Genetics and Molecular Research

Genomic identification, phylogeny, and expression analysis of *MLO* genes involved in susceptibility to powdery mildew in *Fragaria vesca*

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ABSTRACT. The *MLO* (powdery mildew locus O) gene family is important in resistance to powdery mildew (PM). In this study, all of the members of the *MLO* family were identified and analyzed in the strawberry (*Fragaria vesca*) genome. The strawberry contains at least 20 members of the *MLO* family, and the protein sequence contained between 171 and 1485 amino acids, with 0-34 introns. Chromosomal localization showed that the *MLO*s were unevenly distributed on each of the chromosomes, except for chromosome 4. The greatest number of *MLO*s (seven) was found on chromosome 3. A phylogenetic tree showed that the *MLO*s were divided into seven groups (I-VII), four

of which consisted of *MLO*s from strawberry, *Arabidopsis thaliana*, rice, and maize, suggesting that these genes may have evolved after the divergence of monocots and dicots. Multiple sequence alignment showed that strawberry *MLO* candidates related to powdery mildew resistance possessed seven highly conserved transmembrane domains, a calmodulin-binding domain, and two conserved regions, all of which are important domains for powdery mildew resistance genes. Expressed sequence tag analysis revealed that the *MLO*s were induced by multiple abiotic stressors, including low and high temperature, drought, and high salinity. These findings will contribute to the functional characterization of *MLO*s related to PM susceptibility, and will assist in the development of disease resistance in strawberries.

Key words: Strawberry; *MLO*; Bioinformatics; Phylogenic relationship

INTRODUCTION

Powdery mildew (PM) is one of the most important diseases in plants. Four major PM causal agents have been identified in the family Rosaceae: *Podosphaera leucotricha*, *Sphaerotheca pannosa* var. persicae, *Podosphaera tridactyla*, and *Podosphaera aphanis* (syn. *Sphaerotheca macularis* f. sp *fragariae*) (Boesewinkel, 1979; Xiao et al., 2001; Foulongne et al., 2003; Turechek et al., 2004). PM in strawberry is caused by the obligate pathogenic fungus *S. macularis* f. sp *fragariae*, and affects leaves, flowers, and fruit (Amsalem et al., 2006). These four fungi cause similar PM symptoms (Pessina et al., 2014).

MLO (powdery mildew locus O) is a plant-specific gene family that has seven complete transmembrane domains (TM1-TM7) (Büschges et al., 1997; Devoto et al., 1999). In barley, recessive mutations in *MLO* genes confer durable broad-spectrum resistance to all known isolates of the barley PM fungus (Büschges et al., 1997), and were the first genes that are resistant to PM to be cloned (Piffanelli et al., 2004). Further research showed that *MLO* genes encode calmodulin-binding proteins, and that calmodulin binding increases the susceptibility of barley to PM (Kim et al., 2002b). *MLO*s in barley possess the dual function of the regulation of disease resistance and leaf cell death (Devoto et al., 2003). Although *MLO*s were first isolated as PM resistance genes in monocots (Büschges et al., 1997), some members of this family also play a role in modulating hosts' responses to PM in dicots, such as *Arabidopsis* (Consonni et al., 2006) and tomato (Bai et al., 2008). Therefore, *MLO* disease resistance genes are extensively involved in interactions between plants and PM.

Many studies have shown that *MLO*s only exist in plants. *MLO*s have been identified in six plant taxa, including *Arabidopsis*, maize, rice, and grapevine (Devoto et al., 2003; Liu and Zhu, 2008; Feechan et al., 2009). In addition, *MLO*s that modulate hosts' responses to PM have been cloned in several plant species. For example, *Arabidopsis AtMLO02, AtMLO06*, and *AtMLO12* exhibit significantly reduced susceptibility to *Golovinomyces orontii* and complete PM resistance (Consonni et al., 2006). In the tomato, the absence of *MLO*s increases PM resistance (Bai et al., 2008). Therefore, biologically active *MLO*s may be a general requirement for PM pathogenesis in higher plants.

