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Identification and Expression Characteristic Analysis of CML Gene Family of Melon

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Abstract Calmodulin-like (*CML*) is one of the important Ca^{2+} sensors, which plays an important role in plant growth an d development and stress response. In this study, melon (*Cucumismelo* L.) genomic data was used to identify the melon C ML protein family using bioinformatics methods, and its physical and chemical properties, location information, gene struct ure, phylogeny, and promoter were analyzed. The results showed that 60 *CmCML* protein genes were identified in the mel on genomic data, containing 1~4 EF-hand domains, which were unevenly distributed on 12 chromosomes. By analyzing th e evolutionary treewith Arabidopsis *CMLs*, *CmCML* could It is divided into 8 groups, and the number of various groups i s different. It has certain similarities with other plant CML family genes in group classification. In the promoter analysis, abscisic acid, jasmonic acid, auxin, and gibberellin were identified. And drought, low temperature, mechanical damage and other signal response homeopathic elements. Real-time fluorescence quantitative results showed that gene expression of mel on CML protein family was induced by the specialization of Fusarium oxysporum, these results predicted that the transcrip tional regulation of melon CML protein family may participate in the resistance response of melon wilt, which is the gene family. The functional identification of each member laid a theoretical foundation.

Keywords Melon; *CML*; Bioinformatics; Expression analysis

Melon (*Cucumismelon* L.), as an important Cucurbitaceae crop, is widely distributed and has a long history of cultivation. It has high economic and nutritional value. China is a big melon producer, and its output has increased from 2×10^6 tons in the 1950s and 1960s up to nearly 2×10^7 tons, with an average annual growth rate of 3.68%, significantly higher than the world (2.75%). After the 21st century, the status of China's melon industry in the world has been continuously improved, and the total output gradually accounts for more than half of the world (Yang et al., 2019). Melon tastes sweet and juicy. It is rich in soluble sugar, amino acids, organic acids, vitamin C and other nutrients. With high nutritional quality, it can improve human immunity and promote human health (Xia et al., 2017). Biological and abiotic stresses are the main obstacles to melon production. Therefore, it is of practical significance for melon molecular breeding to study the response mechanism of melon to stress and to explore and utilize important gene resources.

As a second messenger, calcium (Ca^{2+}) plays an important role in signal transduction of plant response to stress. It can regulate the activity of a variety of effector proteins and coordinate cell responses (Yang et al., 2019). There are three types of chiral calcium ion sensors in plants (Defalco et al., 2010): one is calmodulin, including calmodulin (*CaM*) and calmodulin-like (*CML*), the other is calcium dependent protein kinase (CDPK), and the third is calcineurin B-like protein (*CBL*).

Calmodulin-like (*CML*), as the most typical calcium binding protein, participates in a variety of physiological and biochemical processes in plants. It is a kind of protein that has been studied more at present. The similarity

between *CML* and *CaM* amino acids is not less than 16% (Perochon et al., 2011). Each protein contains at least one conserved EF-hand chiral domain, and there are no other proven domains. This domain is a helix-ring-helix structure, and one Ca^{2+} can only bind one EF-hand. At the same time, there are differences in chiral molecular sequences among different calmodulin-like molecules. Mccormack et al. (2005) believed that this may reflect the small differences in the response of different family members to Ca^{2+} signals. *CMLs* are ubiquitous in plants. A variety of CML proteins have been identified in the genomes of *Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicum* and *Brassica pekinensis*. They play an important role in growth, biological stress and abiotic stress (Cao et al., 2018). In *Arabidopsis thaliana*, Magnan et al. (2008) found that *CML*9 can enhance its tolerance to salt and drought stress and promote root growth. *CML42* is also related to the formation of cell trichomes (Dobney et al., 2009), while *CML*24 is involved in encoding a potential Ca^{2+} sensor in response to ABA, sunshine and salt stress (Delk et al., 2005).

At present, there is no research on melon CML gene family. In this study, 60 CML family members were identified in the published melon genome by using bioinformatics methods. At the same time, the chromosomal location, gene structure, conserved protein domains, promoters and phylogenetic relationships of melon CML family members were analyzed, and the expression of some CML family genes induced by *Fusarium oxysporum* melon specialization was discussed. We hope that our study can provide reference and data basis for further research of melon CML family genes.

1 Results and Analysis

1.1 Analysis of basic characteristics of *CML* protein gene in melon

In this study, the proteins containing EF-hand but not other domains were selected as melon Calmodulins by bioinformatics. 60 melon calmodulin genes were screened and named *CmCML*1-60 according to their distribution and arrangement on chromosomes (Table 1). According to the analysis, the number of EF-hands of melon CML family members was 1~4, and the protein with 4 domains was the most, and most of them were EF-hand7. Except that the pI of *CmCML*1, 11, 16, 18 and 28 were greater than 7, the remaining 92% of the pI were between 3.99 (*CmCML*40)~6.57 (*CmCML*15), 96.7% of the amino acids were within 300, and the relative molecular weight was generally about 20 000 kD. It can be seen that the melon CML family proteins are mostly small acidic molecular proteins. The subcellular location of the 60 identified *CmCML* showed that *CmCML* were mainly distributed in cell membrane, vacuole, cytoplasm and nucleus. Among them, 53 genes were located on the cell membrane, accounting for 83.3%, followed by vacuole and cytoplasm. Only 4 *CmCML* genes were located in the nucleus, accounting for 6.7%.

1.2 chromosome location of *CML* gene in Melon

According to the location information of *CmCML* on the chromosome, MG2C online software was used to draw the physical map of melon *CML* chromosome and to locate the gene (Figure 1). 60 *CmCML* family members were distributed on 12 chromosomes, and the number of genes on each chromosome was mostly different.

