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Genome-wide analysis of the mildew resistance locus o (*MLO*) gene family in tomato (*Solanum lycopersicum* L.)

Yongbing Chen*, Yan Wang*, Haili Zhang

Wenzhou Vocational College of Science and Technology, Wenzhou, Zhejiang, 325006, People's Republic of China

***Corresponding author: cyb@china.com; wangyan8217@126.com**

Abstract

Mildew resistance locus o (*MLO*), a plant-specific gene family, plays an important role in plant resistance to powdery mildew (PM). In tomato, a *MLO* gene associated with PM has been identified. The recently available tomato genome provides an opportunity to conduct a comprehensive overview of the *MLO* gene family. In the present study, identification and analysis of the *MLO* gene family was conducted using bioinformatic methods. Results demonstrated that there were at least 17 *MLO* gene members with encoded protein lengths between 270 and 591 amino acids. Multiple-sequence alignments showed that they had seven highly conserved transmembrane domains (TMs), a calmodulin-binding domain, peptide domains I and II, and 30 important amino acid residues for *MLO* function. Chromosome location results revealed that *MLO* genes were unevenly distributed in each chromosome. By phylogenetic analysis, the *MLO* genes from tomato and other plant species were divided into seven groups, some of which contained members from *Arabidopsis thaliana*, rice, sorghum, grape, poplar, and tomato, indicating that these genes may have evolved prior to the divergence of monocots and dicots. These findings will facilitate the functional characterization and evolutionary relationship of the *MLO* genes in tomato.

Keywords: Tomato, *MLO*, bioinformatics, phylogenetic relationship.
Abbreviations: MLO_Mildew resistance locus o; TM_transmembrane regions; CaMBD_calmodulin-binding domain; MEME_multiple em for motif elicitation.

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Introduction

Mildew resistance locus o (*MLO*) is an important disease resistance gene in plants, first discovered in barley in 1997 (Büschges et al., 1997). The *MLO* gene encodes a plant specific protein with seven transmembrane domains and a special C-terminal calmodulin-binding domain structure (Devoto et al., 1999). Recessive mutation of *MLO* in barley confers permanent broad-spectrum resistance to all known isolates of the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Büschges et al., 1997). A disease-resistant *MLO* mutant can be obtained by inducing susceptible varieties of wild type *MLO* by mutagenesis. The mutant *MLO* regulates plant disease resistance and death of leaf cells (Büschges et al., 1997, Piffanelli et al., 2002). Many studies have shown that the *MLO* genes belong to a gene family that widely exists in plants. Currently, a total of 15, 9, 12, and 17 *MLO* genes have been found in *Arabidopsis*, maize, rice, and grapes, respectively (Devoto et al., 2003, Liu et al., 2008, Feechan et al., 2009). Furthermore, *MLO* genes have also been cloned in *Arabidopsis*, tomato and pea (Devoto et al., 2003, Bai et al., 2008, Pavan et al., 2011). The *SlMLO01* mutant *MLO* gene was cloned from tomato and exhibited a loss-of-function in resistance to powdery mildew (Bai et al., 2008). In *Arabidopsis*, the *AtMLO2* gene showed significantly reduced susceptibility to *Golovinomyces orontii*. Two other closely related *Arabidopsis* genes, *AtMLO6* and *AtMLO12*, were mutated along with *AtMLO2*, to achieve complete PM resistance (Consonni et al., 2006). The *MLO* genes have also been reported in grapes and were induced by *Erysiphe necator* (Feechan et al., 2009). Thus, some members of MLO gene family may play a role in modulating host response to phytopathogenic PM fungus in dicots.

Apart from the reports on *Arabidopsis*, grape and rice *MLOs* on the whole genome, few studies have been conducted for other *MLO* genes from other plant species. Thus, a comprehensive analysis of the whole *MLO* family in plants is necessary. The available whole tomato genome provides a good platform for understanding the member number, structural characteristics and phylogenetic relationship of the *MLO* gene family (Tomato Genome Consortium 2012). In the present study, the *MLO* gene family was identified in tomato. Moreover, structural characteristics of intron/exon, phylogenetic relationship, and distribution on chromosomes of these genes were analyzed.