Recently, PM disease resistance has been reported in cucumber, wheat, and grapevine (Zhou et al., 2013; Alam et al., 2014; Lu et al., 2014; Zhang et al., 2014). In strawberry (*Fragaria* x *ananassa* Duchesne), cleaved amplified polymorphic sequence markers related to PM resistance have been developed (Heejeong et al., 2015), and interactions between strawberry and PM have been revealed using the RNA-seq method (Sun et al., 2015). Remarkably, the antisense expression of the peach *PpMlo1* gene confers cross-species resistance to PM in *Fragaria* x *ananassa* (Jiwan et al., 2013).

The availability of the strawberry genome sequence provides a good platform for our understanding of the structural characteristics and phylogenetic relationships of the *MLO* gene family at the whole-genome level. In this study, *MLO*s were identified by the integration of intron-exon structural characteristics, phylogenetic relationships, chromosome mapping, and expression analysis, which will provide the foundation for the further cloning of strawberry *MLO* (*FvMLO*) resistance genes.

MATERIAL AND METHODS

Datasets

Information regarding *FvMLO* family members was obtained from https://www.rosaceae.org/, *MLO*s from *Arabidopsis* were obtained from http://www.arabidopsis.org/, and *MLO*s from rice, grapevine, poplar, and sorghum were obtained from https://phytozome.jgi.doe.gov/pz/portal.html.

Identification of *MLO*s in strawberry and other model plants

Strawberry genome sequence data were downloaded from https://www.rosaceae.org/ to establish a local database using the BioEdit software. To identify *MLO*s in the strawberry, two methods were used to search the local database. Firstly, *MLO*s in the strawberry were identified using MLO protein sequences from two model plant species (*Arabidopsis thaliana* and rice) as a query using the BLASTP tool. The query sequences were taken from previously published data on these two plants (Devoto et al., 2003; Liu and Zhu, 2008). Secondly, the hidden Markov models profile of the MLO domain (PF03106) in the Pfam database (http://pfam.sanger.ac.uk/) was exploited for the identification of *MLO*s in the strawberry genome local database using HMMER 3.0 (http://hmmer.org/). The default parameters were used. Subsequently, all of the MLO protein sequences were analyzed to confirm the presence of an MLO domain using the SMART program (http://smart.embl-heidelberg.de/).

Sequence alignment and phylogenetic tree construction

In order to analyze the evolutionary relationships between the members of the *MLO* gene family, MLO proteins from strawberry and *A. thaliana*, grapevine, tomato, and pea were constructed using a phylogenetic tree (Devoto et al., 2003; Bai et al., 2008; Feechan et al., 2009; Pavan et al., 2011). The ClustalX program in BioEdit was used for the multiple sequence alignment of the amino acid sequence of strawberry MLO proteins (FvMLO). The

phylogenetic tree was constructed using the MEGA 5.0 software (Tamura et al., 2011), and was assessed using the bootstrap method (1000 iterations).

Structural analysis of *FvMLO*s

To ascertain the exon/intron positions and phases of the *FvMLO*s, we used the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) to identify exon/intron structures using coding and genomic sequences (Guo et al., 2007).

Chromosomal localization and conserved motif analysis of *FvMLO*s

The chromosomal distributions of the *MLO*s were determined using the Strawberry Genome Annotation Project (https://www.rosaceae.org/), and were displayed using the MapDraw V2.1 software (Liu and Meng, 2003). Conserved motifs of the FvMLO protein were analyzed using MEME (http://meme-suite.org/), and the parameters were set as follows: the minimum length of the conserved motif was 15, the maximum length was 50, and the maximum conserved motif was 10.

Expression profiles of *FvMLO*s under different abiotic stressors

To investigate *FvMLO* expression profiles in stressed (low temperature, high temperature, drought, and high salinity) seedlings, the Genome Database for Rosaceae (http://www.rosaceae.org/) was searched using the identified encoding sequences of the *FvMLO*s. Expressed sequence tags (ESTs) from the stressed seedlings were selected for analysis, and the nucleotide BLAST (BLASTn) program was used to search the ESTs that corresponded to the *FvMLO*s investigated in this study.