*CmCML*1 and 2 were dispersed in the upper and lower arms of chromosome 1, *CmCML*3 was located in the upper arm of chromosome 2, *CmCML*4～6 were concentrated in the lower arm of chromosome 2, *CmCML*7～10 were evenly distributed on chromosome 3, and *CmCML*11～18 were clustered in the lower arm of chromosome 3. *CmCML*19, *CmCML*20～22, *CmCML*23～25, *CmCML*26 and 27 were distributed in the upper and lower arms of chromosomes 4 and 5 respectively, and *CmCML*28～32 was evenly distributed on chromosome 6. *CmCML*39 and 40, *CmCML*41 and 42, *CmCML*43 and 44, *CmCML*45 and 46, *CmCML*52 and 53, and *CmCML*54 were distributed in the upper and lower arms of chromosome 7, 8 and 10, respectively. There is only one chromosome of *CmCML*47 in the upper arm of chromosome 9, of which *CmCML*48～51 were distributed in the lower part. *CmCML*55 and 56, 57 and 58 were evenly distributed in the upper, middle and lower arms of chromosome 11, and *CmCML*59 and 60 were distributed in the middle and lower arms of chromosome 12. The unbalanced distribution of genes on 12 chromosomes indicated that the genetic variation of *CmCMLs* was in the process of evolution.

Table 1 Information of CML gene family in melon

Gene	Gene No.	Number of EF-hands	pI	Number of amino acids	Molecular weight (KD)	Subcellular location
CmCML1	MELO3C018445.2.1	4	8.83	184	21 045.03	Vacuole
CmCML2	MELO3C023443.2.1	1	4.68	175	19 835.55	Cell membrane
CmCML3	MELO3C015602.2.1	4	4.12	149	16 875.72	Cell membrane and Cytoplasm
CmCML4	MELO3C017353.2.1	4	4.35	188	20 615.86	Cell membrane
CmCML5	MELO3C017273.2.1	4	4.18	160	17 393.71	Vacuole
CmCML6	MELO3C026214.2.1	3	4.51	142	15 849.99	Vacuole
CmCML7	MELO3C008204.2.1	4	4.43	140	15 669.84	Cell membrane
CmCML8	MELO3C008466.2.1	3	4.35	161	18 686.25	Cell membrane
CmCML9	MELO3C030156.2.1	2	4.71	83	9 295.51	Cell membrane
CmCML10	MELO3C011166.2.1	2	4.58	174	19 272.09	Cell membrane
CmCML11	MELO3C010876.2.1	3	9.28	167	18 984.7	Cell membrane
CmCML12	MELO3C010875.2.1	1	5.23	87	10 089.26	Cell membrane
CmCML13	MELO3C010870.2.1	3	5.07	137	15 536.75	Cell membrane
CmCML14	MELO3C010869.2.1	1	5.29	87	10 060.3	Cell membrane
CmCML15	MELO3C010868.2.1	1	6.57	91	10 543.03	Cell membrane
CmCML16	MELO3C010865.2.1	1	9.57	81	9 306.69	Cell membrane
CmCML17	MELO3C010864.2.1	1	5.84	87	9 787.95	Cell membrane
CmCML18	MELO3C010863.2.1	2	8.89	92	10 485.81	Cell membrane
CmCML19	MELO3C003889.2.1	3	4.76	176	19 904.67	Cell membrane
CmCML20	MELO3C009858.2.1	2	4.26	118	14 047.69	Cell membrane
CmCML21	MELO3C009818.2.1	2	4.88	168	18 415.68	Cell membrane and Nucleus
CmCML22	MELO3C009094.2.1	3	5.35	248	27 875.74	Cell membrane
CmCML23	MELO3C014698.2.1	3	4.19	113	12 896.33	Cell membrane and Cytoplasm
CmCML24	MELO3C014322.2.1	4	4.10	149	16 983.88	Cell membrane and Cytoplasm
CmCML25	MELO3C014279.2.1	4	4.39	164	19 261.65	Cell membrane and Vacuole
CmCML26	MELO3C004375.2.1	4	4.06	146	16 914.07	Cell membrane and Cytoplasm
CmCML27	MELO3C004465.2.1	4	4.59	225	25 278.25	Cell membrane, Nucleus andVacuole
CmCML28	MELO3C006312.2.1	1	7.63	186	21 597.78	Cell membrane
CmCML29	MELO3C006491.2.1	4	4.08	150	17 057.16	Cell membrane and Cytoplasm
CmCML30	MELO3C006721.2.1	3	4.48	154	16 751.5	Cell membrane
CmCML31	MELO3C020277.2.1	2	4.88	123	13 913.46	Cell membrane
CmCML32	MELO3C020263.2.1	4	4.60	157	17 010.93	Cell membrane
CmCML33	MELO3C032128.2.1	4	4.55	141	16 033.19	Cell membrane
CmCML34	MELO3C016569.2.1	1	4.15	118	13 339.1	Cell membrane
CmCML35	MELO3C025433.2.1	1	4.67	123	13 766.57	Cell membrane
CmCML36	MELO3C013829.2.1	4	4.43	163	18147.28	Vacuole
CmCML37	MELO3C013982.2.1	3	4.82	147	16716.89	Cell membrane and Vacuole
CmCML38	MELO3C014128.2.1	1	4.62	221	25 500.19	Cell membrane
CmCML39	MELO3C016746.2.1	1	5.26	97	10 965.25	Cell membrane
CmCML40	MELO3C010454.2.1	1	3.99	174	18 711.15	Cell membrane
CmCML41	MELO3C017712.2.1	4	4.33	150	17 012.18	Cell membrane
CmCML42	MELO3C017844.2.1	1	4.44	175	20 051.82	Cell membrane
CmCML43	MELO3C007998.2.1	3	4.74	226	25 896.31	Cell membrane
CmCML44	MELO3C020725.2.1	3	4.69	192	21 374.7	Cell membrane
CmCML45	MELO3C026331.2.1	2	6.20	291	31 091.61	Cell membrane and Nucleus
CmCML46	MELO3C026342.2.1	4	4.11	149	16 847.67	Cell membrane and Cytoplasm
CmCML47	MELO3C021998.2.1	4	4.39	181	20 230.89	Cell membrane
CmCML48	MELO3C022853.2.1	2	4.38	84	9 338.29	Cell membrane
CmCML49	MELO3C005073.2.1	4	4.80	375	44 211.46	Cell membrane