Results and Discussion

Identification of the tomato whole genome MLO gene family

In this study, a total of 17 *MLO* proteins (*SlMLO01-SlMLO17*) were retrieved from the tomato genome. Analysis upstream and downstream of the gene showed that among them, *SlMLO14* (Solyc09g018840) was a truncated gene (276 bp) and was therefore excluded in the following analysis. The

Table 1. Members of the *SlMLO* gene family as predicted in the tomato genome sequence.

Name	Gene	Chromosome location	Coding region length (bp)	Amino acid length (aa)	Molecular weight (kDa)	Isoelectric point
SlMLO01	Solyc01g102520	Ch01:83071860-83075439	1428	475	54.40	8.67
SlMLO02	Solyc02g077570	Ch02:37045094-37049936	1128	375	43.71	8.96
SlMLO03	Solyc02g082430	Ch02:40694608-40700995	2157	553	62.84	9.36
SlMLO04	Solyc02g083720	Ch02:41596474-41602413	1602	533	61.01	7.01
SlMLO05	Solyco3g095650	Ch03:50279919-50288063	1554	517	59.93	9.05
SlMLO06	Solyco4g049090	Ch04:38700445-38705951	1878	513	59.06	9.00
SlMLO07	Solyc06g082820	Ch06:44779673-44784035	1536	511	58.78	7.76
SlMLO08	Solyc06g010010	Ch06:4699552-4706571	1434	477	94.97	9.00
SlMLO09	Solyc06g010030	Ch06:4786764-4792828	1776	591	67.34	9.24
SlMLO10	Solyc07g063260	Ch07:62995345-63002900	1692	563	64.55	9.27
SlMLO11	Solyc08g067760	Ch08:53957062-53962884	1599	329	59.85	9.22
SlMLO12	Solyc08g015870	Ch08:6074040-6078983	1515	504	57.47	9.01
SlMLO13	Solyc09g018830	Ch09:17564555-17568214	813	270	30.45	8.95
SlMLO14	Solyc09g018840	Ch09:17567140-17568410	276	91	10.70	5.28
SlMLO15	Solyc10g044510	Ch10:22128868-22135940	1677	558	63.9	8.71
SlMLO16	Solyc11g069220	Ch11:50939533-50946726	1521	506	58.05	9.01
SlMLO17	Solyc00g007200	Ch00:6816892-6823417	1665	281	62.84	8.72

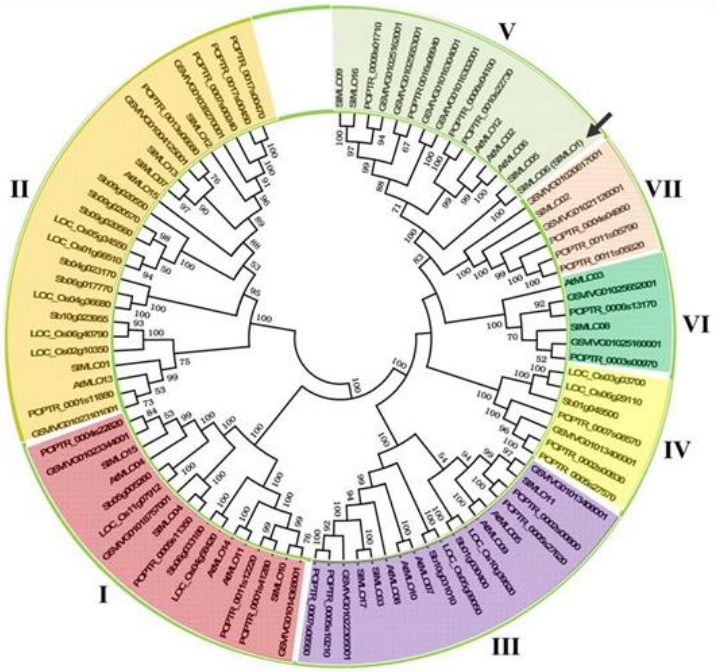

Fig 1. Phylogenetic comparison of MLO genes families from poplar, *Arabidopsis thaliana*, grape, rice, sorghum, and tomato. Arrows represent the MLO genes related to powdery mildew, which were cloned by Bai et al. (2009). Numbers on the branches indicate the percentage of 1000 bootstrap replicates that support the node, with only values > 50% reported.