RESULTS

Identification of *FvMLO*s

We retrieved 20 *MLO* genes from the strawberry genome (*FvMLO01-FvMLO20*). Five of these may have been pseudogenes, as they encoded truncated proteins. The remaining 15 *MLO*s were between 1305 (*FvMLO16*) and 4458 (*FvMLO14*) bp in length, contained between 434 (*FvMLO16*) and 1485 (*FvMLO14*) amino acids, and weighed between 48.67 (*FvMLO16*) and 166.95 (*FvMLO14*) kDa. The isoelectric points of most of the MLO proteins ranged from 7 to 10, with the highest being 10.04 (*FvMLO01*) and the lowest 5.92 (*FvMLO16*) (Table 1).

The phylogenetic tree showed that the *FvMLO*s could be divided into three groups, A, B, and C, which contained 10, 3, and 7 members, respectively (Figure 1). The intron-exon structural analysis revealed that changes in the number of introns varied, suggesting that structures among the members of this gene family are complex. Only three genes (*FvMLO04*, *FvMLO06*, and *FvMLO12*) contained three introns or less.

Table 1. Members of the *FvMLO* gene family.

Gene	Locus name	Transcript name	Genome position	ORF length (bp)	Deduced polypeptide		
					Length (aa)	Mol. wt. (kDa)	pI
FvMLO01	gene10166-v1.0-hybrid	mrna10166.1-v1.0-hybrid	Chr01:697457..707892	2223	740	82.56	10.04
FvMLO02	gene13023-v1.0-hybrid	mrna13023.1-v1.0-hybrid	Chr01:7017413..7022793	1674	557	61.96	8.83
FvMLO03	gene14592-v1.0-hybrid	mrna14592.1-v1.0-hybrid	Chr01:7820289..7827918	2946	981	109.74	5.36
FvMLO04	gene10558-v1.0-hybrid	mrna10558.1-v1.0-hybrid	Chr02:15724387..15725931	1545	514	58.11	9.02
FvMLO05	gene29770-v1.0-hybrid	mrna29770.1-v1.0-hybrid	Chr03:4660882..4663962	1617	538	61.61	8.96
FvMLO06	gene02364-v1.0-hybrid	mrna02364.1-v1.0-hybrid	Chr03:9033592..9034412	516	171	18.98	8.54
FvMLO07	gene31264-v1.0-hybrid	mrna31264.1-v1.0-hybrid	Chr03:9515163..9524099	1704	567	64.80	9.03
FvMLO08	gene03210-v1.0-hybrid	mrna03210.1-v1.0-hybrid	Chr03:10707557..10713141	1644	547	61.76	5.97
FvMLO09	gene28541-v1.0-hybrid	mrna28541.1-v1.0-hybrid	Chr03:17105660..17112982	1146	381	42.99	9.20
FvMLO10	gene10363-v1.0-hybrid	mrna10363.1-v1.0-hybrid	Chr03:25802958..25816253	1353	450	50.09	9.06
FvMLO11	gene10346-v1.0-hybrid	mrna10346.1-v1.0-hybrid	Chr03:25834627..25838931	1158	385	43.88	10.06
FvMLO12	gene29284-v1.0-hybrid	mrna29284.1-v1.0-hybrid	Chr05:19059567..19060154	381	126	13.91	9.78
FvMLO13	gene29285-v1.0-hybrid	mrna29285.1-v1.0-hybrid	Chr05:19060230..19062497	894	297	33.86	7.76
FvMLO14	gene31488-v1.0-hybrid	mrna31488.1-v1.0-hybrid	Chr05:20075140..20093212	4458	1485	166.95	9.06
FvMLO15	gene31498-v1.0-hybrid	mrna31498.1-v1.0-hybrid	Chr05:20171268..20174872	1596	531	60.20	9.21
FvMLO16	gene11028-v1.0-hybrid	mrna11028.1-v1.0-hybrid	Chr05:20490925..20496890	1305	434	48.67	5.92
FvMLO17	gene09651-v1.0-hybrid	mrna09651.1-v1.0-hybrid	Chr06:8541201..8546252	1773	590	67.83	9.55
FvMLO18	gene09653-v1.0-hybrid	mrna09653.1-v1.0-hybrid	Chr06:8563501..8567082	1722	573	65.42	9.14
FvMLO19	gene23198-v1.0-hybrid	mrna23198.1-v1.0-hybrid	Chr07:14517490..14520807	1524	507	58.05	8.88
FvMLO20	gene26428-v1.0-hybrid	mrna26428.1-v1.0-hybrid	Chr07:16370664..16374969	1677	558	63.28	9.39