Continued Table 1

Gene	Gene No.	Number of EF-hands	pI	Number of amino acids	Molecular weight (KD)	Subcellular location
CmCML50	MELO3C005272.2.1	4	4.49	182	19 775.04	Vacuole
CmCML51	MELO3C005319.2.1	4	4.31	144	15 969.01	Cell membrane
CmCML52	MELO3C012195.2.1	4	4.38	161	17 754.9	Vacuole
CmCML53	MELO3C011760.2.1	3	4.41	358	42 038.31	Cell membrane
CmCML54	MELO3C026437.2.1	4	4.37	185	19 993.17	Vacuole
CmCML55	MELO3C020900.2.1	4	5.26	190	21 229.87	Cell membrane
CmCML56	MELO3C020899.2.1	4	4.72	168	19 258.56	Cell membrane
CmCML57	MELO3C013504.2.1	1	4.96	169	19 201.8	Cell membrane
CmCML58	MELO3C022506.2.1	1	5.63	101	11 806.47	Cell membrane
CmCML59	MELO3C004702.2.1	2	5.36	266	29 867.01	Cell membrane and Nucleus
CmCML60	MELO3C001988.2.1	2	4.22	211	24 227.2	Cell membrane

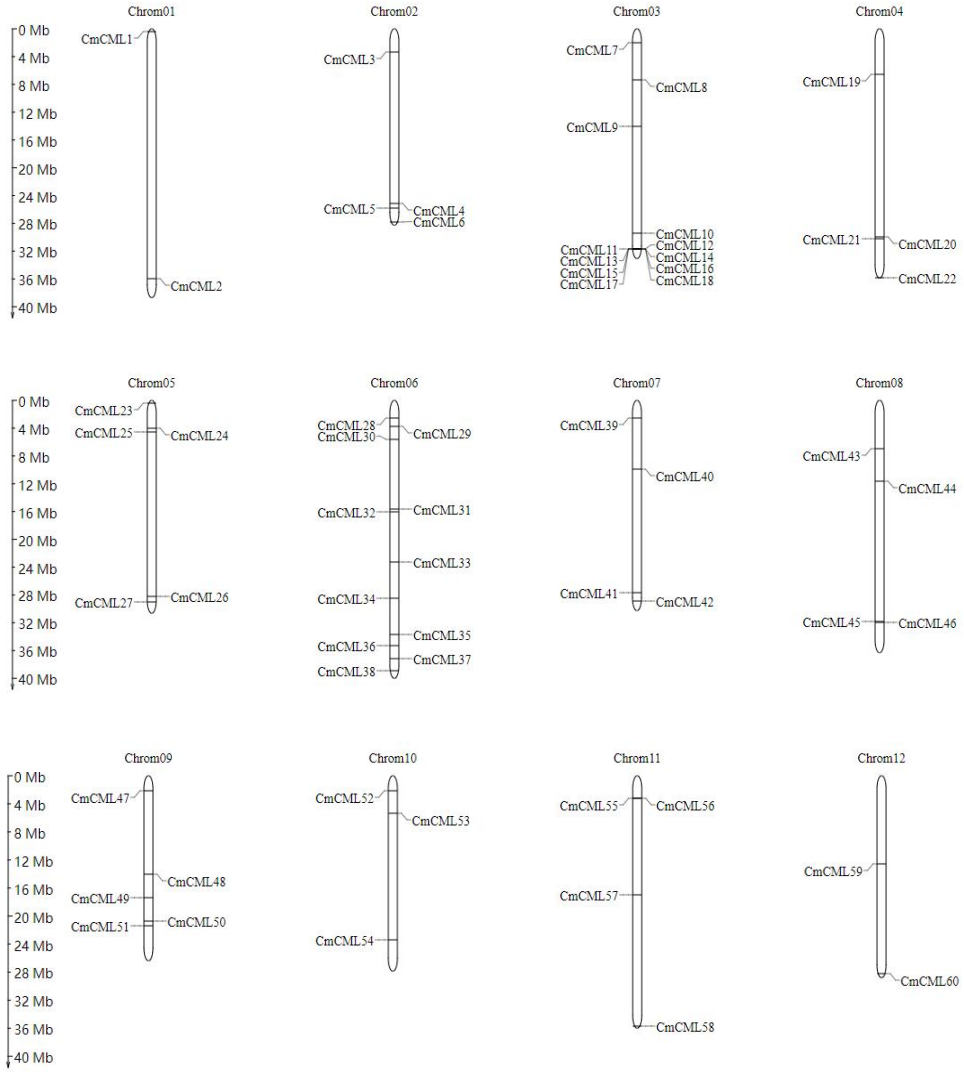

Figure 1 *CmCML* gene location on chromosome

1.3 Analysis on structural characteristics of melon *CML*

1.3.1 Conservative motif analysis of melon CML family

The analysis of conserved domains plays an important role in determining gene functions. In this study, MEME,

an online tool, was used to predict and analyze melon CML family proteins. At the same time, the maximum likelihood method was used to build a phylogenetic tree, and the results of conservative motif prediction were visualized with TBtools (Figure 2). The results showed that the melon CML family had 10 conserved motifs with a width between 15 and 50 amino acids (Table 2). Almost all *CML* proteins contain motif 1~5, which were EF-hand domains. Motif 6 and motif 8 mainly appeared in group I; Motif 10 mainly appears in group II. Members of phylogenetic tree with close relationship often contain the same motif, that is, *CML* proteins of the same group have certain similarity in function.