lengths of the coding regions of the remaining 16 *MLO* genes ranged from 813 bp (Solyc09g018830) to 2157 bp (Solyc02g082430), and the amino acid lengths were between 270 aa (Solyc09g018830) and 591 aa (Solyc06g010030). The molecular weights were between 30.45 KDa (Solyc09g018830) and 94.97 KDa (Solyc06g010010). The isoelectric points of most of the *MLO* proteins were between 7 and 9. Solyc02g082430 had the highest isoelectric point (9.36), while Solyc02g083720 had the lowest isoelectric point (7.01). All *MLO* proteins were alkaline (Table 1).

Phylogenetic relationship of MLO gene family

To analyze the phylogenetic relationship of the tomato *MLO* gene family, *MLO* genes from monocotyledonous rice, sorghum, woody vine grapes, and poplar, and dicotyledonous *Arabidopsis thaliana* (*AtMLO*) and tomato (*SlMLO*) were selected and a phylogenetic tree was constructed (Figure 1) according to the classification method of *Arabidopsis MLO* genes (Devoto et al., 2003). The results illustrated that these *MLO* genes were divided into seven groups (I-VII). Tomato *MLO* genes were distributed in all groups except for IV. In addition to the tomato *MLO* gene *SlMLO6* (i.e., cloned

Table 2. Major MEME motifs in predicted tomato MLO proteins.

Number	Width	Best matching degree
Motif 01	40	VQFLCSYVTLPLYALVTQMGSNMKKAIFDEQVATALRNWH
Motif 02	29	WIVCFFRQFYRSVNKVDYLTLRHGFIMAH
Motif 03	29	FWFNRPRLVLYLIHFVLFQNAFQMAFFAW
Motif 04	40	EALHQLHIFIFVLAVMHVTYCCTTMALGRAKMRQWKCWED
Motif 05	40	HSYFWLPFIPLIIILAVGTKLQHIITQMALEIAERHDVVQ
Motif 06	29	KIKEELMLLGFISLLLTVCQDPISNICI
Motif 07	29	NPNFNFHKYMKRSMEDDFKVVVGISPYLW
Motif 08	40	TPTWAVAMVCTVIVAISIAIERIIHYLGKWLKKKNKKPLY
Motif 09	29	THEYQFSNDPERFRFTRETSFGRRHLNFW
Motif 10	15	WYEYGLKSCFHDNYE

Fig 2. Multiple sequence alignment of *SlMLO* genes with MLO proteins from other plant species constructed using Clustal X software. The positions of the seven TM domains (TM1 to TM7) and the approximate position of CaMBD are indicated by lines under the sequences. Two conserved peptide domains within the highly polymorphic C-termini are highlighted using Roman numerals I and II. Black dots under the aligned sequences indicate the position in barley MLO of the 30 residues conserved in 38 MLO proteins from barley, rice, maize, wheat, *Arabidopsis*, and *Physcomitrella patens*.

SIMLO01), the V group *MLO* genes also included cloned *AtMLO02*, *AtMLO06* and *AtMLO12* in *Arabidopsis*, which are a specific group with powdery mildew resistance (Bai et al., 2008, Consonni et al., 2006). The V class genes also contained three tomato *MLO* genes (*SIMLO05*, *SIMLO09* and *SIMLO16*), four poplar *MLO* genes (POPTR_0009s01710, POPTR_0016s09940, POPTR_0008s04100 and POPTR_0010s22730) and four grape *MLO* genes (GSVIVG01025162001, GSVIVG01025653001, GSVIVG01016304001 and GSVIVG01016302001). Given that the putative functions of these *MLO*s in group V were derived from *Arabidopsis*, tomato, and pea, we inferred that this clade was significant for cucumber because these genes are required for PM susceptibility (Consonni et al., 2006; Bai et al., 2008; Pavan et al., 2011). This clade may be a dicot-specific group. Phylogenetic trees not only analyze phylogenetic relationships of gene families, but can also identify homologous genes. There were eight pairs of orthologous genes and nineteen pairs of paralogous genes in the phylogenetic tree. The eight orthologous gene pairs included LOC_Os04g36680/Sb06g01770, LOC_Os06g40790/Sb10g023955, LOC_Os11g07912/Sb05g005260, LOC_Os04g58420/Sb06g033180, LOC_Os05g09050/Sb10g031010, LOC_Os10g39520/Sb01g30400, GSVIVG01026552001/POPTR_0006s13170, and GSVIVG01013408001/-SlMLO11.

