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Article

Genome-Wide Reidentification and Transcriptome Analysis of *MADS-Box* Gene Family in Cucumber

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Abstract: MADS-box transcription factors play an important role in plant growth and development. Although previous studies have carried out genome-wide analysis of the MADS-box family in cucumber, with the update of the genome data of Cucurbitaceae, this paper re-identified and analyzed the differences of the family genes in different genome versions. The results showed that a total of 48 *CsMADS-box* genes were identified in the V3 version of cucumber, while three of the 43 genes identified in the V1 version were duplicated. The V1 version actually has only 40 genes. Additionally, we analyzed the variability in protein sequences and found that the amino acid sequences of 14 genes showed no differences between the two versions of the database, while the amino acid sequences of 29 genes exhibited significant differences. Further analysis of conserved motifs revealed that although the amino acid lengths of 15 genes had changed, their conserved motifs remained unchanged; however, the conserved motifs of 12 genes had altered. Furthermore we found that motif1 and motif2 were present in most proteins, indicating that they are highly conserved. Gene structure analysis revealed that most type I ($M\alpha$, $M\beta$) *MADS-box* genes lack introns, whereas type II (MIKC) genes exhibit a similar structure with a higher number of introns. Chromosomal localization analysis indicated that *CsMADS-box* genes are unevenly distributed across the seven chromosomes of cucumber. Promoter region analysis showed that the promoter regions of *CsMADS-box* genes contain response elements related to plant growth and development, suggesting that *CsMADS-box* genes may be extensively involved in plant growth and development. Different *CsMADS-box* genes exhibit specific high expression in roots, stems, leaves, tendrils, male flowers, female flowers, and ovaries, suggesting that these genes play crucial roles in the growth, development, and morphogenesis of cucumber. Moreover, 26, 18, 8, and 10 *CsMADS-box* genes were differentially expressed under high temperature, NaCl and/or silicon, downy mildew, and powdery mildew treatments, respectively. Interestingly, *CsMADS07* and *CsMADS16* responded to all tested stress conditions. These findings provide a reference and basis for further investigation into the function and mechanisms of the *MADS-box* genes for resistance breeding in cucumber.

Keywords: gene reidentification; cucumber; MADS-box; transcription factor

1. Introduction

The MADS-box gene family is one of the most widely studied transcription factor genes in plants. It is characterized by a highly conserved DNA-binding MADS domain at the N-terminus, containing 56-60 amino acids [1,2]. Plant *MADS-box* genes can be divided into two categories according to their evolutionary lineages: type I and type II. Type II genes are also known as MIKC genes because they share a common structure of four domains. In addition to the MADS (M) domain, MIKC-type genes also contain three other conserved domains: I, K and C domains [3]. The I domain

is responsible for the specificity of DNA binding dimer formation, the K domain is involved in protein-protein interaction, and the C domain is involved in transcriptional activation [4–6]. Based on its structural characteristics, MIKC can be further divided into two types: MIKC* and MIKC^C [7]. Compared with MIKC^C-type proteins, MIKC*-type proteins tend to have longer I domains and fewer conserved K domains. MIKC^C-type MADS-box genes are the most representative class of *MADS-box* genes, which play important and diverse roles in plant growth and development [3,8]. MIKC^C-type MADS-box genes can be divided into 12 subclasses according to their phylogenetic relationships in *Arabidopsis thaliana* [9]. Compared with the type II lineage group, the type I gene has a simpler gene structure and lacks the K domain. They are thought to share a common ancestor with type I genes from animals and fungi, but their functions are generally not well understood [3,10]. Type I MADS-box genes can be further subdivided into M α , M β , M γ and M δ in plants [11,12].

MADS-box genes play an important role in plant development. The most important role of is as a major component of the well-known ABCDE model, which describes its function in regulating floral organs. Different combinations of A, B, C, D and E functions of *MADS-box* genes determine different floral organ characteristics: sepals (A + E), petals (A + B + E), stamens (B + C + E), carpels (C + E) and ovules (D + E). Class A genes mainly control the development of calyx, corolla and floral organs. B-type genes control the development of corolla and stamens, and also affect the development of calyx in a few plants. Class C genes control the development of three-wheeled floral organs of stamens, pistils and ovules, while only two-wheeled structures of stamens and pistils are controlled in some plants. Class D genes control the development of ovules, and class E genes are involved in the formation of floral organs during each round of flower development, and form a ' tetramer model complex with class A, B, and C genes. In *Arabidopsis*, the corresponding functional genes are class A, APETALA 1 (AP 1); class B, PISTILATA (PI) and AP 3; class C, AGAMOUS (AG); class D, SEEDSTICK / AGAMOUS-LIKE 11 (STK / AGL 11); and E, SEPALLATA (SEP 1, SEP 2, SEP 3 and SEP 4) [3,13–15].

In addition to its important role in determining floral organ traits, MADS-box genes have also been found to be involved in the regulation of flowering time and flower initiation, such as SOC1, FLC, AGL and SVP [16–23]; and in fruit formation (FUL) [24,25] and root development (AGL12 and AGL17) [26,27].

MADS-box genes have been demonstrated to participate in the nutrient growth processes and various stress responses in different plants such as *Arabidopsis* [28], rice [29], wheat [30,31], and cabbage [32]. Therefore, the MADS-box protein family is an important transcription factor family in plant growth and development, influencing almost the entire process of plant growth and development. Model plant species of the MADS-box family have been extensively studied, including rice (*Oryza sativa*), cabbage (*Brassica rapa*), poplar (*Populus trichocarpa*), wheat (*Triticum aestivum* L.), banana (*Musa acuminata*), and others.

Cucumber (*Cucumis sativus* L.) is an economically and nutritionally important vegetable crop cultivated worldwide. It is loved by people because of its good taste, high nutritional value and economic value. Although *MADS-box* genes exist as a superfamily, little is known about MADS-box genes in cucumber. Therefore, it is of great significance to identify the MADS-box gene family of cucumber. In previous studies, 43 MADS-box genes were identified in the V1 genome of cucumber [33]. With the update of the Cucurbitaceae genome database, the cucumber genome version has been updated to the V3 version. Therefore, it is necessary to re-identify and modify the members of the MADS-box gene family in cucumber. This work is helpful for a more comprehensive study of cucumber MADS-box gene family members and provides a basis for further functional analysis to elucidate their roles in cucumber development.

2. Methods

2.1. Identification of MADS-Box Genes in Cucumber

The cucumber genome data were downloaded from the Cucurbit Genomics Database (<http://cucurbitgenomics.org/>) and NCBI (<https://www.ncbi.nlm.nih.gov/>). The hidden Markov model (HMM) profile files of the MADS-box conserved domain (PF00319) were downloaded from the Pfam database (<http://pfam.xfam.org/>). The MADS-box genes of cucumber were identified from the genome database using HMMER 3.0 with the default parameters and a cutoff value of 0.01. All CsMADS-boxs were further examined to confirm the MADS-box conserved domain through the CDD, Pfam, and SMART online tools. All CsMADS-box genes were named according to their locations on seven cucumber chromosomes.

2.2. Gene Structure and Motif Analysis

The CDS sequences and genomic data for CsMADS-box genes retrieved from the C. sativus genome database (<http://cucurbitgenomics.org/organism/20>) were visualized using the Gene Structure Display Server online tool (<http://gsds.cbi.pku.edu.cn/>) [34]. The conserved motifs of CsMADS-box proteins were then identified with MEME 4.9.1 (<http://meme-suite.org/>) [35] and visualized with WebLogo (<http://weblogo.berkeley.edu/logo.cgi>) [36]. The total number of motifs (nmotifs) is 10, the minimum length of motifs (minw) is 6 amino acids, and the maximum length of motifs (maxw) is 10 amino acids.

2.3. Phylogenetic Analysis and Multiple Sequence Alignment

The protein sequences of MADS-box in cucumber were uploaded to the MEGA software (v7.0) to be aligned using ClustalW, and the phylogenetic relationships among all MADS-box proteins were examined via the neighbor-joining method with 1000 bootstrap replicates. Then, the phylogenetic trees were landscaped in Evolview (<https://evolgenius.info/evolview-v2/#login>).

2.4. Gene Duplication Analysis and Genome Distribution

CsMADS-box loci were extracted from the cucumber genome database (<http://cucurbitgenomics.org/organism/20>) and their locations on chromosomes were visualized using MapChart software [37].

2.5. Analysis of Promoter Regions of CsMADS-Box Genes

The 1.5-kb sequences upstream of the initiation codons (ATG) of CsMADS-box genes were obtained from the cucurbit genomics data website (<http://cucurbitgenomics.org/organism/20>), and analyzed for cis-elements in the promoter region using the online tool PlantCARE [38].