ORF = open reading frame; aa = amino acid; Mol. wt. = molecular weight; pI = isoelectric point.

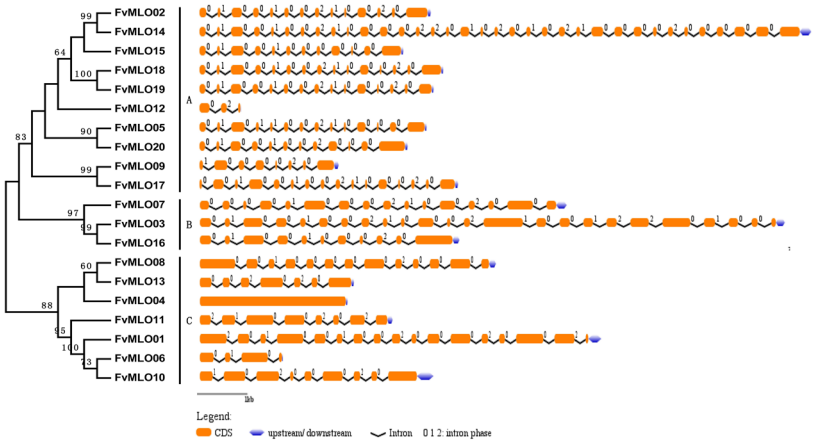

Figure 1. Phylogenetic analysis and intron/exon configurations of *FvMLO* genes. Introns and exons are drawn to scale, with the full transcript regions of their respective genes. Boxes and lines indicate exons and introns, respectively. 0, intron phrase 0; 1, intron phrase 1; 2, intron phrase 2.

Evolutionary relationships between *MLO*s in different plant species

The phylogenetic tree showed that *MLO*s could be divided into seven groups (I-VII), with each group containing a different number of *MLO*s (Figure 2). Group V contained the *Arabidopsis AtMLO02*, *AtMLO06*, and *AtMLO12* genes, which are a specific group of PM resistance functions, as well as a tomato PM resistance gene (*SlMLO*) and four *FvMLO*s (*FvMLO05*, *FvMLO18*, *FvMLO19*, and *FvMLO20*), suggesting that these genes may be candidate genes for resistance to PM.

The phylogenetic tree was also used to identify homologous genes, and contained six pairs of orthologous genes and five pairs of paralogous genes. The former included one from wheat, one from strawberry, and four from *A. thaliana*. The latter included two from *A. thaliana* and strawberry, one from barley and rice, one from strawberry and grapevine, and one each from rice and maize. The woody grapevine *MLO*s shared a common ancestry with the monocot maize and rice *MLO*s. Three plant taxa (strawberry, grapevine, and poplar) had orthologous genes, indicating a common ancestry. In addition, three groups (I, II, and III) contained *MLO*s from both monocots and dicots, suggesting that *MLO*s were present before the divergence of monocots and dicots.

Multiple sequence alignment

In order to analyze the sequence characteristics of MLO proteins that are related to PM susceptibility, a multiple sequence alignment of MLO proteins from strawberry, *Arabidopsis*, tomato, and pea was conducted (Figure 3) (Consonni et al., 2006; Bai et al., 2008; Pavan et al., 2011). All of the FvMLO proteins had seven highly conserved transmembrane domains (TM1-7), which is a significant feature of the *MLO* gene family (Devoto et al., 2003). A calmodulin-binding domain (CaMBD) that consists of an α-helix with 10-15 extended amino acids (Kim et al., 2002a,b) in the C-terminus is highly conserved in FvMLOs. Panstruga (2005) identified two other conserved regions in the C-terminus of MLO proteins (I and II), which

play important roles in modulating PM infection. These two peptide domains were located at the end of the C-terminus. Peptide domain I is characterized by conserved serine and threonine residues, while peptide domain II contains the consensus sequence D/E-F-S/T-F. In this study, we found that the peptide domains I and II were poorly conserved in the strawberry.

