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A N I N T E R N A T I O N A L J O U R N A L

Genome-wide Identification, Classification and Expression Analysis of the *Mildew Resistance Locus O* (*MLO*) Gene Family in Sweet Orange (*Citrus sinensis*)

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ABSTRACT

Mildew resistance Locus O (MLO), a gene family specific to plants, plays significant roles in the resistance to powdery mildew (PM) and response to a variety of abiotic stresses, plant growth and development. Despite their importance as barley, rice, wheat, few studies are reported in dicots except Arabidopsis; no global analysis has been performed in the burgeoning model fruit plant sweet orange (Citrus sinensis). The recent release of the genome sequences of C. sinensis provides an opportunity to conduct a comprehensive overview the evolution and features of the MLO gene family in sweet orange. In this study, amount to 14 members of the Citrus sinensis MLO gene (CisMLO) family according to their gene structures, conserved motifs, and similitude among their presumptive Arabidopsis and rice orthologs were identified in silico. Based on these analyses, all CisMLOs were grouped into six clades and expanded partly due to one tandem duplication and two segmental duplication events. Survey of their chromosomal distributions uncovered that 14 CisMLOs are localized across 6 chromosomes. Multiple-sequence alignments showed that 11 of them shared seven highly conserved transmembrane domains (TMs), while all of the sweet orange MLO proteins except CisMLO4/14 had a calmodulin-binding domain for MLO function. Expression analysis demonstrated that the MLO gene family has a diverse tissue-specific expression profiles in the sweet orange development and plays potential critical roles in stress responses. These findings will facilitate further studies of evolutionary pattern and biological functions of MLO genes in sweet orange.

Key words: *Citrus sinensis*; *MLO* gene family; powdery mildew; abiotic stress

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INTRODUCTION

Sweet orange is an important fruit tree widely cultivated in China. However, powdery mildew (PM) pathogen *Acrosporium tingitaninum* Carter is a major fungal threat to citrus production in areas of south and southwest China [1]. Agricultural average productivity losses caused by PM have been estimated that up to 10-30% for citrus [2]. Chemical prevention of PM has become the majority of fungicides traditionally utilized in the agricultural environment [3]. Consequently, the development of new citrus cultivars with enhanced genetic resistance to PM would be widely conceived as a valuable tactic to minimize agricultural costs and to deal with public interest in human health and global environment [4].

The plant-specific *Mildew resistance Locus O* (*MLO*) gene family comprising several transmembrane domains (TMs), topologically redolent of G-protein coupled receptors in metazoan [5]. Particular homologs of the *MLO* family serve as perceptivity roles towards the PM fungi. Furthermore, their deactivation, through silencing or loss-of-function mutations, has been associated with a specific form of PM resistance, interpreted as *mlo* resistance [6]. This is connected with the increasement of exocytosis defense pathways at plant-pathogen interaction sites, which are perceived to result in the prevention of fungal penetration into host plants [7]. Originally discovered in *Hordeum vulgare* (barley), *mlo* immunity has also been found to exist in several plant species later, particularly *Arabidopsis* [8], tomato [9, 10]), pea [11], pepper [12] and bread wheat [13]. This consequently led to the breeding protocol founded upon the methodical inactivation of *MLO* susceptibility genes across planted varieties infected by the PM fungi [6, 9, 14]. Proof of formalization of this approach has been recently conducted by the experiment, showing the introduction of PM resistance in *Triticum aestivum* (bread wheat) following targeted mutagenesis of 3 *MLO* homoeoalleles by the CRISPR-Cas9 and TALEN system [13]. Similarly, knockdown through RNA interference of *MdMLO19* and *VvMLO7* in combination with *VvMLO6* and *VvMLO11* decreased PM severity in apple [15] and grapevine [16]. Rather than the majority of hereditary sources of PM resistance, experimental results clearly show that *mlo* resistance is not limited to special fungal strains and is supremely durable. For instance, loss-of-function mutations of barley *MLO* endow resistance to PM fungus *Blumeria graminis* f. sp. *hordei*, and was successfully applied in barley breeding nearly 40 years ago [17]. Similarly, knockdown through RNA interference of *PhMLO1* in petunia increases resistance to PM [18]. Furthermore, PM resistance of pea *er1* initialing from the loss of function of *PsMLO1* was first reported since 1948 and is the sole resistance source internationally used for breeding objectives [11, 19].

