MeJA signal transduction pathways. *AtCAMTA1* induces the expression of photosynthesis-related genes and changes membrane integrity by generating ABA under drought stress [45]. Interestingly, the expression of *CsCAMTA4* is up-regulated under 48 h salt and drought stresses, but it has no obvious response to ABA treatment, indicating that *CsCAMTA4* may improve the drought tolerance of cucumber seedlings through an ABA-independent pathway. The detailed signal process of *CsCAMTA* genes under various environmental stimuli requires further study.

As a post-transcriptional modification mechanism, alternative splicing increases the complexity of gene expression and the diversity of protein expression to a certain extent. In the analysis of gene families, the phenomenon that a gene generates multiple transcripts through alternative splicing often occurs, including in the pepper *WRKY* family, *Arabidopsis PPR* family, and human *KIR* family [46–48]. The *CAMTA* gene family is no exception. In previous studies, Wei et al. [21] found that under cold stimulation, the expression patterns of alternative spliceosomes of poplar *PtCAMTAs* differ between different tissues and different varieties, which indicates that alternative splicing may play a key regulatory role in plant development and the response to environmental stimuli. Previous studies have shown that *CAMTA* plays an important role in the regulation of salt and drought stresses [18,20]. Therefore, we analyzed the expression patterns of *CsCAMTAs'* alternative spliceosomes under salt and drought stresses. According to its expression results, we found that the expression level of *CsCAMTAs'* spliceosome at different time points under stress is various. Most of the spliceosome tends to be inhibited by short-term stress and induced by long-term stress, such as *CsCAMTA1.4*, *CsCAMTA2.3*, and *CsCAMTA3.2*, etc. This is contrary to the tendency that *PtCAMTA1.2*, *2.3*, and *6.2* in poplar leaves are

induced by short-term stress treatment [21]. This difference may be because the poplar and cucumber respond differently to stress: under short-term stress, plants may down-regulate *CsCAMTAs* to maintain the balance of plant growth and metabolism, while under long-term stress, *CsCAMTAs* are up-regulated to participate in a series of metabolic pathways to maintain plant growth and development. However, the response mode of *PtCAMTAs* may be the opposite. Some spliceosomes have different major genes under different stresses. *CsCAMTA3.1* is the major gene under salt stress, while under PEG treatment, the effect of *CsCAMTA3.2* is more significant (Figures 8 and 9). This phenomenon also occurs in *Brassica rapa BrRS2Z5* [49], probably because different stresses may induce the expression of the different spliceosomes to respond to the corresponding stress more efficiently [50]. However, the specific model needs further experimental verification.

5. Conclusions

In general, in this study, we identified four cucumber *CAMTA* gene family members and analyzed their gene structure, conserved domains, and phylogenetic relationships. The results show that the *CsCAMTA* gene family is highly conserved in the evolutionary process. The analysis of the cis-acting shows that the cucumber *CAMTA* genes have a genetic basis for responding to multiple hormones and stress. Tissue-specific analysis indicates that *CsCAMTAs* are expressed in multiple tissues, but the expression levels are different, indicating that different *CsCAMTAs* may be involved in the growth and development of cucumbers in different periods. The expression patterns under hormonal and abiotic stresses indicate that the participation of *CsCAMTAs* in the plant response to stress may be through stress-related hormonal signal pathways, and alternative splicing may also be involved. Our research provides evidence for the involvement of the *CAMTA* gene family in cucumbers' growth and development and response to stress, and lays a theoretical foundation for further exploration of the functions of *CsCAMTAs*.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11091827/s1, Table S1: Primer sequence for real-time RT-PCR.

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