1.3.2 Melon *CML* intron identification

Among the 60 identified *CmCML*, 19 family members contained introns, of which *CmCML*3, 46 and 55 contained only one intron, and *CmCML*57 contained the most introns, which was 9 (Figure 3). These introns may play an important role in regulating gene expression and promoting transcriptional expression. In this study, 68.3% of *CmCML* lack intron structure, which was also the main reason for the small length of melon CML family genes.

1.4 Genetic evolution analysis of melon CML family

In order to reveal the homologous evolution relationship of *CmCMLs*, a phylogenetic tree was constructed with reference to *Arabidopsis thaliana* CML gene family. Phylogenetic analysis was carried out on 60 proteins of melon *CML* family and 50 proteins of *Arabidopsis thaliana* CML family (Figure 4). According to the genetic relationship and sequence similarity, the CML gene family was divided into 8 groups, I~VIII. Each group contained 4, 6, 14, 15, 6, 5, 6 and 4 members respectively. The genes of each group had high homology and close evolutionary relationship.

1.5 Promoter analysis

In this study, the online software PlantCARE was used to analyze the sequence of 2000 bp upstream of the *CmCML* start codon, and the main promoters were displayed and plotted (Figure 5). A variety of *cis*-elements responding to environmental and hormonal signals were identified in the *CmCML* family. It can be seen that its expression regulation mechanism was complex. Melon CML family contained a variety of hormone response elements, including ABRES, TGA element, TCA element and ERE. TCA element was also involved in salicylic acid response. At the same time, it was found that *CmCML* could also respond to drought, light, temperature, hypoxia and salt stress.

Table 2 Prediction of Conserved Motifs of *CML* Gene in Melon

Name	Number of amino acids	Best possible match
Motif 1	21	CKEMIREVDLDGDGVISFEEF
Motif 2	21	LKEAFKVFDKDGDGFISAEEL
Motif 3	40	EQIAELKEAFKKFDKNGDGKITIEELGALLRSLGQNPTEE
Motif 4	26	QEMINEVDSDGDGLIEFDEFVNLMEK
Motif 5	15	KHVMRSLGEKLTEEE
Motif 6	47	PIIGKIHPSEHYYAKQQAEYIISQADSDKDGRLTLTEMIENPYVFYS
Motif 7	41	VPEFAMNPLSQRLLKMVDGLNFKDFVAFLSAFSAKASVQRK
Motif 8	29	TTVQRHRRLRLRSNFTFTPSLHRQQPVPY
Motif 9	17	HTKSTPIQPYPPTPPPY
Motif 10	50	GPFMSDEQRKQVLSQLLQEAGYTRNVQLTQDDFVKVLGNSGLKMEVEVPV