Interestingly, when displayed in the evolutionary trees of MLO protein family, all MLO isoforms from dicots experimentally shown to be necessary for PM immunity group in the clade V in the scientific documents [8-10, 20-22]. Furthermore, clade IV harbors all featured monocot PM perceptivity proteins [23]. These show that phylogenetic studies on MLO proteins may predict factor candidates for PM perceptivity. Previous research has also identified a calmodulin-binding domain (CaMBD) and two conserved regions (I and II) characteristic of barley MLO orthologs that modulate PM infection in the C-terminus of MLO proteins [24, 25]. Peptide domain I is situated at about 15 to 20 residues downstream of the CaMBD and is featured by conserved residues of threonine and serine. Peptide domain II is situated at the distal end of the C-terminus and harbors the consensus tetra-peptide sequence D/E-F-S/T-F.

Apart from the distinguished function of MLOs in response to pathogen defense, MLO proteins are involved in a variety of processes of plant development. Such as, *Arabidopsis* AtMLO4/11 are together functioned in root architecture regulation [26], and AtMLO7 takes part in pollen tube reception at the female gametophyte in *Arabidopsis* [27]. In rice, OsMLO12 is involved in pollen hydration through interaction with calmodulin in the cytosol [28]. However, *SlMLO1* mutant showed no abnormal phenotype in tomato [10]. Thus, further studies are needed to characterize the functions of the MLO homologs.

MLO genes are only identified in plants and moss. Currently, a list of MLO isoforms variable between 14 and 21 has been identified in most diploid species with completed genome sequences, such as, *Arabidopsis* (15 genes) ([5, 26]), tomato (17 genes) [29] grapevine (17 genes) ([21, 22]), peach (16 genes) [30], strawberry (17 genes) [31], apple (21 genes) [31] and cucumber (14 genes) [32, 33]. However, the largest MLO protein members (39 genes) were found so far in soybean [34, 35] recently. To date, *MLO* like gene in sweet orange has not been reported, a comprehensive analysis of the whole *MLO* family in this species is necessary.

The newly available sweet orange genome from the *Citrus sinensis* Annotation Project (CAP; http://citrus.hzau.edu.cn/orange/) [36] was exploited to provide a broad overview of the member number, structural characteristics and phylogenetic relationship of the *MLO* gene family. In this study, we attempt to identify the members of the *MLO*-like gene family in the *C. sinensis* genome. Moreover, structural characteristics of exon/intron, phylogenetic relationship, and distribution on chromosomes of these genes were characterized and analyzed, and these have been used to perform a comprehensive study to assess the possible functions of *MLO* genes in growth and development process of sweet orange. The results provide a solid foundation for functional genomic characterizations of the *MLO* gene family of sweet orange in the future.

MATERIAL AND METHODS

Retrieve *MLO* genes in sweet orange

The sequences of 15 *Arabidopsis* [26, 37] and 12 rice [38] MLO proteins were extracted from the TIGR database (http://rice.plantbiology.msu.edu) and the TAIR database (https://www.arabidopsis.org/browse/genefamily/mlo.jsp). To identify all the *MLO* genes in sweet orange, BLASTP searches were conducted in the sweet orange genome database (http://citrus.hzau.edu.cn/cgi-bin/orange/blast) [36], and NCBI database (http://www.ncbi.nlm.nih.gov/) with the *Arabidopsis* and rice MLO proteins as queries, using default parameters. We selected sequence as a candidate CisMLO protein if it satisfied with $E<10^{-10}$. And then, the full-length cDNA sequences of *CisMLO* genes were extracted from the sweet orange genome database. Due to the variation in *MLO* sequences, we additionally retrieved the sweet orange sequencing database to get more detailed information of the potential *CisMLO* genes using the keyword "MLO". Finally, all putative non-overlapping MLO protein sequences were submitted to the SMART (http://smart.embl-heidelberg.de/) and Pfam database (http://pfam.sanger.ac.uk/) to verify the existence of the MLO domain (PF03094), respectively. Information about full-length coding and the amino acid sequence was also received by BLAST program for each *MLO* gene from CAP. NCBI ORF (open reading frame) finder (https://www.ncbi.nlm.nih.gov/orffinder) was applied to uncover the presumptive ORFs. The exon/intron gene organization of the 14 *CisMLO* genes were determined by aligning the cDNA sequences against their corresponding gDNA sequences based on Gene Structure Display Server (GSDS) 2.0 (http://gsds.cbi.pku.edu.cn/) [39].