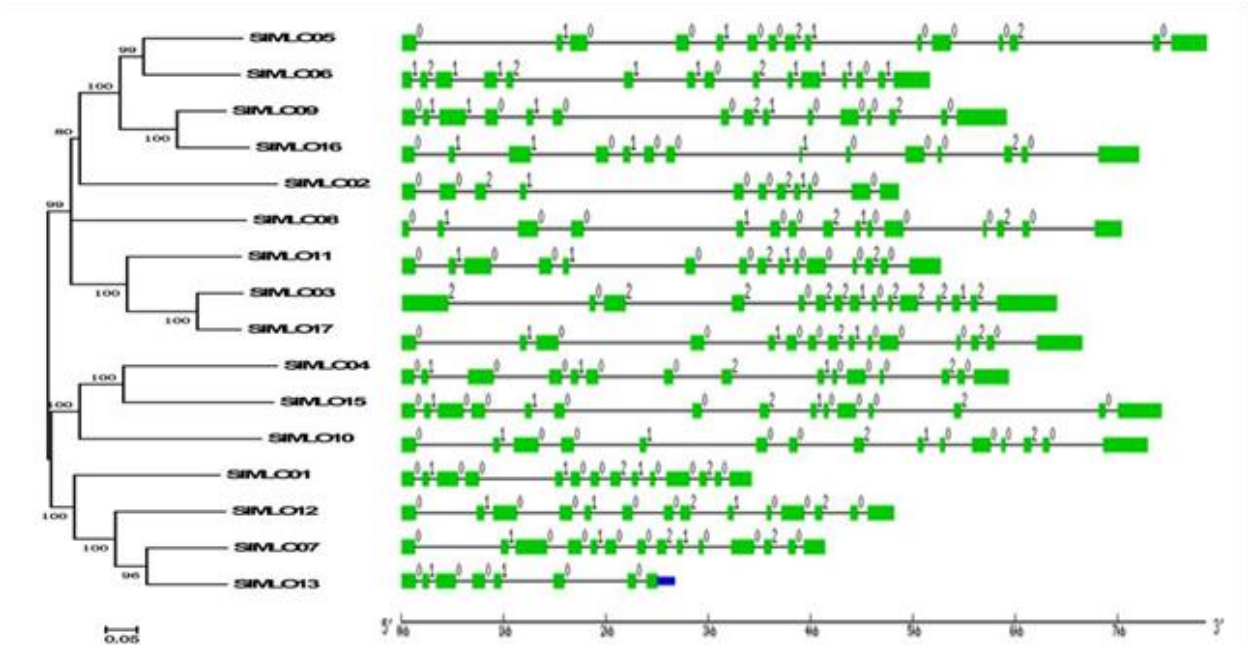

Fig 3. Phylogenetic analysis and intron/exon configurations of *MLO* genes in tomato. A phylogenetic tree of *MLO* genes was constructed using MEGA 5.0. Introns and exons are drawn to scale with the full encoding regions of their respective genes. Boxes indicate the exon, and lines indicate the intron. 0 = intron phrase 0; 1 = intron phrase 1; 2 = intron phrase 2.