2.6. Transcriptome Analysis of CsMADS-Box Genes in Cucumber

The expression patterns of the CsMADS genes were analyzed using the transcriptomic data of the roots, stems, leaves, flowers, ovaries, and tendrils of cucumber. The published RNA-Seq data (SRA046916) [39] were downloaded from the Cucurbit Genomics Database (<http://cucurbitgenomics.org/>). The remapped clean tags and the recalculated FPKM values were cited to analyze the expression patterns of the CsMADS. The genome-wide expression of the CsMADS genes was shown on a heatmap using TBtools [40]. The heatmap values were calculated according to the following steps: the fold change values of the FPKM value of the treatment group and the control group were calculated first, and then the logarithm based on two of the fold change values were taken.

2.7. Transcriptome Analysis of CsMADS in Response to Abiotic and Biotic Stresses

The publicly available transcriptomic data of cucumber treated with salt (GSE116265) [41], heat (GSE151055) [42], DM (SRP009350) [43], and PM (GSE81234) [44] were downloaded from NCBI (<https://www.ncbi.nlm.nih.gov/>) to analyze the expression patterns of CsMADS under different stresses. After aligning the gene IDs to the cucumber genome, the genome-wide expression of the

CsMADS genes was shown on a heatmap using TBtools [40]. For the transcriptome analysis of the CsMADS, a threshold of FDR (or p-value) ≤ 0.05 and an absolute value of $\log_2$ (fold-change) ≥ 1 or $\log_2$ (fold-change) ≤ -1 were used to define DEGs.

3. Results

3.1. Characterization of MADS-Box from Chinese Long 9930 (V3 Version)

In previous studies, we identified 43 *MADS-box* genes in the cucumber V1 genome [33]. With the update of the cucumber family genome database, the cucumber genome version has been updated to V3. Therefore, we re-identified and revised the *MADS-box* gene family members in cucumber. In the cucumber V3 version, we identified a total of 48 *MADS-box* genes, and found that *Csa014213* and *Csa025232* aligned to the gene *CsaV3_6G006010* in the cucumber V3 version database; similarly, *Csa014249* and *Csa026408* aligned to *CsaV3_1G009750*; *Csa014140* and *Csa025231* aligned to *CsaV3_6G006020*. *Csa014213* and *Csa025232* have the same CDS sequence. *Csa014140* and *Csa025231* also have the same CDS sequence. The CDS sequence similarity between *Csa014140* and *Csa025231* is as high as 96%. These results indicate that there are some errors in the *MADS-box* genes identified in the V1 version. Therefore, there are actually 40 *MADS-box* genes in the V1 version, 48 in the cucumber V3 version, and the 48 *MADS-box* genes are divided into 14 subfamilies (Figure 1). The eight newly added genes are *CsaV3_6G052910*, *CsaV3_6G051220*, *CsaV3_5G040310*, *CsaV3_5G040370*, *CsaV3_6G051590*, *CsaV3_3G009400*, *CsaV3_3G016620* and *CsaV3_UNG063480* (Table 1).

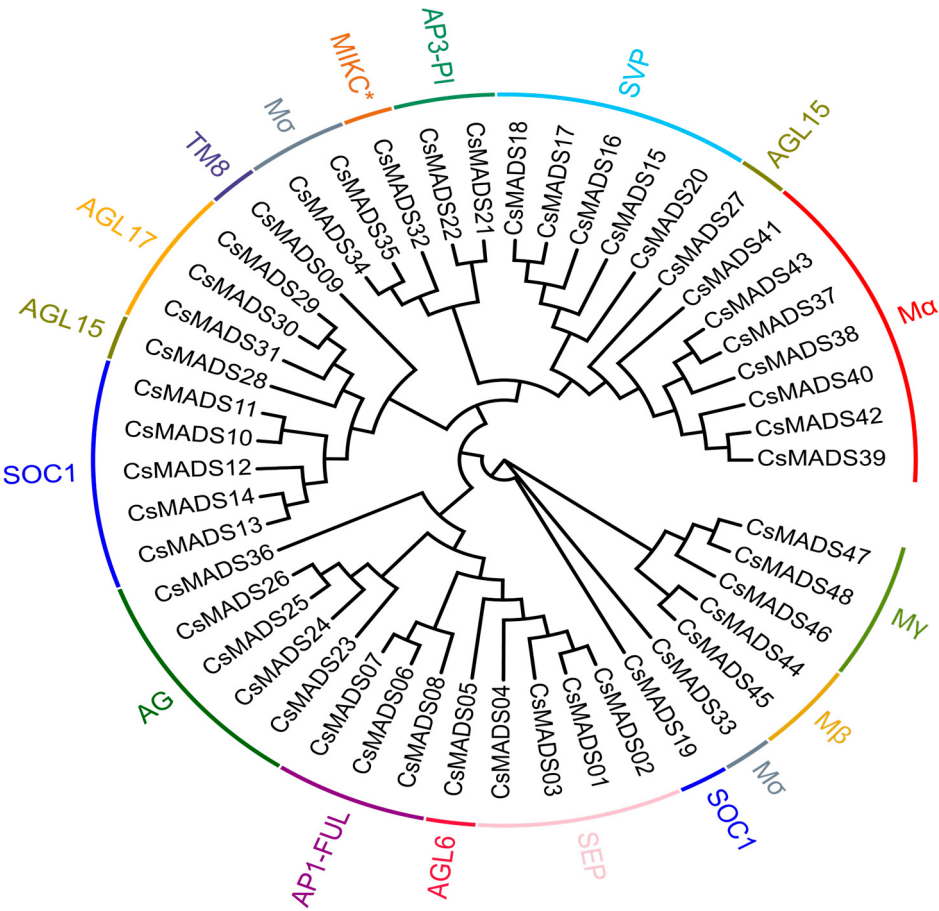


Figure 1. The phylogenetic tree of the MADS-box proteins from cucumber : Different colors represent different subgroups of the MADS-box family.

Table 1. Comparison of the number of MADS-box families in the. Cucumber V1 and V3 versions.

Serial No.	Gene name	Gene ID(V3)	Gene ID(V1)	Chromosomal location	Group	Subfamily
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1	CsMADS01	CsaV3_4G010090.1	Csa004117	4	MIKC	SEP
2	CsMADS02	CsaV3_6G008200.1	Csa008448	6	MIKC	SEP
3	CsMADS03	CsaV3_1G006210.1	Csa004591	1	MIKC	SEP
4	CsMADS04	CsaV3_6G033790.1	Csa013129	6	MIKC	SEP
5	CsMADS05	CsaV3_6G006010.1	Csa014213, Csa025232	6	MIKC	AGL6
6	CsMADS06	CsaV3_4G010080.1	Csa004120	4	MIKC	AP1-FUL
7	CsMADS07	CsaV3_1G006220.1	Csa004592	1	MIKC	AP1-FUL
8	CsMADS08	CsaV3_6G033800.1	Csa013130	6	MIKC	AP1-FUL
9	CsMADS09	CsaV3_1G009750.1	Csa014249, Csa026408	1	MIKC	TM8
10	CsMADS10	CsaV3_5G005600.1	Csa012493	5	MIKC	SOC1
11	CsMADS11	CsaV3_3G016650.1	Csa021114	3	MIKC	SOC1
12	CsMADS12	CsaV3_3G009400.1		3	MIKC	SOC1
13	CsMADS13	CsaV3_6G006020.1	Csa014140, Csa025231	6	MIKC	SOC1
14	CsMADS14	CsaV3_5G003360.1	Csa012879	5	MIKC	SOC1
15	CsMADS15	CsaV3_6G045010.1	Csa012099	6	MIKC	SVP
16	CsMADS16	CsaV3_2G030300.1	Csa003859	2	MIKC	SVP
17	CsMADS17	CsaV3_4G014770.1	Csa017496	4	MIKC	SVP
18	CsMADS18	CsaV3_7G006940.1	Csa003446	7	MIKC	SVP
19	CsMADS19	CsaV3_5G040310.1		5	MIKC	SVP
20	CsMADS20	CsaV3_6G052910.1		6	MIKC	SVP
21	CsMADS21	CsaV3_3G045590.1	Csa017887	3	MIKC	AP3-PI
22	CsMADS22	CsaV3_4G028010.1	Csa011135	4	MIKC	AP3-PI
23	CsMADS23	CsaV3_6G015770.1	Csa000681	6	MIKC	AG
24	CsMADS24	CsaV3_1G032920.1	Csa017355	1	MIKC	AG
25	CsMADS25	CsaV3_6G051220.1		6	MIKC	AG
26	CsMADS26	CsaV3_5G040370.1		5	MIKC	AG
27	CsMADS27	CsaV3_4G028880.1	Csa021473	4	MIKC	AGL15
28	CsMADS28	CsaV3_3G031900.1	Csa020302	3	MIKC	AGL15
29	CsMADS29	CsaV3_3G048150.1	Csa002117	3	MIKC	AGL17
30	CsMADS30	CsaV3_4G014700.1	Csa017500	4	MIKC	AGL17
31	CsMADS31	CsaV3_6G044810.1	Csa012111	6	MIKC	AGL17
32	CsMADS32	CsaV3_4G030750.1	Csa015983	4	MIKC	MIKC*
33	CsMADS33	CsaV3_6G008210.1	Csa008449	6	Mσ	
34	CsMADS34	CsaV3_5G032860.1	Csa000939	5	Mσ	
35	CsMADS35	CsaV3_1G005580.1	Csa004560	1	Mσ	
36	CsMADS36	CsaV3_3G016620.1		3	AG	
37	CsMADS37	CsaV3_4G000010.1	Csa021069	4	Mα	
38	CsMADS38	CsaV3_1G032570.1	Csa017317	1	Mα	
39	CsMADS39	CsaV3_1G015790.1	Csa007119	1	Mα	