Sequence alignment and phylogenetic analysis of the *MLO* genes

Selected sequences were aligned using program ClustalX 2.1 [40]. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis version (MEGA) Version 6.06 software [41] by employing the neighbor-joining (NJ) method with 1000 replicates for statistical reliability. All the other parameters were taken to the default settings.

TMs, chromosomal locations and gene duplication analysis of *CisMLO* genes

The presence and number of TMs were predicted using the online software TOPCONS (http://topcons.cbr.su.se) [42]. Molecular weight and the isoelectric point of the deduced polypeptides were calculated using ExPasy (http://web.expasy.org/compute_pi). The subcellular location was predicted with the WoLF PSORT programs (http://www.genscript.com/wolf-psort.html) and CELLO v.2.5 (http://cello.life.nctu.edu.tw) [43]. To identify the locations of *MLO* genes on sweet orange chromosomes, the positions of these genes were retrieved from the sweet orange genome data downloaded from the CAP database (http://citrus.hzau.edu.cn/orange/) [44]. The 14 sweet orange *MLO* genes were mapped to the chromosomes using MapChart [45]. Moreover, the gene duplication events of *CisMLOs* were also defined according to the criteria: (i) the alignment was covered >70% of the longer gene; (ii) the aligned region had an identity >70%; (iii) only one duplication event was counted for tightly linked genes. A block of duplications was defined if more than one gene was involved in the duplication [46]. The data of non-synonymous substitutions per non-synonymous site (*K*a) and synonymous substitution per synonymous site (*K*s) of duplicated genes were analyzed using DnaSP 5.10 [47]. The ratio of *K*a/*K*s between paralogs

was calculated to define the pattern of selection. To trace the dates of duplication events, the *K*s value was translated into duplication time in million years ago using a synonymous mutation rate of λ substitutions per synonymous site per year. The duplication time (*T*) were calculated by the equation $T = Ks/2\lambda \times 10^{-6}$, for sweet orange, the $\lambda = 2.53 \times 10^{-9}$ substitutions/synonymous site/year [48].

Protein motifs and structure analysis

Analysis for conserved functional motifs in the MLO proteins was performed using MEME Suite Version 4.10.2 (http://meme-suite.org/tools/meme) [49]. The settings were: any number of repetitions of a single motif, the optimum width of a motif with 6 to 25 amino acids, and the maximum number of motifs up to 35 amino acids. The program of MAST was used to retrieve conserved motifs in protein databases. The details of amino acid sequence logo of 15 most frequent motifs were illustrated in Supplementary Fig. S1.

Identification and Analysis of the Promoter Regions of *CisMLOs*

To explore probable stress-related *cis*-elements in promoter sequences of the 14 *CisMLO* genes, 1500-base-pair of gDNA sequence upstream of the transcriptional start site was extracted from the *C. sinensis* genome database (http://citrus.hzau.edu.cn/cgi-bin/orange/) [36]. The PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [50, 51] program was applied to analyze the *CisMLO* gene promoter sequence and identify the *cis*-elements. Regulatory elements existed in the database and proven to participate in the regulation of gene expression during the course of pathogen infection and under abiotic stress conditions were chosen for further investigation.

Expression Analysis of sweet orange *MLO* Genes

To investigate the expression profiles of all *CisMLOs* in four distinct tissues, i.e., callus, flower, leaf and fruit, the normalized RNA-Seq RPKM (reads per kilobase exon model per million mapped reads) values of all *CisMLOs* were downloaded from the online database of the CAP [44], and imagined by the heat maps with transformed $\log_2$ values using MultiExperiment Viewer (MeV) Version 4.9.0 software (http://www.tm4.org/mev.html) [52].