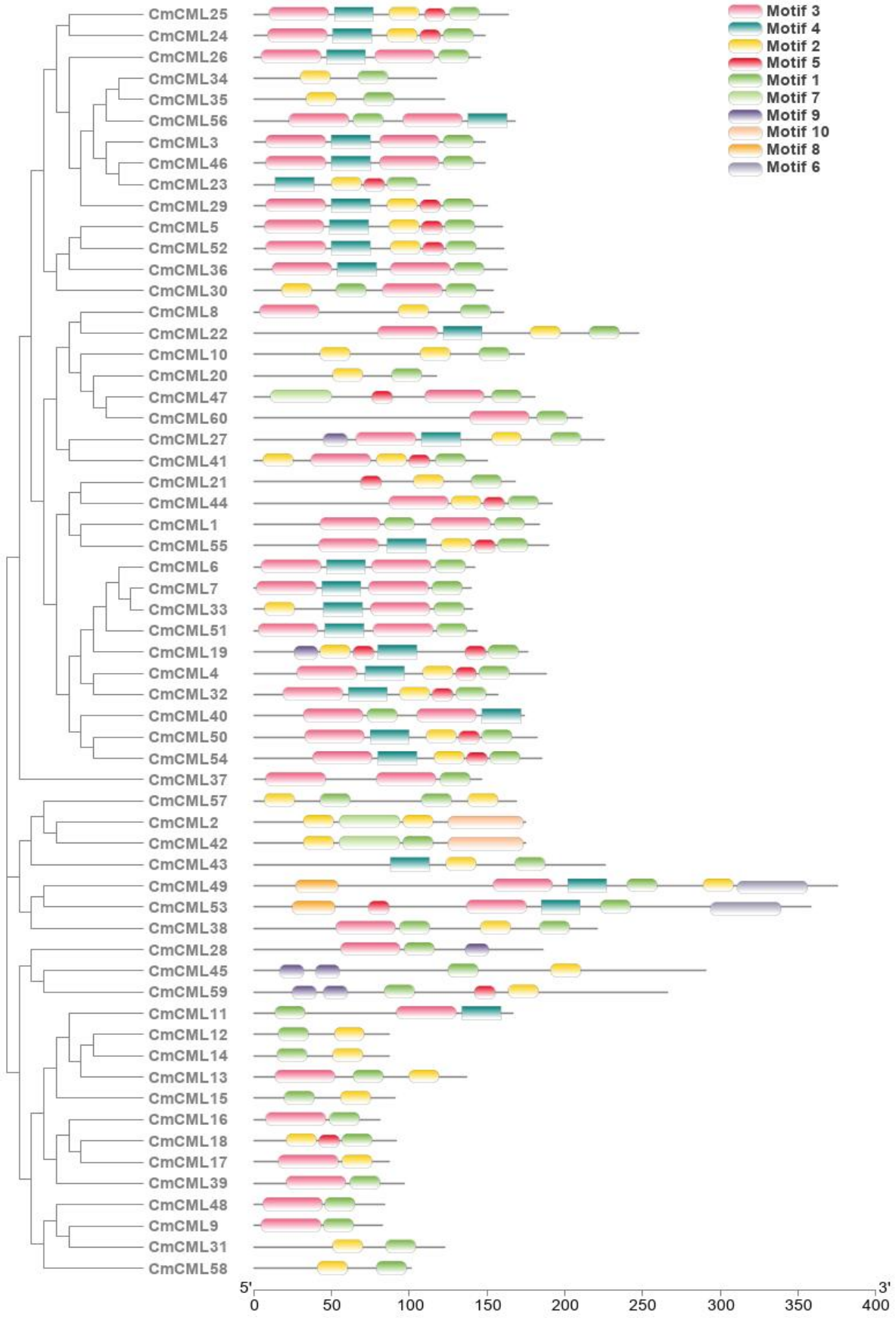

Figure 2 The prediction of conserved motifs in CmCML proteins

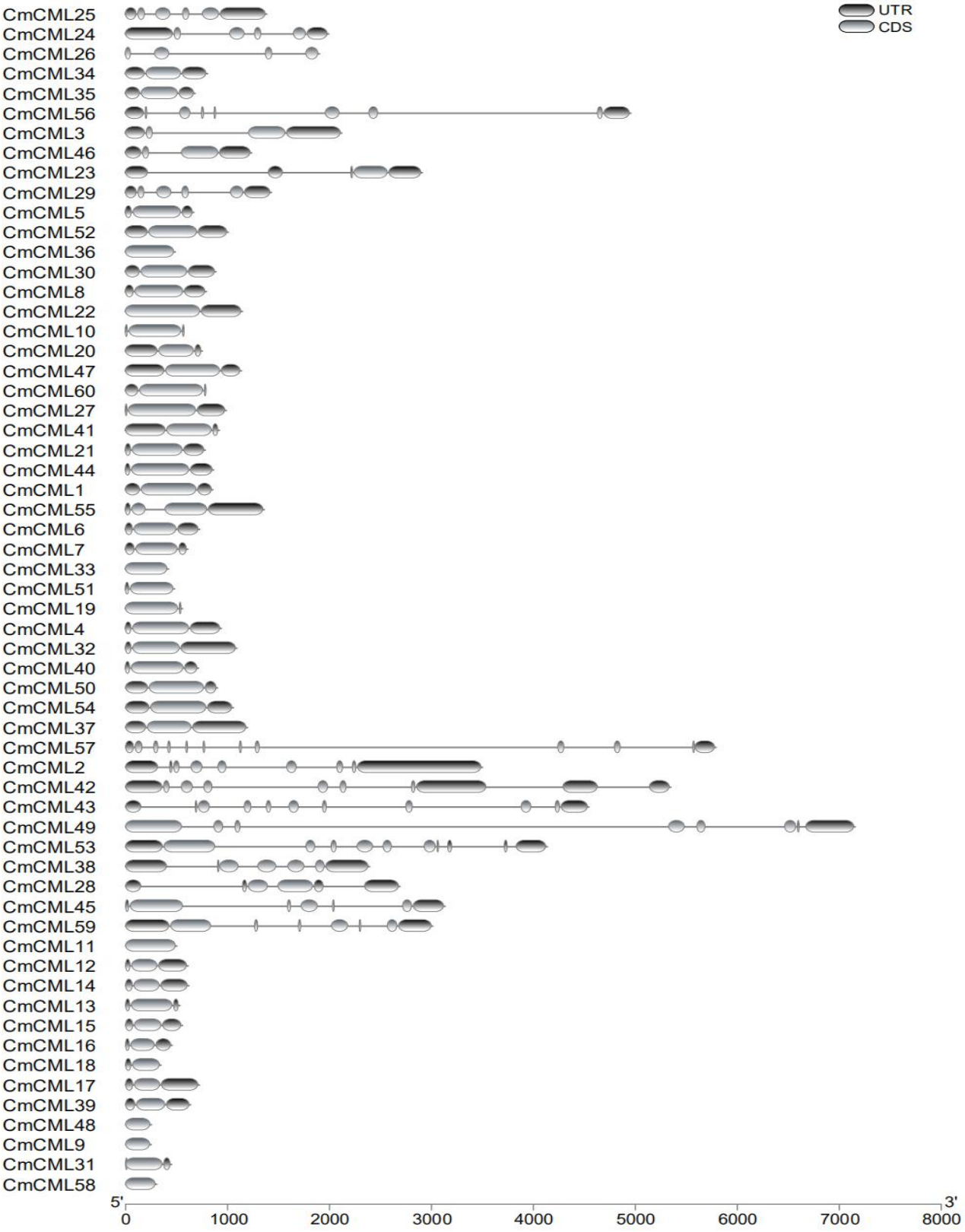

Figure 3 The gene structure of CML gene family in melon

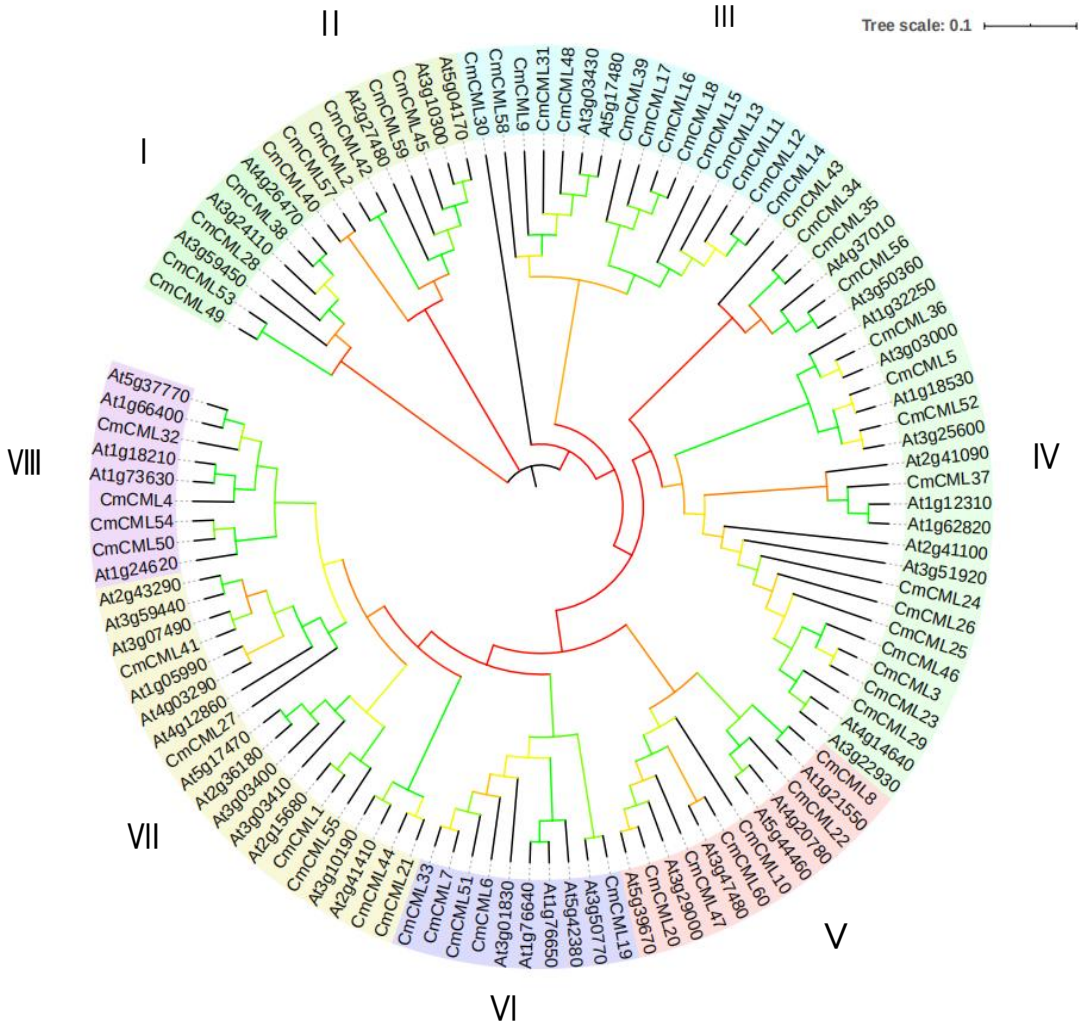

Figure 4 Phylogenetic tree of melon and *Arabidopsis thaliana* CML family

1.6 Analysis of gene expression characteristics of melon CML family induced by *Fusarium oxysporum* melon specialization

The expression characteristics of some genes of melon CML family induced by *Fusarium oxysporum* melon specialization were analyzed by fluorescence quantitative PCR. The expression levels of *CmCML29*, *CmCML35* and *CmCML45* were up-regulated under *Fusarium oxysporum* melon specialization, and the other genes were down-regulated. The expression levels of *CmCML12*, *CmCML29* and *CmCML35* genes in resistant and susceptible varieties were significantly different (Figure 6).

2 Discussion

A total of 60 melon CML family proteins were identified in this study, which were consistent with the studies on 50 *Arabidopsis thaliana CML* (Delk et al., 2005), 52 *Lycopersicon esculentum CML* (Munir et al., 2016), 68 *Glycine max CML* (Chen et al., 2015) and 60 *Gossypium CML* (He 2015, Huazhong Agricultural University, pp.3-7). Melon *CML* proteins were mostly acidic small molecular proteins (Table 1), with large differences in the number of exons between genes (Figure 3), and relatively complex gene structures. Therefore, *CmCML* was easy to be cut during replication, resulting in instability of gene structure.

Figure 5 Major cis-acting element 2 000 bp upstream of *CML* start codon in melon