40	CsMADS40	CsaV3_2G016620.1	Csa020265	2	Mα
41	CsMADS41	CsaV3_3G045410.1	Csa017909	3	Mα
42	CsMADS42	CsaV3_1NG063480.1			Mα
43	CsMADS43	CsaV3_6G051590.1		6	Mα
44	CsMADS44	CsaV3_3G020270.1	Csa014962	3	Mβ
45	CsMADS45	CsaV3_2G019470.1	Csa017249	2	Mβ
46	CsMADS46	CsaV3_3G038110.1	Csa002566	3	My
47	CsMADS47	CsaV3_1G038060.1	Csa001552	1	My
48	CsMADS48	CsaV3_1G017300.1	Csa007130	1	My

3.2. Analysis of the Differences of Amino Acid Sequences of MADS-Box Family Members Between V1 and V3 Versions

Due to the difference in the number of members of the *MADS-box* gene family in the V1 and V3 versions, we analyzed the differences in the protein sequences of the 43 genes in the V1 version between the two versions. The results showed that there were no differences in the amino acid sequences of 14 genes in the two versions of the database. They were *Csa004117*, *Csa008448*, *Csa014140*, *Csa025231*, *Csa012879*, *Csa012099*, *Csa017355*, *Csa000939*, *Csa021069*, *Csa017317*, *Csa020265*, *Csa017909*, *Csa002566* and *Csa001552*. The amino acid sequences of 29 genes were significantly different between the two versions of the data (Table 2). See Dataset S1 for amino acid sequence information.

Table 2. Comparison of the amino acid number of MADS-box in the Cucumber V1 and V3 versions.

gene name	V1	V3	氨基酸序列相似度	gene name	V1	V3	氨基酸序列相似度
Csa004117	245	245	100%	Csa011135	191	211	
Csa008448	241	241	100%	Csa017355	254	254	100%
Csa004591	250	255		Csa021473	255	286	
Csa013129	227	241		Csa020302	204	250	
Csa014213	171	246		Csa002117	91	225	
Csa025232	171	246		Csa017500	122	235	
Csa004120	197	555		Csa012111	204	228	
Csa004592	248	261		Csa015983	323	350	
Csa013130	201	223		Csa008449	107	181	
Csa014249	203	202		Csa000939	339	339	100%
Csa026408	210	203		Csa004560	313	355	
Csa012493	182	282		Csa021069	228	228	100%
Csa021114	177	183		Csa017317	173	173	100%
Csa014140	221	221	100%	Csa007119	202	602	
Csa025231	221	221	100%	Csa020265	187	187	100%
Csa012879	222	222	100%	Csa017909	225	225	100%
Csa012099	228	228	100%	Csa014962	216	250	
Csa003859	245	230		Csa017249	294	202	
Csa017496	67	173		Csa002566	387	387	100%
Csa003446	67	221		Csa001552	225	225	100%
Csa017887	244	276		Csa007130	219	213	
Csa000681	135	373					

3.3. Analysis of Protein Motif Difference of MADS-Box

In order to explore whether the difference of amino acid sequence leads to the change of protein conserved motifs, we compared and analyzed the protein conserved motifs of MADS-box family members in V1 and V3 versions. The results showed that the amino acid length of 15 family members changed, but their protein motifs did not change. They are CsMADS05, CsMADS06, CsMADS07, CsMADS09, CsMADS10, CsMADS16, CsMADS21, CsMADS22, CsMADS28, CsMADS32, CsMADS33, CsMADS39, CsMADS44, CsMADS45 and CsMADS48, respectively. The protein motifs of 12 family members changed, including CsMADS03, CsMADS04, CsMADS08, CsMADS11, CsMADS17, CsMADS18, CsMADS23, CsMADS27, CsMADS29, CsMADS30, CsMADS31, CsMADS35. Similarly, we analyzed the conserved motifs of the eight newly identified proteins, and found that CsMADS42 contains only two motifs (motif1, motif2), and CsMADS43 contains three motifs (motif1, motif2, motif6) (Figure 2). In addition, the conserved motifs of the remaining six proteins contain 5-6 motifs. Moreover, we found that motif1 and motif2 are present in most proteins, indicating that motif1 and motif2 are highly conserved (Figure 2). The amino acid sequence for each motif is presented in the Figure S1.

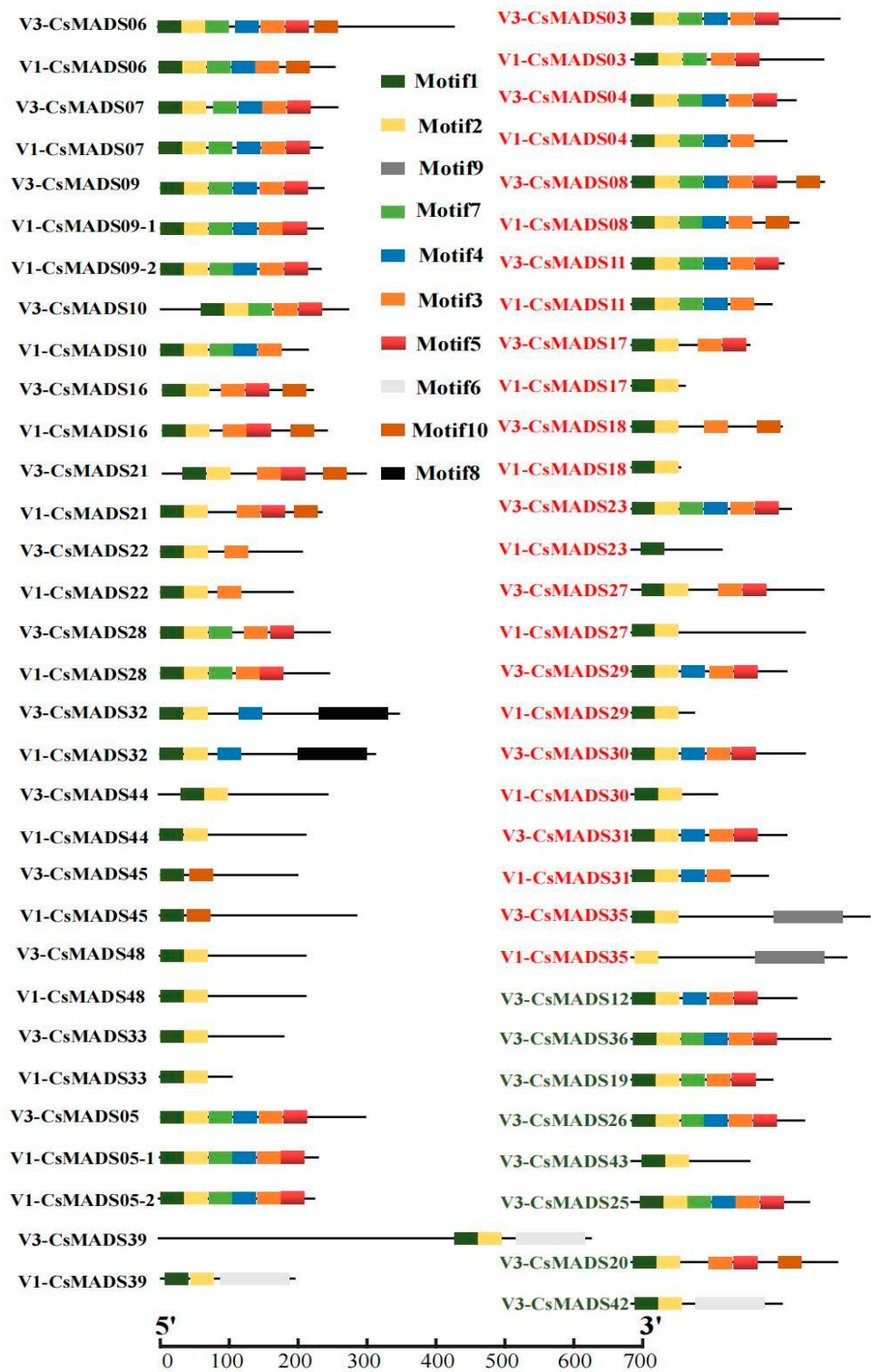


Figure 2. Analysis of Differences in Protein Motifs of MADS-box in Versions of V1 and V3.