RESULTS

Identification, chromosomal locations and evolutionary analysis of the *CisMLO* genes

To verify all potential genes of the MLO family in the sweet orange genome, we used BLASTP and the keywords "MLO" to search the CAP (http://citrus.hzau.edu.cn/orange/) [44] with the 15 *A. thaliana* and 12 rice MLO proteins as a retrieve. Adopt this method, 14 potential *MLO* genes, named *CisMLO1* to *CisMLO14*, were identified. The information of MLO family genes in sweet orange, including locus name, gene localization, amino acid length, *Arabidopsis* and rice orthologs, are listed in Table 1. The CisMLO proteins are rich in leucine, which consists of 9.3-13.5% of the total number of amino acid residues, a similar result was reported in 2012 [35]. It has been documented that proteins rich in leucine residues responsible for the mechanisms of defense response [53, 54].

The genomic locations of *CisMLOs* were illuminated on chromosomes of sweet orange available at CAP [44]. *CisMLOs* were widely scattered among all the chromosomes except chromosomes 4, 6, and 9. The spread of the genes was irregular: chromosome 3 harbors four *MLO*s, chromosome 1 has three *MLO*s, two genes on chromosome 5, 7 and 8, and one gene is localized to chromosome 2. Interestingly, most *MLO*-like genes are distributed among the genome, but in four cases two genes are located close together, i.e., the pairs *CisMLO2*/*3*, *CisMLO7*/*8*, *CisMLO9*/*10*, and *CisMLO13*/*14* (Fig. 1). With the exception of *CisMLO9*/*10*, the genes in other three cases of tight linkage, grouped in different clades, were not tandem repeats (Fig. 1).

To identify the evolutionary relationships among candidate CisMLO proteins and cluster them within the established clades, multiple sequence alignments and phylogenetic analysis of these CisMLOs as well as MLOs of *Arabidopsis* and rice were further conducted (Fig. 2 A; Supplementary Table S1). 14 CisMLOs were clustered into six clades in light of the classification standards of MLO proteins in *Arabidopsis* [5]. In detail, CisMLO1, 2, and 6, which were clustered with three AtMLOs and two OsMLOs, belonged to clade I (Fig. 2 A). Clade II, the largest grouping of sweet orange MLO proteins, was comprised of CisMLO5, 9, 10, and 11, clustered with three *Arabidopsis* and six rice MLO-like proteins (Fig. 2 B). The

clade III included CisMLO3 and 8. CisMLO7 belonged to clade IV, which was formerly reported to contain only monocot homologs acting as PM susceptibility factors [5], similar to that observed in the dicot grapevine (VvMLO14) [21] and soybean [34]. CisMLO4, 12, and 13 fell into clade V along with *Arabidopsis* MLO homologs formerly linked to PM responses. CisMLO14 and AtMLO3 belonged to clade VI [21] (Fig. 2 A). By phylogenetic analysis, the *MLO* genes from sweet orange, *Arabidopsis thaliana,* and rice divided into six groups, indicating that these genes may have evolved either before (clades I–IV) or after (clades V and VI) the divergence of monocots-dicots.

Analysis for the TMs and conserved motif structures in the *CisMLO* genes

In addition, the TMs of all *CisMLOs* were investigated. Supplementary Fig. S2 showed the alignment of sweet orange MLO proteins. Results demonstrated that all MLO proteins except CisMLO6/10/11 had seven TMs and were highly conservative, which are considered to be remarkable features of *MLO* gene family [5]. Previous research has also identified a calmodulin-binding domain (CaMBD) and two conserved regions (I and II) that modulate PM infection in the C-terminus of MLO proteins [24, 25]. Peptide domain I is situated at about 15 to 20 residues downstream of the CaMBD and is featured by conserved residues of threonine and serine. Peptide domain II is situated at the distal end of the C-terminus and harbors the consensus tetra-peptide sequence D/E-F-S/T-F. As regard to this tetra-peptide sequence, CisMLO3/7 contain D-F-S-F, which is same as HvMLO and AtMLO2/6, while CisMLO12/13 contain different sequences (E-F-S-F). All clade V of sweet orange MLO proteins except CisMLO4 had these two peptide domains, which had potential conservative functions (Supplementary Fig. S2).