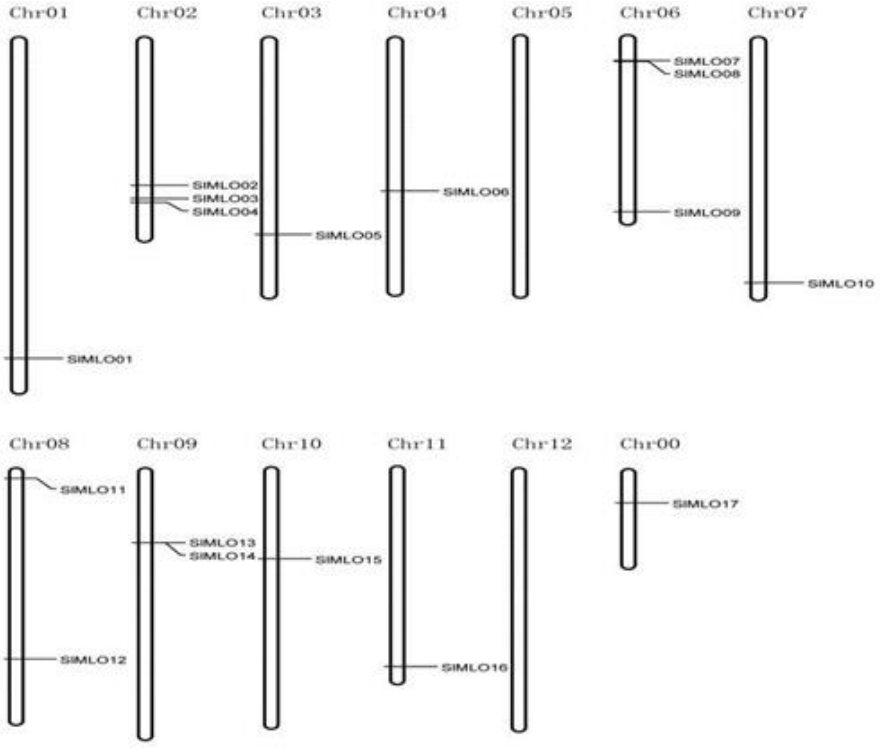

Fig 4. Position of MLO genes on tomato chromosomes. Chromosome numbers are indicated at the top of the chromosome. Chr00 represents sequence scaffolds.

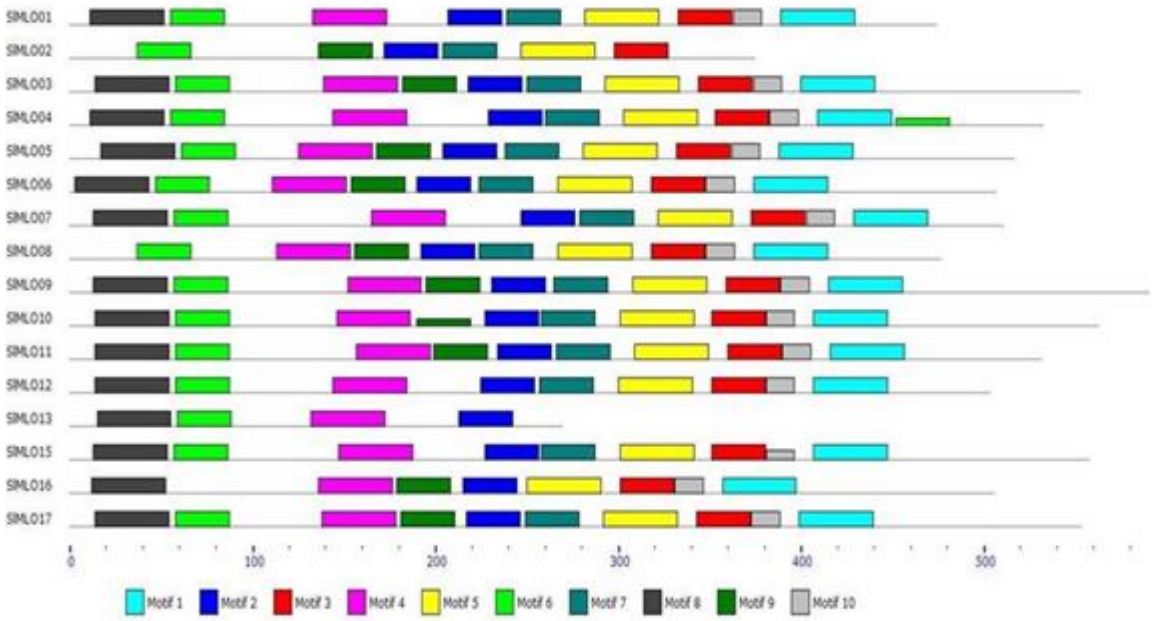

Fig 5. Distribution of conserved motifs in the *MLO* gene family members. All motifs were identified by MEME using the complete amino acid sequences of 17 *SlMLO* genes. Motif sizes are indicated at the bottom of the figure. Different motifs are indicated by different colors numbered 1-10.