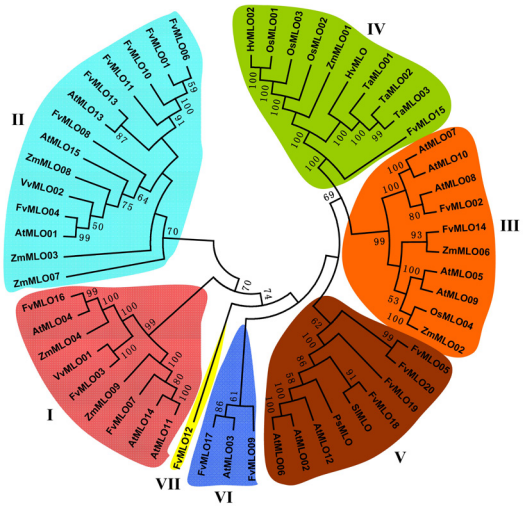

Figure 2. Phylogenetic comparison of *MLO* genes from *Arabidopsis*, rice, grapevine, and strawberry. Numbers on the branches indicate the percentage of 1000 bootstrap replications that support the node (only values greater than 50% are presented).

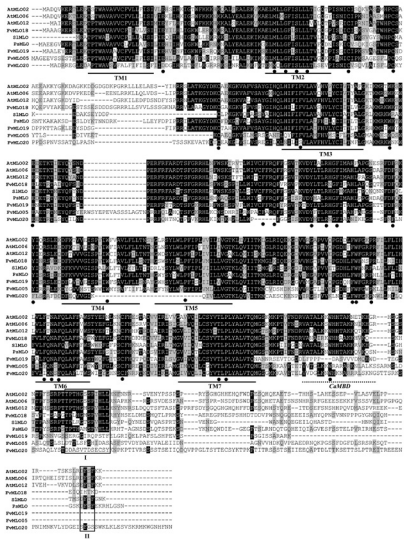

Figure 3. Multiple sequence alignment of MLO proteins from the *MLO* genes in Group V (Figure 1) in the strawberry, with selected MLO proteins involved in PM susceptibility in pea (PsMLO), tomato (SlMLO), and *Arabidopsis* (AtMLO02, AtMLO06, and AtMLO12). Positions of the seven transmembrane domains (TM1-TM7) inferred from the experimentally determined topology of HvMLO and the approximate position of CaMBD are indicated by lines under the sequences. Two conserved domains (I and II) within the highly polymorphic C-termini are highlighted.

Chromosomal localization

In order to elucidate the chromosomal distribution of *FvMLO*s, we conducted *in silico* mapping. The *FvMLO*s were on six of seven chromosomes (Figure 4). There were no *FvMLO*s on chromosome 4, and only one (*FvMLO04*) on chromosome 2. Three and five *FvMLO*s were on chromosomes 1 and 5, respectively, and two were on each of chromosomes 6 and 7. The remainder was on chromosome 3.

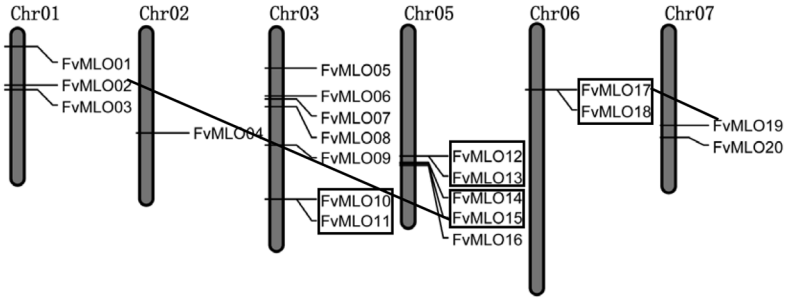

Figure 4. Mapping of *MLO* genes on strawberry chromosomes. Chromosome numbers are indicated at the top of the chromosomes; those located on sequence scaffolds are not shown. Straight lines connecting the *MLO* genes indicate duplicated chromosomal segments; tandem duplicated gene clusters are indicated by boxes.