3.4. CsMADS Multiple Sequence Alignment

MADS-box genes have a highly conserved DNA binding domain, namely MADS box. By analyzing the amino acid sequence, we found that CsMADS contains a highly conserved MADS box (Figure 3).

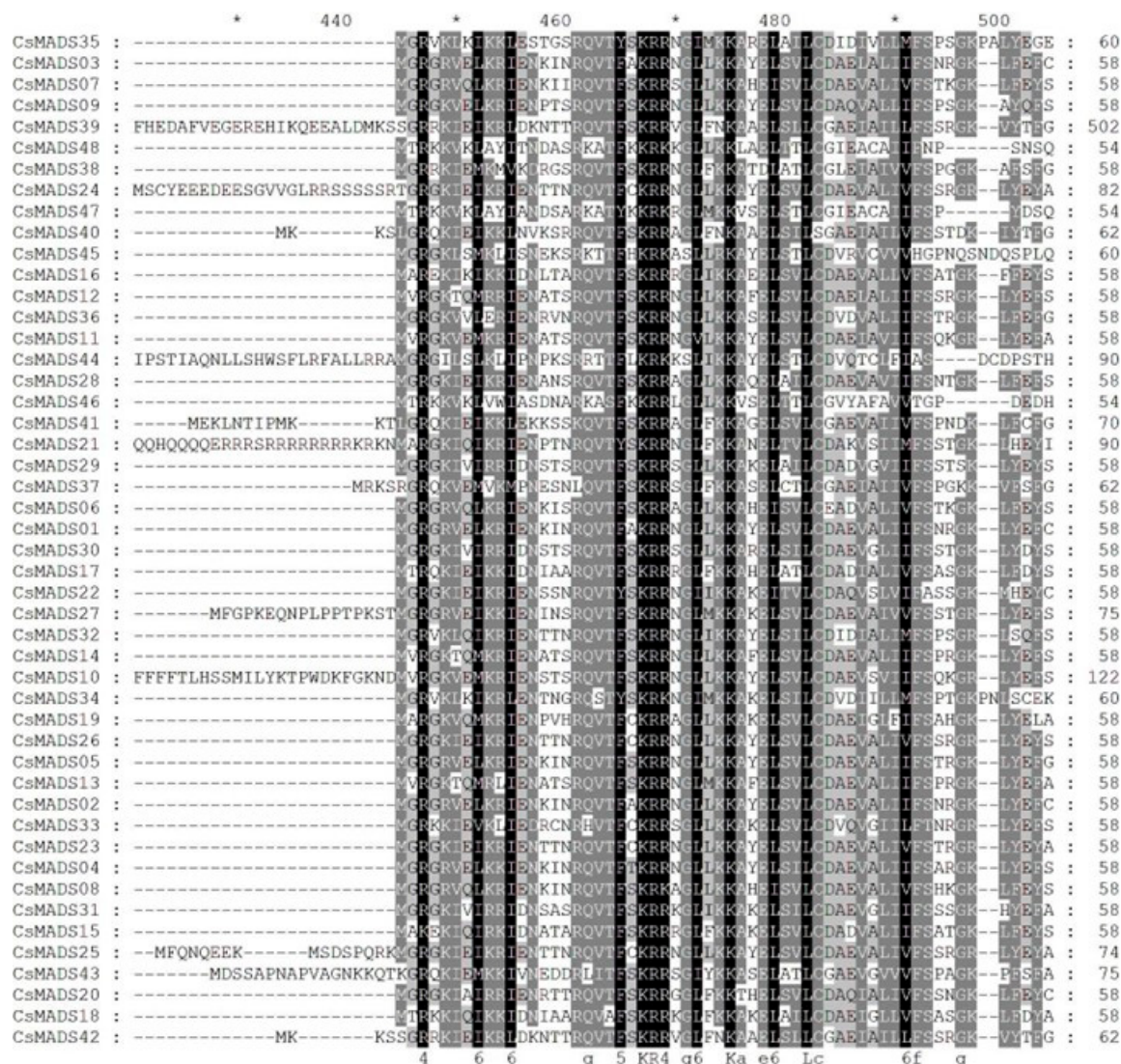


Figure 3. The multiple protein sequence alignment of the domains of MADS from cucumber. Conserved sequences are highlighted by black and grey shading; the black shading represents completely conserved sequences, while the grey shading represents incompletely conserved sequences.

3.5. Phylogenetic Relationship and Gene Structure Analysis of MADS-Box Genes

The genome annotation information of V1 version cannot be obtained. Therefore, it is impossible to analyze the difference of the gene structure of the *MADS-box* family in the V1 and V3 versions, so only the structure of the *CsMADS* in the V3 version is analyzed. The results showed that the number of introns of *CsMADS* varied greatly, ranging from 0 to 10. *CsMADS38*, *CsMADS40*, *CsMADS41*, *CsMADS44*, and *CsMADS46* have no introns, and they all belong to type I genes and belong to the $M\alpha$ and $M\beta$ subfamilies. Type II genes, also known as MIKC genes, contain a large number of introns (*CsMADS01*-*CsMADS32*) and have similar structures (Figure 3).

3.6. Chromosome Distribution Analysis of *CsMADS*-Box Genes

The chromosome distribution analysis of cucumber *CsMADS* gene showed that the distribution of *CsMADS* gene on 7 chromosomes was uneven (Figure 3). Specifically, there are 9 *CsMADS* genes on chromosome 1 and chromosome 3, 3 *CsMADS* genes on chromosome 2, 8 *CsMADS* genes on chromosome 4, and 5 *CsMADS* genes on chromosome 5. The number of *CsMADS* genes on chromosome 6 is the largest, with 12 *CsMADS* genes, and the number of *CsMADS* genes on chromosome 7 is the smallest, with only one *CsMADS* gene (Figure 5).

3.7. Analysis of Cis-Acting Elements in Gene Promoter Region

Analysis of the 1500 bp upstream sequence of the *CsMADS* gene revealed a variety of cis-acting elements (Figure 4). For example, hormone response elements: ABRE, CGTCA-motif, GARE-motif, TCA-element, TATC-box, TCA-element, TGACG motif, etc. Stress corresponding components : MBS, MBSI, etc. And other important response elements in plant growth and development : ARE, CAT-box, LTR, GC-motif, O2-site, RE-element, TC-rich. It shows that the MADS-box family is widely involved in the growth and development of plants (Figure 6).