Moreover, the exon/intron structures of all *CisMLOs* and the conserved motifs of their coding proteins were investigated. Various gene structures were analyzed by comparing the predicted CDS with the genomic sequence of *CisMLO* genes in the CAP database (Fig. 3 B). Conserved motifs of sweet orange MLO proteins were further analyzed with MEME version 4.10.2 online tools (http://meme-suite.org/tools/meme) [49]. The results showed 15 conserved motifs in sweet orange MLO proteins, and their lengths were 15 (motif 14), 21 (4, 5, 12, 13 and 15) and 25 (1-3, 6-11) amino acids (Fig. 3 C). The distributions of these conserved motifs in the CisMLOs were further analyzed. As demonstrated in Supplementary Fig. S2, five CisMLOs, i.e., CisMLO3/7/8/12/13, contained all 15 conserved motifs. CisMLO6/9/10/11/14 were the genes with the most deletions, containing only 12 conserved motifs. 8 out of 15 conserved motifs (motifs 1, 2, 4-6, 8, 13 and 14) were situated within the MLO domain (Fig. 3 C) for all members of the sweet orange MLO family.

Duplications of *CisMLO* genes

Next, to estimate the dates of the duplication events, we used the DnaSP 5.10 [47] to calculate *K*s and *K*a. The estimated dates of duplication events were measured using *K*s. The results of analysis between tandem and segmental duplication events are showed in Table 2. The segmental duplications of *CisMLO3*/*8* and *CisMLO10*/*11* (Fig. 1) in sweet orange originated from 142.29 million years ago (Mya) (*K*s = 0.72) to 158.10 Mya (*K*s = 0.80), with the mean being 150.20 Mya (*K*s = 0.76). The *K*s of tandem duplication of the *CisMLO9*/*10* (Fig. 1) was 0.55, dating the tandem duplication event at 108.70 Mya. The above divergence time estimations indicated that all the duplications of MLO genes in sweet orange occurred after the monocots/dicots split (approximately 200 Mya) [55]. The *K*a/*K*s ratio of one tandem duplication pair and two segmental duplication pairs were all more than 0.3, showing that palpable functional tributary might have happened after duplication blocks. All these data indicate that the sweet orange *MLO* family tandem/segmental expansion can be traced back to recent duplication blocks. Furthermore, these *MLO* genes maintained their function after duplication. It should arouse our interest to discover whether the few sequence differences result in a functional variety, or these genes are originally redundant in function.

***In silico* stress-responsive *cis*-element analysis of *CisMLO* genes**

To discover potential stress-related *cis*-elements in the promoter regions of the *CisMLO* genes, 1.5 kb upstream promoter sequences of the *CisMLO* genes were submitted to program PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Various biotic stress-responsive regulatory elements, for example, pathogen-related *cis*-elements TC/AT-rich repeat and W box/Box-W1 elements

[56] (Fig. 4; Supplementary Table S2), were observed. Many abiotic stress mediated *cis*-elements were also identified, containing wound response element (WUN motif), drought stress-related MYB binding site (MBS), heat shock element (HSE), and low temperature-responsive element (LTR) (Fig. 4). Moreover, many elements probably involved in reaction to hormones. For instance, auxin (TGA-element and AuxRE), abscisic acid (ABA) (ABRE), Ethylene (ET) (ERE), methyl jasmonate (MeJA) (CGTCA and TGACG motifs), gibberellin (GARE motif and TATC box) and salicylic acid (SA) (TCA-element), were detected as well (Fig. 4). All *CisMLOs* had at least one stress or hormone-responsive *cis*-element, in addition, 3 of the 14 *CisMLO* genes (*CisMLO4*/*5*/*11*) contained four different hormone-responsive elements each. Furthermore, 11 genes harbored pathogen elicitor response elements, and among the different motifs, the W box/Box-W1 motif was found in 6 genes; AT-rich repeat motif was detected in 3 genes; however, TC-rich repeat motif was present in 11 genes. Moreover, *CisMLO1*/*6*/*8*/*11*/*13* had four different abiotic stress response elements each. Finally, 10 *CisMLO* genes contained more than 10 biotic and/or abiotic stress- or hormonal-response *cis*-elements, indicating these *CisMLOs* might variously participate in these pathways.