Segmental and tandem duplication played important roles in *MLO* family expansion during evolution (Cannon et al., 2004). In the present study, four pairs of genes (*FvMLO10* and *FvMLO11*, *FvMLO12* and *FvMLO13*, *FvMLO14* and *FvMLO15*, and *FvMLO17* and *FvMLO18*) exhibited tandem duplication (Figure 4). In addition, evidence of segmental genome duplication was found in the *FvMLO02* and *FvMLO17* chromosomal regions (Figure 4), which exhibited synteny with the surrounding genomic regions that contained *FvMLO15* and *FvMLO19*, respectively.

Conserved motifs of FvMLO proteins

The FvMLO proteins had 10 types of conserved motifs; four motifs contained over 40 amino acids (Motif 01, Motif 03, Motif 04, and Motif 06), and the remainder contained between 21 and 30 amino acids (Table 2). We analyzed the distributions of these conserved motifs (Figure 5), and found that most of the FvMLO proteins contained all motif types, but not three of them (FvMLO06, FvMLO11, and FvMLO12). The distributions of the conserved motifs were consistent with the phylogenic relationships between the FvMLO proteins.

In silico analysis of *FvMLO* expression using EST libraries

To evaluate the expression profiles of *FvMLO*s under different stressors, a BLAST search was performed using the *FvMLO* coding sequences in the Genome Database for Rosaceae. Only 11 predicted genes had corresponding ESTs in the database (Table 3). Of these, three (*FvMLO01*, *FvMLO10*, and *FvMLO11*) were induced by high salinity, four (*FvMLO03*, *FvMLO07*, *FvMLO09,* and *FvMLO19*) by low temperature, four (*FvMLO04*, *FvMLO12*, *FvMLO13*, and *FvMLO18*) by high temperature, and *FvMLO04* by high temperature and drought.

Table 2. Motif compositions of FvMLO proteins.

Motif	Width	Best possible match
Motif 01	44	QYGFDSCFMENIEYIIPRLVMGVFVQFLCSYSTLPLYAIVTQMG
Motif 02	41	DPVVQPSDDHFWFNRPRWVLHLIHFILFQNAFEMAFFFWIW
Motif 03	30	LEKIKEELMLLGFISLLLTVFQDPIAKICI
Motif 04	43	CKKGKVPFVSYNGLHQLHIFIFVLAIFHVVYCCLTMALGMAKM
Motif 05	29	CFFRQFYGSVTKVDYMTMRHGFINAHCA
Motif 06	41	LEYTPTWAVATVCFVIVFISIIIEHGIHCLGKWLKRRRKKA
Motif 07	27	NFDFHKYMKRSLEDDFKVVVGISPIMW
Motif 08	21	GTKLQHIITQMAHEIAEKHNV
Motif 09	28	SNMKKAIFDEHVQEGLKGWHKDAKKHQA
Motif 10	28	EYQFSNDPNRFRYTHQTSFGKRHLKFWT