The nineteen paralogous gene pairs were: POPTR_0005s-10210/POPTR_0007s08560, POPTR_0001s41280/ POPTR_0011s12220, POPTR_0017s00450/ POPTR_0017s00470, POPTR_0002s00800/ POPTR_0005s27620, POPTR_0002s83001/ POPTR_0005s27570, POPTR_0011s05790/ POPTR_0011s05820, POPTR_0008s04100/ POPTR_0010s22730, LOC_Os01g66510/ LOC_Os05g34550, LOC_Os03g03700/LOC_Os06g29110,Sb09g020560/Sb09g020570, GSVIVG01016304001/GSVIVG01016302001, SlMLO07/ SlMLO13, SlMLO3/ SlMLO17, SlMLO05/SlMLO06, SlMLO09/ SlMLO16, AtMLO11/AtMLO14, AtMLO07/ AtMLO10, AtMLO05/AtMLO09, and AtMLO02/AtMLO06.

Multiple sequence alignments of tomato MLO proteins and analysis of exon/intron structure

To analyze the sequence characteristics of the candidate tomato powdery mildew resistance genes, multiple sequence alignments of these candidate proteins as well as the amino acid sequences of powdery mildew resistance genes in *Arabidopsis* and pea were conducted (Figure 2). Results demonstrated that all *MLO* gene family proteins had seven transmembrane regions (TM1-7) and were highly conservative, which are considered to be remarkable features of *MLO* gene family (Devoto et al., 2003) 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 (Kim et al., 2002a, b, Panstruga, 2005). Peptide domain I is located approximately 15–20 residues downstream of the CaMBD and is characterized by conserved serine and threonine residues. Peptide domain II is located at the distal end of the C-terminus and contains the consensus sequence D/E-F-S/T-F. All V group *MLO* genes had these peptide domains (I and II), which had potential conservative functions. Except for *SlMLO16*, the other *MLO* genes were highly conserved in the seven transmembrane regions. Both TM2 and TM4 in *SlMLO16* had deletions, and *SlMLO05* and *SlMLO06* had partial sequence deletions. Multiple sequence alignment of these *MLO* proteins revealed the presence of 30 amino acid residues, which have been previously identified as invariable in 38 *MLO* genes from various species (Elliott et al., 2005). To further reveal the amino acid sequences of *MLO* proteins, a clustering tree of tomato *MLO* proteins was constructed using MEGA5.0. These gene structure diagrams (Figure 3) were drawn by the online tool GSDS. Results showed that the number of tomato *MLO* gene introns ranged from seven to fourteen; *SlMLO13* had seven introns and *SlMLO02* had ten introns. In addition, four *MLO* genes (*SlMLO16*, *SlMLO01*, *SlMLO12*, and *SlMLO07*) contained thirteen introns. The remaining *MLO* genes contained fourteen introns, accounting for 62.5% (10/16) of all *MLO* genes.

Chromosomal localization of SlMLO genes

Chromosome distributions of the 17 *MLO* genes were analyzed according to the tomato genome sequencing information in the Solanaceae genome website. As illustrated in Figure 4, these genes were located on ten chromosomes of tomato, but there was no gene in the fifth or twelfth chromosome. Furthermore, one gene *SlMLO17* was located in Chroo (it is unknown in which chromosome it was located), and most genes were located at the two ends of the chromosomes. There were three *MLO* genes distributed in chromosome 2 and 8, two *MLO* genes distributed in chromosome 8 and 9, and only one *MLO* gene in each of the remaining chromosomes. It was reported that gene families can arise through tandem duplication of chromosomal regions, resulting in a clustered occurrence of family members, or through segmental duplication, resulting in a scattered occurrence of family members (Schauser et al., 2005). According to criteria, we inferred that the expansion of the cucumber *MLO* gene family mainly resulted from segmental duplications.