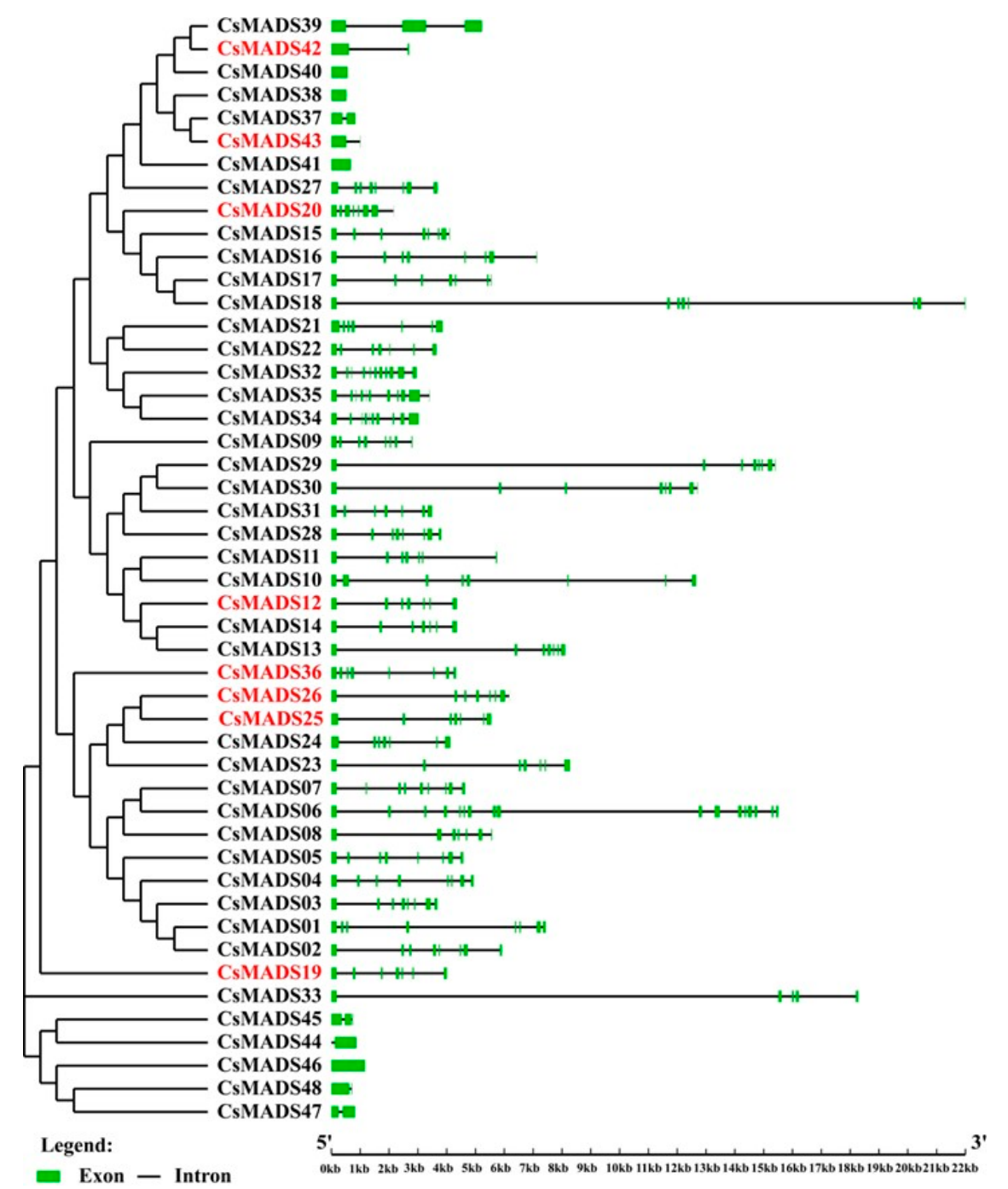


Figure 4. Phylogenetic tree and gene structure of MADS family members in *C. sativus*. The phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap replicates, based on the alignment of the identified MADS proteins in *C. sativus*. The gene structures of the identified 48 MADS genes in *C. sativus* were generated utilizing the Gene Structure Display Server v.2.0. In the structures, the red represents the new genes in versions of V3, the yellow box represents the exon, and the black line represents the intron.

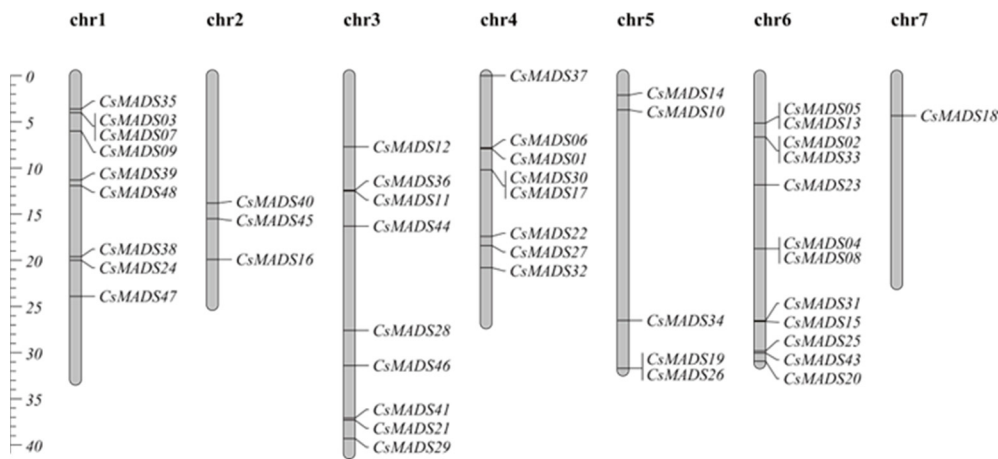


Figure 5. Chromosomal location of CsMADS genes.

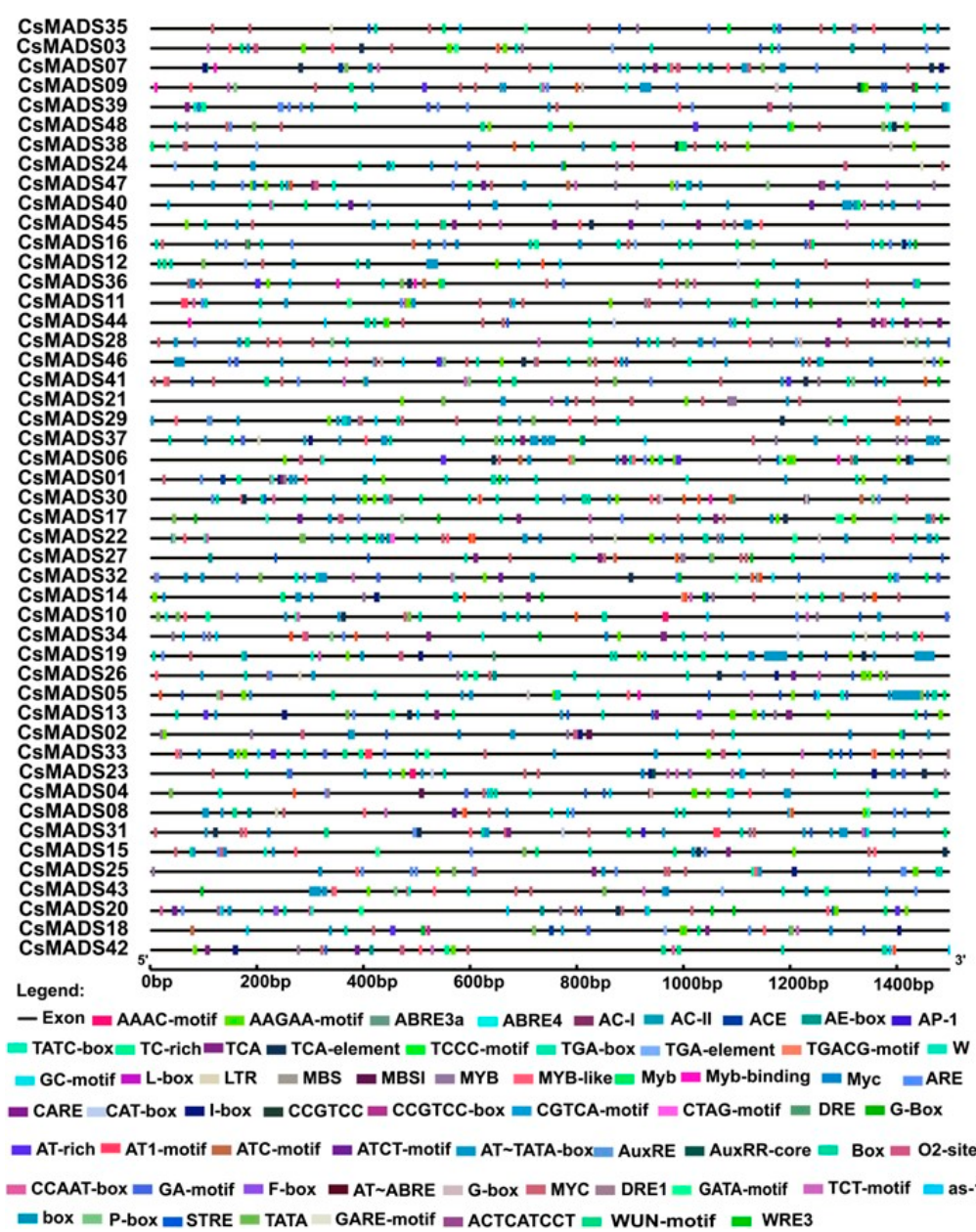


Figure 6. Predicted cis-elements in promoter regions of CsMADS genes. The promoter region was defined as a 1.5 kb sequence upstream of the translation initiation codon of the MADS gene. Identification of cis-acting

elements using the online tool Plant CARE. Different types of cis-acting elements are represented by closed boxes of different colors.