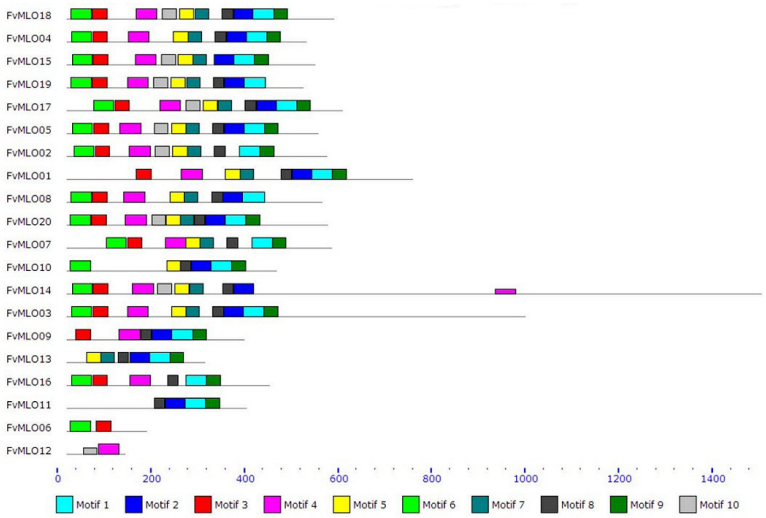

Figure 5. Motif structure of *MLO* genes in the strawberry.

Table 3. Expressed sequence tag (EST)-derived expression profiles of *FvMLO* genes in stressed seedlings.

Name	High salinity	Low temperature	High temperature	Drought
FvMLO01	+			
FvMLO02				
FvMLO03		+		
FvMLO04			+	+
FvMLO05				
FvMLO06				
FvMLO07		+		
FvMLO08				
FvMLO09		+		
FvMLO10	+			
FvMLO11	+			
FvMLO12			+	
FvMLO13			+	
FvMLO14				
FvMLO15				
FvMLO16				
FvMLO17				
FvMLO18			+	
FvMLO19		+		
FvMLO20				

DISCUSSION

In order to analyze the phylogenic relationships between *MLO*s, dicots, monocots, and woody plant *MLO*s were investigated in this study. According to a previous classification of *Arabidopsis MLO*s, *FvMLO*s could be divided into seven groups (I-VII). Only three groups (I, II, and III) contained *MLO*s from all of the species tested, indicating that the members of these three groups were more conserved than those in the remaining four groups. In addition, six pairs of orthologous genes and five pairs of paralogs were identified in the phylogenetic tree, suggesting a common ancestry of *MLO*s in these plant species. Six pairs of orthologous genes were in *Arabidopsis* with four pairs, as well as in wheat with one pair and strawberry with one pair; therefore, wheat, strawberry, and *Arabidopsis* have orthologous genes, indicating that *MLO*s underwent gene duplication after the divergence of the monocots and dicotsl.

Given that Group V, which included *Arabidopsis AtMLO02*, *AtMLO06*, *AtMLO12*, pea *PsMLO*, and tomato *SlMLO*, indicates the putative functions of *MLO*s in many plant species, we inferred that this group is significant for strawberry, as these *MLO*s are required for PM susceptibility (Consonni et al., 2006; Bai et al., 2008; Pavan et al., 2011). Therefore, this group may be dicot-specific. Four *MLO*s (*FvMLO05*, *FvMLO18*, *FvMLO19*, and *FvMLO20*) were in Group V, suggesting that they may be candidate PM resistance genes. In addition, in the multiple sequence alignment, these genes not only contained seven highly conserved transmembrane domains and the CaMBD domain of the *MLO* gene family, but also two conserved peptide regions (I and II) in the C-terminal.

At present, two types of gene duplication events have been identified during the evolution of gene families: tandem and segmental duplication. The former may result in a clustered occurrence of family members, whereas the latter may result in a scattered occurrence of family members (Schauser et al., 2005). Both types of duplication event were observed in this study, indicating that the expansion of the *FvMLO* family mainly resulted from segmental and tandem duplication.

Many sequences that are available from EST libraries (e.g., http://www.rosaceae.org/) can provide useful information regarding gene expression analysis. In this study, 12 ESTs were identified for 11 *FvMLO*s, and most of them were expressed under different stressors (Table 3), indicating that *FvMLO*s may be involved in plant growth and development. The large number of *FvMLO*s found in this study will provide useful information for further experimental verification.

In conclusion, we conducted a genome-wide identification and analysis of *FvMLO*s using bioinformatics. At least 20 *FvMLO*s were identified, and their structures, phylogenies, and sequence characteristics were analyzed. These results will provide a foundation for the breeding of strawberries that are resistant to PM.

Conflicts of interest

The authors declare no conflicts of interest.

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