Only one EF-hand domain can be predicted in *CMLs*, indicating the importance and necessity of this domain. EF-hand plays an important role in Ca^{2+} binding and transport. Through comparison, it was found that *CmCML* contained 1~4 EF-hand domains, which were similar to the characteristics of *Arabidopsis thaliana*. Therefore, the whole *CML* gene families of melon and *Arabidopsis thaliana* were analyzed by phylogenetic tree and divided into 8 subfamilies (Figure 4), indicating that melon CML family had many protein members, different origins and diverse functions. Through analysis, it was found that *CML*I, IV, V, VI and VIII contain roughly the same amount of *CML* in the two species, but only in the three groups of *CML*II, *CML*III and *CML*VII had great differences, indicating that *CML* had a relatively close evolutionary relationship among plant genes. In the same subfamily or smaller branch structure, different genes basically played similar functions (Wang et al., 2018). In promoter analysis (Figure 5), about 97% of melon *CMLs* contain MYC, so it was predicted that CML family proteins had strong drought resistance. In addition, *cis*-elements and a large number of hormone response elements under hypoxia, low temperature and salt stress had been identified. It can be seen that hormones play a very important

role in plant stress resistance. Based on the fluorescence quantitative PCR technology, it was found that the expression of some melon CML family genes changed significantly under the induction of *Fusarium oxysporum* melon specialization (Figure 6). Therefore, it can be inferred that *CmCMLs* are conducive to inhibiting the occurrence of Melon Fusarium Wilt and alleviating continuous cropping obstacles, but the specific mechanism and mode of action need to be further studied.