3.8. Expression Patterns of CsMADS in Different Tissues

This part aims to study the role of *MADS* genes in cucumber development by analyzing public RNA-seq data of different tissues. The expression patterns of the *CsMADS* gene family were detected using data from the Cucurbitaceae Genomics Data website and the NCBI SAR database, and heat maps were generated to visualize the expression levels of each tissue. The results showed that compared with other tissues, the expression of most *CsMADS* genes in cucumber stems was relatively lower (Figure 7). In contrast, *CsMADS10*, *CsMADS12*, *CsMADS16* and *CsMADS31* were highly expressed in tendrils. *CsMADS03*, *CsMADS22*, *CsMADS32*, *CsMADS34*, *CsMADS36* and *CsMADS40* were highly expressed in female flowers, indicating their potential roles in flower differentiation and development. *CsMADS14*, *CsMADS19*, *CsMADS29*, *CsMADS30*, *CsMADS37*, *CsMADS38*, *CsMADS41*, *CsMADS44*, *CsMADS45* and *CsMADS46* were mainly expressed in roots, indicating that they may be involved in regulating ion transport in the underground part of cucumber (Figure 7).

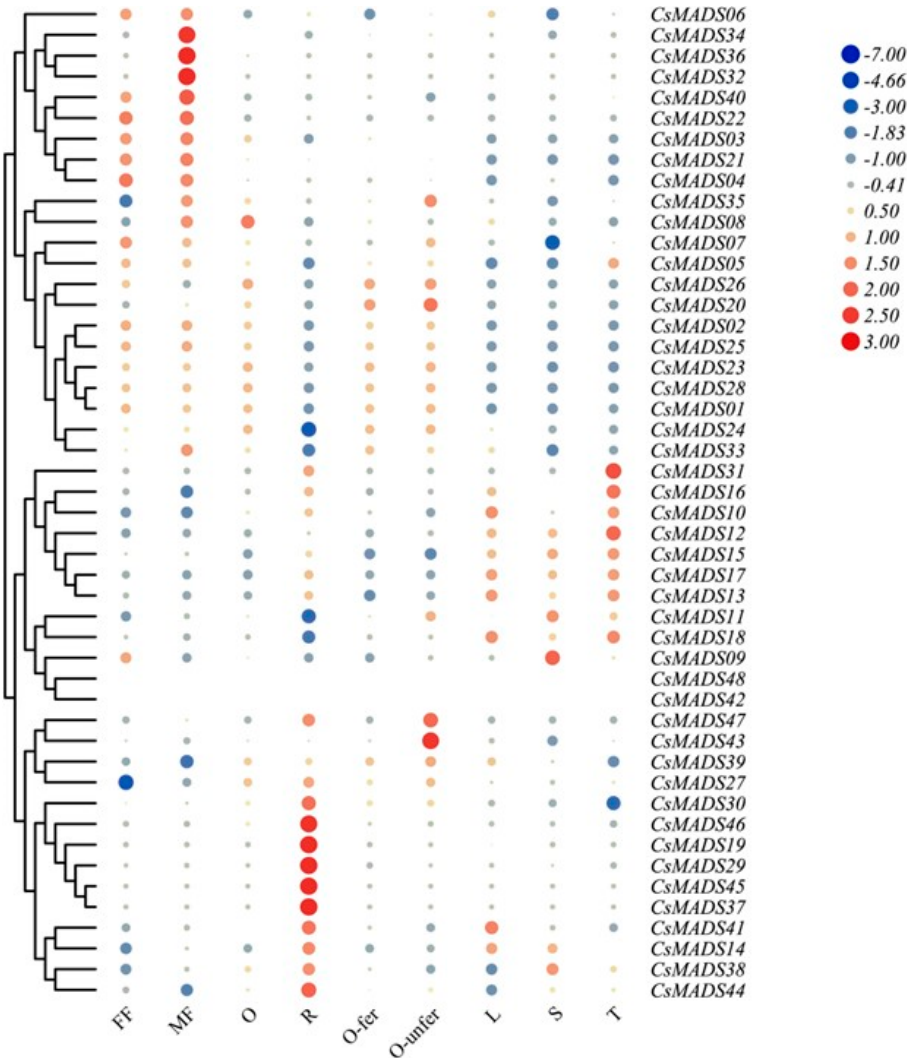


Figure 7. Temporal-spatial expression of cucumber MADS genes. (a) Heatmap displaying the expression profile of *CsMADS* genes in nine different cucumber tissues. The RNAseq datasets with accession number PRJNA80169 were obtained from the Cucurbit Genomics Data website. The color scale represents Log₂(FPKM) values, where blue and red indicate low and high expression levels, respectively. The FPKM values of *CsMADS* genes can be found in Table S1. R: root; S: stem; L: leaf; FF: female flower; MF: male flower; O: unexpanded ovary; O-fer: expanded fertilized ovary; O-unfer: expanded unfertilized ovary; T: tendril.

3.9. Expression Profiles of CsMADS Genes Under Abiotic and Biotic Stresses

Although MADS genes in many species have been identified to be involved in a variety of stress responses, they have not been studied in cucumber. In this study, based on the public transcriptome information, the expression patterns of CsMADS genes under different stress conditions such as salt, heat, downy mildew (DM, *Pseudoperonospora cubensis*) and powdery mildew (PM, *Podosphaera fusca*) were analyzed to explore the role of CsMADS genes under different stress conditions.

Firstly, the role of CsMADS gene in salt stress was analyzed. The results are presented in the form of heat maps(Figure 8). Under NaCl treatment, most genes were up-regulated; only a small number of genes were down-regulated, including CsMADS13, CsMADS25 and CsMADS40. Silicon (Si) is considered to be an essential element for plant growth and development, and plays a significant role in promoting plant growth and development and enhancing stress resistance. We found that only under the condition of Si treatment, gene expression also has many changes. The expression of CsMADS06, CsMADS09, CsMADS16, CsMADS19, CsMADS21, CsMADS37 and CsMADS40 was up-regulated (Figure 8). In contrast, the expression of CsMADS12, CsMADS13, CsMADS28 and CsMADS30 was up-regulated. It is worth noting that the expression of CsMADS06, CsMADS07, CsMADS09, CsMADS16, CsMADS29 and CsMADS40 was up-regulated under the treatment of NaCl and silicon(Si), suggesting that they play an important role in the process of plant resistance to salt stress (Figure 8).

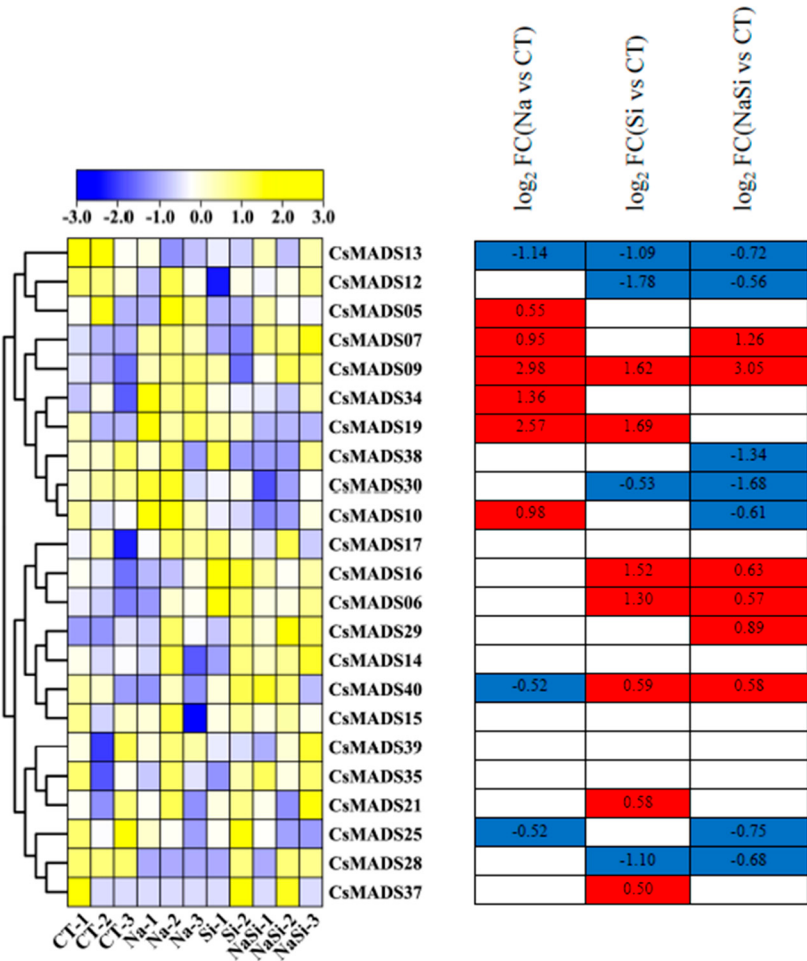


Figure 8. Expression profiles of CsMADS genes in response to salt stress treatments: A range of -3.00 to 3.00 was artificially set with the color scale limits according to the normalized values. The color scale shows increasing expression levels from blue to yeollow. The FPKM value of CsMADS genes under salt are listed in Table S2.