Analysis of the expression of *CisMLO* genes based on the sweet orange EST database

To predict the function of *CisMLOs*, RNA-seq data were downloaded from *C. sinensis* genome database [36, 44] and the expression patterns of *CisMLOs* in various tissues (callus, leaf, flower, and fruit) were analyzed and demonstrated in Fig. 5. A hierarchical classification was conducted according to the expression data of each *CisMLOs* in four different tissues. Based on the hierarchical classification results, we clustered genes of each clade to three assigned expression pattern groups (Fig. 5 A-C). The group A is the smallest with only three members, *CisMLO10/12/13*, showed prevalent higher expression level in callus as compared to other tissues. Whereas the members of group B, *CisMLO4/5/7/8/9*, showed no significant difference expression between tissues. The group C containing genes, *CisMLO1/2/3/6/11/14*, showed relatively higher expression level in flower, leaf, fruit tissues than in callus. It should be noted that *CisMLO3* and *CisMLO11* were highly expressed in all samples analyzed, with the highest expression in flower (Fig. 5). Most importantly, the different expression patterns of *CisMLOs* among the four tissues showed that these genes might participate in a broad range of biological processes in citrus, and the interesting challenge for the future is to determine the specific functions in conjunction with individual *CisMLO* genes in sweet orange.

DISCUSSION

We retrieved *MLO*-like genes in the sweet orange genome via an *in silico* approach and identified 14 potential *CisMLOs*, unevenly situated across 6 of the 9 chromosomes (Table 1; Fig. 1). By phylogenetic analysis, the *MLO* genes from sweet orange and other plant species were divided into six clades, some of which contained members from *A. thaliana* and rice (Fig. 2), indicating that these genes may have evolved after the divergence of monocots/dicots. Most *CisMLO* homologs seemed to be widely scattered within the sweet orange genomes (Fig. 1), illustrating segmental duplication as the predominant evolutionary pattern for the citrus *MLO-like* gene family. However, we also found a case of adjacent homologs (*CisMLO9/10*) (Fig. 1), which are consistent with the data of tandem duplication events. The results above indicate that the sweet orange MLO family tandem or segmental expansion can be traced back to recent duplication blocks. A similar conclusion was made and supported by Jiwan et al. [30] and Acevedo-Garcia et al. [20], respectively.

A massive amount of former researches demonstrated that phylogenetic analysis enables functional prediction of many novel genes because orthologs usually keep the same biological role during the course of evolution. For example, phylogenetic analyses of the ARF and NAC families of citrus with their corresponding orthologs with clear functions in *Arabidopsis* resulted in an approximately complete match between sequence homology and expression profiles or biological roles [57, 58]. And then, a phylogenetic analysis associating sweet orange, *Arabidopsis* and rice MLO protein family would not only help illuminate the evolutionary relationships of MLO proteins but would also grant the insight to the potential biological roles of the CisMLO proteins derived from the clustering clades currently documented in *Arabidopsis* and rice (Table 1; Fig. 2).