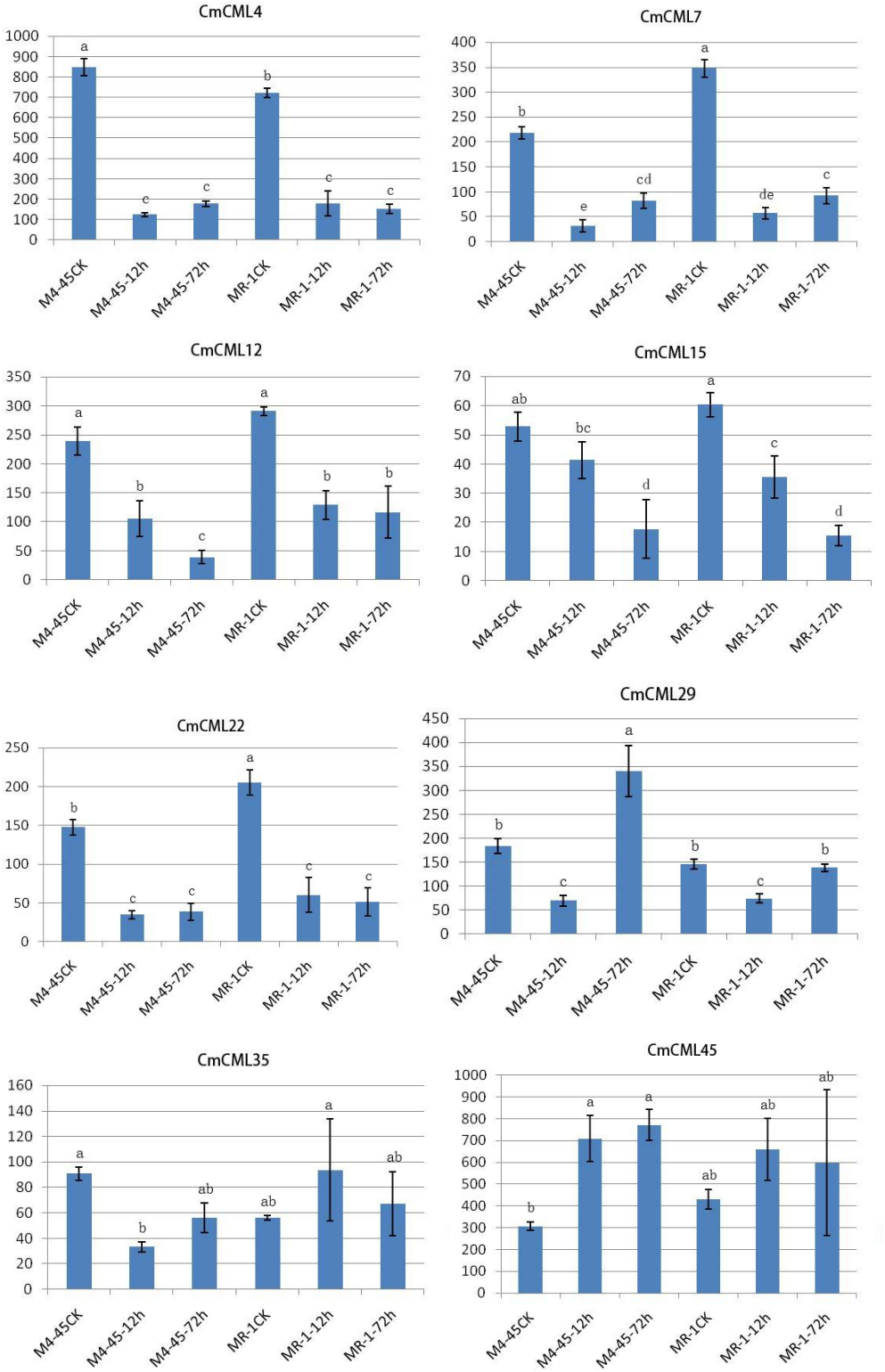

Figure 6 Expression characteristics of CML family genes induced by *Fusarium oxysporum*

CMLs were the most conserved calcium sensing proteins in all eukaryotes (Yang et al., 2019). They play a key role in the cellular signal network by regulating various targets, thus responding to plant stress response and phylogeny (Perochon et al., 2011). This study preliminarily clarified the basic bioinformatics characteristics of melon *CML* gene, verified that *CML* gene was involved in the formation of Melon Fusarium Wilt resistance, and provided data reference and theoretical basis for further study on the biological function of the family members.

3 Materials and Methods

3.1 Identification and physicochemical properties analysis of melon *CML* protein gene

In order to screen and obtain CML family genes in melon, the TAIR genome database (https://www.arabidopsis.org/) was used to download 50 *Arabidopsis thaliana CML* genes, and the Cucurbitaceae database (http://cucurbitgenomics.org/) was used to obtain the candidate protein coding sequences of melon CML family genes by blast search and comparison (E-value was e^{-5}, Identity>60%). Then the online database Pfam and SMART were used to analyze and identify the above candidate proteins (E-value<1.0), and the genes without domains were eliminated. Finally, the candidate proteins were manually screened and identified by Markov model. A series of bioinformatics analyses were carried out on all the identified members of the melon calmodulin family, and ExPASy (http://web.expasy.org/protparam/) was used to analyze the biochemical properties such as isoelectric point, amino acid number and relative molecular weight.

3.2 Prediction of gene structure of melon CML family members

MG2C online software (http://mg2c.iask.in/mg2c_v2.0/) was used to draw the physical map of chromosome and locate the genes. Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) online software was used to predict subcellular localization. Finally, TBtools local tools were used to analyze and map the gene structure.