We also analyzed the response of *CsMADS* genes to heat stress. The expression of *CsMADS04*, *CsMADS06*, *CsMADS16*, *CsMADS29*, *CsMADS30*, *CsMADS35*, *CsMADS39*, *CsMADS41*, *CsMADS42* and *CsMADS43* were significantly up-regulated in both three-hour and six-hour heat and high temperature treatments. The expression of *CsMADS08*, *CsMADS10*, *CsMADS12*, *CsMADS15*, *CsMADS18*, *CsMADS19*, *CsMADS21*, *CsMADS28*, *CsMADS33* and *CsMADS46* were significantly down-regulated. In addition, the results showed that the expression of *CsMADS07* was significantly down-regulated after 3 hours of high temperature treatment, but significantly up-regulated after 6 hours of high temperature treatment (Figure 9).

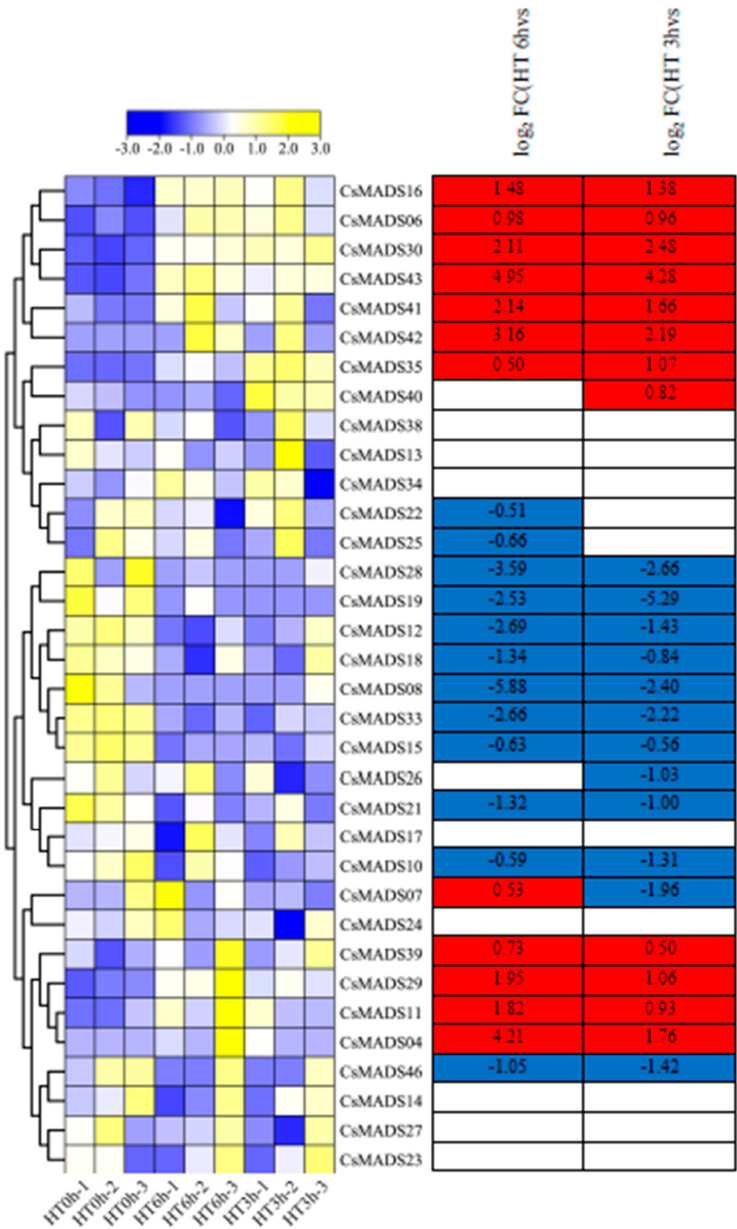


Figure 9. Expression profiles of *CsMADS* genes in response to heat stress treatments: A range of -3.00 to 3.00 was artificially set with the color scale limits according to the normalized values. The color scale shows increasing expression levels from blue to yeellow. The FPKM value of *CsMADS* genes under heat stress are listed in Table S3.

In order to explore the role of *CsMADS* in biological stress resistance, we used RNA-Seq database to analyze the expression of *CsMADS*. The results showed that *CsMADS17*, *CsMADS39* and *CsMADS48* were significantly up-regulated in susceptible and resistant cucumber lines after

inoculation with powdery mildew (PM). On the contrary, the expression of *CsMADS09* was down-regulated (Figure 10A). In addition, we found that the expression of *CsMADS25* was significantly up-regulated in susceptible cucumber lines, indicating that the gene may play an important role in the mechanism of cucumber resistance to powdery mildew.

In the transcriptome data of cucumber seedlings inoculated with DM, only 8 *MADS* genes were detected. *CsMADS06* and *CsMADS07* were up-regulated at most treatment time points, while *CsMADS10*, *CsMADS13* and *CsMADS16* were down-regulated at most treatment time points (Figure 10B).

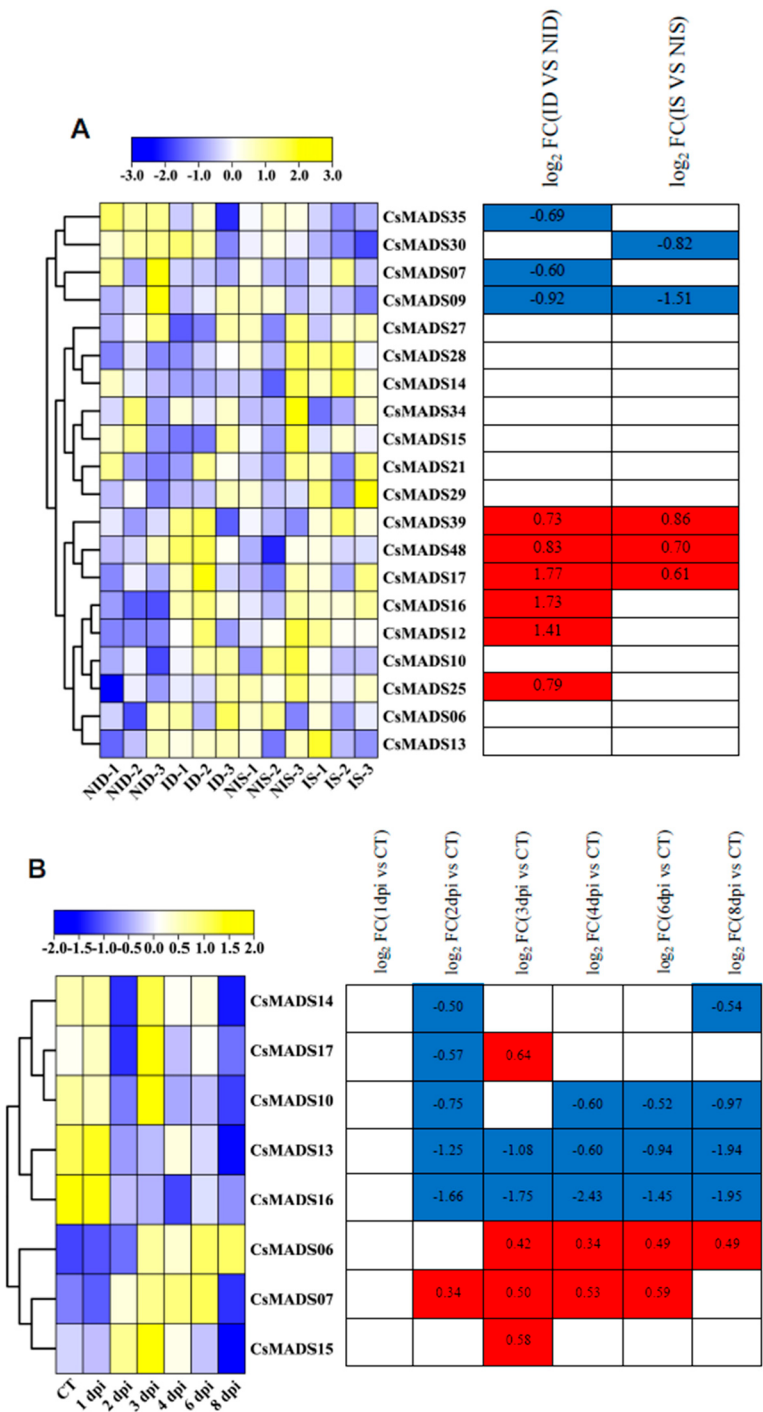


Figure 10. Expression analysis of *CsMADS* under biotic stresses: The transcriptional levels of *CsMADS* genes after infection with powdery mildew (PM) for 48 h (A) and with downy mildew (DM) for 1–8 days post-inoculation (B) are shown on the heatmaps. A range of –3.00 to 3.00 was artificially set with the color scale limits according to the normalized values. The color scale shows increasing expression levels from blue to red. ID, PM-

inoculated susceptible cucumber line D8 leaves; NID, non-inoculated D8 leaves; IS, PM-inoculated resistant cucumber line SSL508–28 leaves; NIS, non-inoculated SSL508–28 leaves; CT, without inoculation; DPI, days post inoculation; FC, fold-change. The FPKM value of CsMADS genes under powdery mildew (PM) and downy mildew (DM) are listed in Table S4.