Analysis of tomato MLO genes conserved motif

Conserved motifs of tomato *MLO* proteins were analyzed with MEME online tools. The results showed ten conserved motifs in tomato *MLO* proteins, and their lengths were 15 (Motif 10), 29 (Motifs 02, 03, 06, 07 and 09) and 40 (Motifs 01, 04, 05 and 08) amino acids (Table 2). The distributions of these conserved motifs in the tomato *MLO* proteins were further analyzed. As demonstrated in Figure 5, seven *SlMLO* genes contained all ten conserved motifs (Motifs 03, 05, 06, 09, 10, 11, 17). Motif 13 was the gene with the most deletions, containing only four conserved motifs. Motif 02 and Motif 16 had four and two conserved motif deletions, respectively. The remaining six genes had only lost one conserved motif (Motifs 01, 04, 07, 08, 12, and 15). There were two conserved motifs for Motif 09 on the *SlMLO04* protein.

Materials and Methods

Retrieval of different plant MLO gene family

Information on the tomato *MLO* gene family members was obtained from two databases, (http://solgenomics.net and http://mips.helmholtz-muenchen.de/plant/tomato/searchjsp/index. jsp). Information on the *Arabidopsis thaliana MLO* gene family members was obtained from the *Arabidopsis* Information Resource (TAIR) website (http://www.arabidopsis.org/). Information on the rice *MLO* gene family members was obtained from previously published data (Liu et al., 2008). Information on the *MLO* gene family members in grapes, poplar and sorghum was obtained from the database http://www.phytozome.net/index.php.

Isolation of MLO genes in tomato

Two methods were used to identify tomato *MLO* genes. Firstly, the keyword "MLO" was applied to search the databases above. Secondly, a BlastP search was conducted in the tomato database using the amino acid sequence of the *MLO* gene from *Arabidopsis thaliana*. Combining the results of the two methods, redundant sequences were excluded. The remaining genes were submitted to the PFAM database (http://pfam.janelia.org/) to verify the presence of the *MLO* domain.

Phylogenetic analysis

The searched amino acid sequences of *Arabidopsis* and tomato *MLO* proteins were aligned using Clustal X (Ramu et al., 2003). A phylogenetic tree was constructed with the amino acid sequence using the Neighbor-Joining method in MEGA 5.0 (Tamura et al., 2011). Bootstrapping (1000 replicates) was used to evaluate the degree of support for a particular grouping pattern in the phylogenetic tree. Nodes with bootstrap rates of less than 50% were removed, and the length of each branch was displayed.

Analysis of tomato MLO gene structure

The exon/intron structures of tomato MLO genes were obtained using the online Gene Structure Display Server (GSDS: http://gsds.cbi.pku.edu.cn) with both coding sequences and genomic sequences (Guo et al., 2007).

Chromosome distribution of MLO genes in tomato

Chromosome distribution diagrams of the *MLO* genes were drawn with MapDraw V2.1 (Liu et al., 2003) according to the obtained tomato *MLO* gene information from the Solanaceae genome database (http://solgenomics.net/).

Analysis of conserved motifs of MLO genes in tomato

The conserved motifs of the tomato *MLO* proteins were analyzed by Multiple Em for Motif Elicitation (MEME) online tools (http://meme.nbcr.net/meme/cgi-bin/meme.cgi). The parameters were set as follows: minimum length of the conserved motif was 15, maximum length was 40, and maximum number of conserved motifs was 10. Other parameters were default values.

Conclusions

In the present study, the identification and analysis of tomato *MLO* genes at the whole genome level was conducted by bioinformatic methods. A total of 17 *MLO* genes were obtained. Gene structure, phylogenetic relationship, and sequence characteristics were investigated. These results not only provide insight into the evolutionary relationship of the *MLO* gene family, but also lay a foundation for cloning tomato powdery mildew resistant genes.

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