In the 14 sweet orange MLO proteins, CisMLO1/2/6 assembled with *Arabidopsis* AtMLO4/11/14 into clade I (Fig. 2), AtMLO4/11 referred to the regulation of root thigmomorphogenesis in *Arabidopsis*, however, the closely paralogous *AtMLO14* gene does not participate in this plant growth process, since no obvious variant phenotype was displayed in the *AtMLO14* mutant [26, 59]. CisMLO3/8 were assembled in clade III together with the five *Arabidopsis* proteins AtMLO5/7/8/9/10 representing the functional clade with AtMLO7 protein responsible for similar functions of pollen tube perception of the female germ cell in *Arabidopsis* [27]. Importantly, three homologs, *CisMLO4/12/13*, were found to code for predicted proteins that assembled in clade V (Fig. 3). All dicot MLO homologs in clade V have experimentally proven hitherto to be necessary for PM pathogenesis, which thus provided a critical role for identification the candidate genes in sweet orange with similar functions in PM susceptibility [5]. Consequently, the clustering in clade V is the main selection criterion for *MLO* genes participated in PM pathogenesis, which provides an initial important clue for the reduction in the number of candidates for further functional experiment design. Interestingly, one homolog from sweet orange, CisMLO7, was found to group in clade IV, which includes all monocot MLO proteins modulating PM infection [5] (Fig. 2). Consistent with this finding, VvMLO14 in the dicot species grapevine, also assembles in clade IV [21]. Finally, clade VI, containing AtMLO3 and CisMLO14, which was considered as a single divergent lineage in former studies [21], more detailed functions of AtMLO3 and CisMLO14 will be revealed by the further investigation. These results strongly indicated the existence of sweet orange-specific *MLO* gene that was either lost in *Arabidopsis* and rice or acquired in the sweet orange lineages after an evolutionary splitting event from the most recent common ancestor. These results further indicated that functional prediction based on evolutionary analysis might be convenient for us to efficaciously choose candidate genes, which could then be given priority to the functional investigation in future.

Further study revealed that most of the *CisMLOs* promoter contains a variety of biotic- (W box / Box-W1, TC-rich repeat and AT-rich repeat), abiotic- (HSE, LTR, MBS, WUN motif), and hormonal- (ABRE, CGTCA/TGACG motif, AuxRE, TGA-element, ERE, TCA-element, CGTCA/TGACG motif, TATC box, GARE motif) responsive related *cis*-regulatory elements (Supplementary Table S2). Therefore, *CisMLOs* may play roles as an important cross-talk node in both stress and hormone signaling processes. The *MLO* genes are reported to take part in programmed cell death, and in the PM pathogen responses [12, 24], the biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis*, as well as the hemibiotrophic bacterial pathogens, for example, *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* [60, 61]; wounding [21, 37, 62]; phytohormone [24, 26, 63] and abiotic stresses [63, 64]. As a rule, however, the paralogs in MLO family, as illuminated by their clustering pattern (Fig. 3 A), did not show similarities in their *cis*-elements (Fig. 4). For instance, the paralogous members of *CisMLO3*, *7* and *10* shared no pathogen-responsive elements (Fig. 4). The paralogs in *CisMLO* family, therefore, harbor the MLO domain, but their biological roles seem to have expanded during the course of evolution. These findings also suggest that *CisMLO4*/*12*/*13* likely play roles in PM responses similar to that of their counterparts in other plant species, additional experiments are necessary to explore the accurate roles of these genes, harboring many pathogen-responsive elements, in response to biotic stress stimuli, especially in the emerging commercially important citrus.

CONCLUSION

Here, systemic investigations including chromosomal distribution, phylogenetic relationship, domain composition, gene structure, evolutionary features, expression patterns, and the *cis*-elements of all members of the sweet orange *MLO* family were conducted. Homology search using *AtMLOs* as query sequences verified a total of 14 *CisMLO* genes clustered into 6 clades and located unevenly across 6 of the 9 *C. sinensis* chromosomes. Comparative studies revealed that 3 paralogous pairs were amplified by tandem or segmental duplication types. In addition, the gene specific promoter *cis*-elements analysis indicated that these proteins might take part in various functions, such as response to biotic/abiotic stresses and developmental processes, several of them are probably related to the responses to fungal pathogens. A further in-depth discussion of the expression patterns based on the sweet orange EST database provided insights into the probable functional divergence among members of the *MLO* family.

Although the distinct roles of sweet orange *MLO* genes remain largely unclear, and many novel researches are necessary to elucidate their exact functions, the findings from this study may contribute valuable information for future breeding PM resistance in citrus, and isolation and functional validation of the individual biological processes-linked *CisMLO* genes.

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