4. Discussion

The *MADS-box* gene is an important transcriptional regulator in eukaryotes, which is involved in the regulation of growth and development and signal transduction processes. It has been widely identified in many species [32,33,51,52]. Although the *MADS-box* gene of cucumber has been previously identified, its comprehensive identification and characterization are limited due to the low quality of the genome. In addition, the function of MADS-box family in signal transduction and response to different stress conditions is also relatively insufficient. With the update of the Cucurbitaceae genome database, the cucumber genome has been upgraded. Therefore, it is necessary to re-identify and modify the members of the *MADS-box* gene family in cucumber to further explore its variation and potential functions, and to elucidate its role in cucumber development.

In previous studies, 43 *CsMADS-box* genes were identified in the cucumber V1 version database[33], while 48 *CsMADS-box* genes were identified in the cucumber V3 version database. By analyzing the 43 *CsMADS-box* genes in the V1 version, we found that *Csa014213* and *Csa025232* had the same CDS sequence, as well as *Csa014249* and *Csa026408*. In addition, the CDS sequence similarity of *Csa014140* and *Csa025231* is as high as 96%, indicating that they may actually be the same gene (Table 1). These results indicate that there are some errors in previous studies, so it is necessary to re-identify and correct the members of the *MADS-box* gene family in cucumber.

By analyzing the differences in protein sequences and motifs, we found that more than half of the amino acid sequences were significantly different between the two versions of the data (Table 2). Specifically, there were no differences in the amino acid sequences of 14 genes, which were *Csa004117*, *Csa008448*, *Csa014140*, *Csa025231*, *Csa012879*, *Csa012099*, *Csa017355*, *Csa000939*, *Csa021069*, *Csa017317*, *Csa020265*, *Csa017909*, *Csa002566* and *Csa001552*. In addition, the amino acid length of 15 family members changed, but their protein motifs did not change (CsMADS05, 06, 07, 09, 10, 16, 21, 22, 28, 32, 33, 39, 44, 45 and 48). Besides, we found that motif1 and motif2 are highly conserved in most *CsMADS* genes (Figure 2). Type I ($M\alpha$, $M\beta$ and $M\gamma$) MADS-box genes usually lack introns or have only one intron, and their gene structure is very simple (Figure 4). In contrast, the gene structure of type II genes (MIKC and $M\delta$) seems to be more complex, including multiple exons and introns. Studies have shown that genes containing multiple introns are usually more conserved [45], so type I genes may not be as conserved as type II MADS-box genes. In addition, studies have shown that a small number of introns help genes respond quickly to various stresses and activate down-regulated genes[46]. The presence of introns may lead to alternative splicing, thereby delaying the response to stress, and type I genes may respond to stress earlier. Chromosomal localization analysis showed that *CsMADS-box* genes were unevenly distributed on seven chromosomes of cucumber (Figure 5).

We also identified 10 important cis-acting elements of the MADS-box family, most of which are related to plant hormones. Hormone response elements (ABRE, CGTCA-motif, GARE-motif, TCA-element, TATC-box, TCA-element, TGACG motif) were highly enriched in the promoter region of *CsMADS* gene, indicating that *CsMADS* gene may be involved in the regulation of various hormone responses (Figure 6). Meristem response elements (ARE, CAT-box, LTR, GC-motif, O2-site, RE-element, TC-rich) are mainly identified in type II genes, suggesting that type II *MADS-box* genes have a function in determining meristem and floral organ identity in cucumber, which was in accordance with *Callicarpa americana* [47].

Understanding gene expression is essential to reveal the molecular mechanism of biological development [48]. *MADS-box* genes are widely thought to be associated with floral organ development and identity determination in plants. Previous studies have shown that AOAMOUS (AG) and APETALA1 (AP1) gene family members are mainly expressed in flowers, fruits and buds

in species such as *Arabidopsis* [49], tomato [50], cotton [51], watermelon [52] and soybean [53]. The results in this study are consistent with previous observations. AG gene family members (*CsMADS23,24,25,26* and *36*) and AP1 gene family members (*CsMADS06,07* and *08*) are mainly expressed in cucumber flowers. Moreover, we found that 10 other *MADS* genes (*CsMADS14,19,29,30,37,38,41,44,45* and *46*) were highly expressed in roots, indicating that they may play an important role in plant growth and ion transport (Figure 7).

In addition to regulating the characteristics of floral organs and their meristems in plant flower development, *MADS-box* genes have also been found to be involved in a variety of stress responses [54]. For example, *DgMADS114* and *DgMADS115* can enhance the resistance of transgenic *Arabidopsis* to PEG, NaCl, ABA and high temperature stress [55]. Under ABA treatment, the expression levels of *MsMADS001* and *MsMADS075* gradually increased with time, while *MsMADS075* showed a trend of increasing first and then decreasing under drought treatment, which was consistent with the results of RNA-Seq and qRT-PCR analysis, indicating that they may play an important role in stress response [56]. In addition, the expression level of *TaMADS19* was significantly increased after inoculation with wheat stripe rust. After inoculation with powdery mildew, the expression of *TaMADS117* was significantly reduced. The expression levels of *TaMADS121*, *93* and *21* were significantly increased under phosphorus deficiency stress. Under high temperature stress, the expression levels of *TaMADS63* and *41* were significantly reduced [57]. In this study, we found that the expression levels of *26,18,8* and *10 CsMADS-box* genes had significant change after high temperature, NaCl, silicon, downy mildew and powdery mildew treatments (Figures 8–10). Only two genes, *CsMADS07* and *CsMADS16*, showed responses to all stress conditions, indicating that they played an important role in the multiple stress resistance of cucumber. In summary, the results of this study revealed the important role of *CsMADS* genes in cucumber growth and development, and provided new gene resources for cucumber stress resistance breeding.

5. Conclusion

In this study, we re-identified and revised the members of the *MADS-box* gene family. In the updated version (V3), 48 *CsMADS-box* genes were identified, 8 more than the V1 version. In different versions, most amino acid sequences are significantly different. Further analysis of conserved motifs revealed that the conserved motifs of 12 genes were changed. In addition, we found that motif1 and motif2 are highly conserved. Gene structure analysis showed that most of the type I genes did not contain introns. The gene structure of type II is similar, and the number of introns is large. The *CsMADS-box* gene was located on seven chromosomes of cucumber. The promoter region of *CsMADS-box* gene contains response elements and other response elements related to plant growth and development. The expression pattern analysis of *CsMADS-box* in different tissues showed that they were likely to be involved in the growth and morphogenesis of cucumber. Furthermore, transcriptome data under different stress conditions revealed the expression of *CsMADS-box* under abiotic and biotic stresses, and found that *CsMADS07* and *CsMADS16* responded to all four stresses. This study provides data and theoretical reference for the potential role of *CsMADS* in cucumber stress resistance breeding.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org. Figure S1: The conserved motif LOGO of cucumber *CsMADS* proteins; Table S1: The FPKM values of *CsMADS* genes; Table S2: The FPKM value of *CsMADS* genes under salt treatments; Table S3: The FPKM value of *CsMADS* genes under heat treatments, Table S4: The FPKM value of *CsMADS* genes under powdery mildew and downy mildew; Dataset S1: The amino acid sequences of the *CsMADS* proteins.

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Z.R.; visualization, L.W. and Z.R.; supervision, L.W. and Z.R.; project administration, L.W. and Z.R.; funding acquisition, Z.W. and Z.R. All authors have read and agreed to the published version of the manuscript.

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