3.3 Conservative motif analysis of melon CML family

MEME online tool was used to predict and analyze the conserved motifs of melon CML family genes (Bailey et al., 2009). The parameters were set as follows: the minimum width of conserved motifs was set to 6, the maximum was set to 50, the number of conserved motifs was set to 10, and other parameters were set by default. Download the result file mast.xml. The phylogenetic tree of melon CML family genes was constructed by using the maximum likelihood method (ML). The amino acid replacement model was LG+G, and the phylogenetic analysis was carried out. The Bootstrap method was used to repeat 1000 times to evaluate the support rate of each node, and the Partial deletion value was set to 80%. The results of prediction and conservative motifs were visualized by TBtools (https://www.biorxiv.org/content/10.1101/289660v2.abstract).

3.4 Phylogenetic analysis of melon *CML*

the MUSCLE program in MEGAX was used to perform multiple sequence alignment for the identified melon CML family genes (60) and *Arabidopsis thaliana* CML family genes (50), where the Max Iterations parameter was set to 100, and other parameters were set by default. The Neighbor-Joining (NJ) method was adopted. The Bootstrap value was set to 1000, and the Partial deletion value was set to 80%. Other parameters were set by default.

3.5 Prediction of melon *CML cis*-elements

2 000 bp upstream DNA sequences of 60 CML family members were extracted from melon genome database as promoter sequences and submitted to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The possible *cis*-acting elements in the promoter sequence were analyzed.

3.6 Analysis of gene expression characteristics of melon CML family induced by *Fusarium oxysporum* melon specialization

3.6.1 Activation of *Fusarium oxysporum* melon specialization

The activated pathogens were cultured in PDA medium and Armstrong medium. The specific method was as follows: the mycelium was inoculated on PDA (200 g potato, 20 g glucose, 20 g agar, 1 l distilled water) potato culture medium, dark cultured at 25 ℃ for 7 days, cut into 0.5 cm^2 cubes after the culture, inoculated on

Armstrong (1.1 g KH_2PO_4, 1.6 g KCl, 5.9 g $Ca(NO_3)_2$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.2 mg $ZnSO_4$, 0.2 mg $MnSO_4$, 0.2 mg $FeCl_3$, 20 g glucose, Inoculate in Armstrong (1.1 g KH_2PO_4, 1.6 g KCl, 5.9 g $Ca(NO_3)_2$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.2 mg $ZnSO_4$, 0.2 mg $MnSO_4$, 0.2 mg $FeCl_3$, 20 g glucose, add distilled water to 1 L) liquid medium, and culture at 27 ℃ and 120 rpm for 3 days.

3.6.2 Inoculation of melon seedlings

The tested melon varieties were M4-45 and MR-1. Before sowing, the soil and seeds were disinfected. When the tested seedlings grew to the third leaf stage, the root soaking inoculation method was adopted, and the inoculation concentration was 1×10^6/mL spore suspension. Seedlings with the same growth were selected to cut the roots and soak the roots in the bacterial solution for 1 quarter of an hour, and shake them for 2~3 times. After root soaking, it was planted in a nutrient bowl, and the one inoculated with clean water was used as the control. Buckle the shed to keep warm and moist. Root tissues were taken at 0 h (control), 12 h and 72 h after inoculation. The samples were named M4-45CK, M4-45-12h, M4-45-72h, MR-1CK, MR-1-12h and MR-1-72h. Each treatment was repeated for 3 times, with 10 strains for each repetition, a total of 18 samples. Clean it repeatedly with distilled water, dry the residual distilled water on the surface with filter paper, and then freeze it with liquid nitrogen at -80 ℃.

3.6.3 Analysis on gene expression characteristics of melon CML family

Total RNA was extracted with Trizol reagent of Invitrogen company, and cDNA synthesis was performed with the reverse transcription kit TRUEscript 1st Stand cDNA Synthesis Kit of Aidlab company. Melon actin gene was used as the internal reference gene (Sense:5'-TCTATTCCAGCCATCTCTC-3', Antisense:5'-GACCCTCCAATCCAAAC-3"), and candidate gene specific primers were designed with Primer Premier v6.0 software (Table 3), QRT PCR experiments were conducted on iQ5Gradient Real Time PCR system (Bio-Rad, CA, USA). The reaction conditions were as follows: 95 ℃ for 30 s, 95 ℃ for 15 s, 60 ℃ for 30 s, 72 ℃ for 30 s, and for 40 cycles, 55 ℃~95 ℃, +0.5 ℃/cycle, 5 s, and stored at 4 ℃. The reaction system was carried out according to the instructions of SYBR Green Realtime PCR Master mix (TOYOBO, OSAKA, JAPAN), and the results were determined by relative quantity $2^{-\Delta\Delta CT}$ method, and the variance analysis was performed on SAS local software at significant level α=0.05.

Table 3 Primer sequence used for qRT-PCR

Gene ID	Sense primer	Antisense primer
CmCML4	GACCCTAACCAGATTCCCACC	TTCACATTTCCGTCCCCAT
CmCML7	TTTCTTACTTACCCTTGGCTCG	AACATCCTCTTCAAACTCTTCGTC
CmCML12	ACTCACAACCCCAAACGCAATA	CTGAAACCTTTCGGCATAATCG
CmCML15	CCTTGCCTTCCATTTCCTTC	ATCCATCGCCGTCGTCATC
CmCML22	GCTCTGGGGCTTCTTGGGTT	GTGTCATTTAGCGACTGGTGC
CmCML29	AAACCGATGCCGAAGAAGA	GTTGGGCTAAAAGGGGAAAA
CmCML35	TGACTTCCAACAACCACATCCC	GTCCCCTTCCCTCAACATTC
CmCML45	ACCCTTTTGCTTCACTCTTGC	AATAAACCCACTCCCATCCTG

Authors' Contributions

LL and WXZ were the executors and designers of this experimental study; LL, SXY and SL completed writing the first draft of the manuscript; GP and LY directed the writing of the manuscript; WXZ, the conceiver and principal of this study, directed the data analysis and revision of the manuscript. All authors read and approved the final manuscript.

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